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Chlorotoxin as a Drug Delivery System to treat Glioblastoma

by

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Abbreviations

2xYT	2x Yeast Extract Tryptone
AKT	Protein kinase B
AOM	Azoxymethane
AIC	5-Aminoimidazole-4-Carboxamide
APE-1	Abasic Site Endonuclease
BBB	Blood Brain Barrier
BER	Base Excision Repair
bFGF	Basic Fibroblast Growth Factor
Bp	Base Pairs
CAM	Chick Chorioallantoic Membrane
CT	C-Terminal
CITx	Chlorotoxin
CREB	cAMP response element-binding protein
Da	Dalton
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DCM	Dichloromethane
DMBA	7, 12-Dimethylbenz (a) anthracene
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nuclease
DTT	Dithiothreitol
ECM	Extracellular Matrix
E.coli	Escherichia coli
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EphA3	Ephrin Type-A Receptor 3
Fas-L	Fas Ligand

Fas-R	Fas Receptor
FEN-10	Flap Endonuclease-1
FPLC	Fast Protein Liquid Chromatography
GBM	Glioblastoma
GFP	Green Fluorescent Protein
GuHCl	Guanidinium Hydrochloride
HBSS	Hank's Balanced Salt Solution
HGFR	Hepatocyte Growth Factor Receptor
HPLC	High Performance Liquid Chromatography
HT	6x Histidine Tag
IGFII	Insulin Like Growth Factor II
iMRI	Intra-Operative Magnetic Resonance Imaging
IPTG	Isopropyl β -D-1-thiogalactopyranoside
kb	Kilobases
kDa	Kilo Dalton
KLD	Kinase, Ligase and DpnI
LB	Lysogeny Broth
M6P	Mannose-6-Phosphate
MGMT	O ⁶ -Methylguanine-DNA Methyltransferase
MMP-2	Matrix Metalloproteinase-2
MMR	DNA Mismatch Repair
MTIC	5-(3-Methyltriazene-1-yl)-Imidazole-4-Carboxamide
mTOR	Mammalian Target of Rapamycin
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NNK	Nicotine-derived Nitrosamine Ketone
NP	Nano-particles
OS	Overall Survival
PI3K	Phosphoinositide 3-Kinase
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PDGFR	Platelet Derived Growth Factor Receptor
PFS	Progression Free Survival

PMF	Peptide Mass Fingerprint
PNK	Polynucleotide Kinase
POH	Perillyl Alcohol
PTEN	Phosphatase and Tensin Homolog
RPM	Rotations Per Minute
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulfate
SOC	Super Optimal Broth with Catabolite Repression
Sulfo-NHS	<i>N</i> -hydroxysulfosuccinimide
TAE	Tris-Acetate-EDTA
TEMED	Tetramethylethylenediamine
TEA	Triethylamine
TEV	Tobacco Etch Virus
TMZ	Temozolomide
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
XRCC1	X-ray Repair Cross-Complementing 1

Abstract

This thesis describes the generation of a number of new conjugates and their evaluation against brain tumour derived cell lines. Chlorotoxin (CITx) is a 36-amino acid protein found in the venom of the deathstalker scorpion (*Leiurus quinquestriatus*). CITx binds to brain tumours and can stop invasion and metastasis of glioma cells, such as glioblastoma multiforme (GBM). Perillyl alcohol (POH) is a monoterpene derived from the mevalonate pathway. It is an effective chemotherapeutic agent against several cancers such as breast, liver and pancreatic by causing the cells to undergo apoptosis. Temozolomide (TMZ) is a DNA methylating agent which adds a methyl group at the N^7 or O^6 position of guanine in the DNA causing GBM cells to undergo apoptosis. TMZ is currently used as the main chemotherapeutic drug for GBM. The aim of this research was to first purify recombinant CITx-constructs, followed by synthesis of cytotoxic ligands that will be conjugated to CITx and finally, test these CITx-ligand conjugates for cytotoxicity against GBM cells. In this research, CITx constructs were successfully cloned and purified. The CITx inhibited U87 cell migration while CITx conjugated to fluorescein showed binding of this protein on the surface and inside U87 cells within 40 min after exposure. POH was modified to create linkers enabling conjugation of the ligand to CITx. POH was finally conjugated to CITx through a cleavable ester link. The CITx-POH conjugate showed no cytotoxic activity upto a concentration of 10 μ M. Instead a variant of TMZ, known as N^3P -TMZ, which has a propargyl group instead of a methyl group at the N^3 position of temozolomide, was synthesised in order to assess its cytotoxicity against GBM cells. The N^3P -TMZ requires modification to allow conjugation to CITx which can then create a cytotoxic drug which should specifically target GBM.

1

Introduction

1.1 Glioblastoma

Glioblastoma Multiforme (GBM) is a malignant primary central nervous system cancer. It accounts for at least 70% of adult brain tumours with a median survival of only 15 months.¹ GBM has been shown to recur following resection, even when the resection was expanded to normal brain tissue surrounding the tumour mass.² As such, treatment of GBM has evolved into a three-stage process:³

1. Maximal Surgical Resection
2. Radiation Therapy
3. Chemotherapy using Temozolomide (TMZ)

GBM can be further split into two categories: primary, which accounts for 80% of all tumours and secondary, which normally occurs in younger patients (mean age of 45 compared to 62 years for primary GBM) and usually develops from low grade oligodendroglioma and astrocytoma.³

The aetiology of GBM is currently unknown. The only known risk factor is exposure to high dosage of ionised radiation.⁴ There has been no link of GBM to environmental conditions such as smoking or dietary conditions.⁵

1.1.1 Treatment of glioblastoma

Unlike other tumours, chemotherapeutic treatment of brain tumours faces several hurdles, the main one being the relative impermeability of the blood brain barrier (BBB) to chemotherapeutic drugs. The BBB is a semipermeable, highly selective membrane and the weight, charge and lipophilicity of potential drugs can affect their ability to pass through it.⁶ The BBB stops nearly all of the large molecules (over 400 Da) and more than 98% of small molecule drugs from entering the central nervous system.^{7,8}

As such, the current treatment for brain tumours involves surgery where the maximum possible resection is carried out to result in the longest possible progression-free survival (PFS) and overall survival (OS).⁹ Surgical treatment can be followed by radiotherapy, using a total dosage of 60 Gy and then chemotherapy.¹⁰ Currently TMZ is the preferred drug with a dosage of 150-200 mg/m²/day (Figure 1.1).¹¹ Treatment with TMZ helps with OS but does not completely eradicate the cancer. TMZ causes DNA methylation which leads to DNA mismatch and later cell death. Unfortunately, protection pathways *O*⁶-methylguanine-DNA methyltransferase (MGMT) system exist in the cancer cells which can fix damage caused by TMZ. Furthermore, extended exposure to TMZ has led to more of these pathways being upregulated and an increasing in TMZ resistance has been observed, leading to an increase in tumour recurrence.¹² This has led to the need to identify novel chemotherapeutic drugs to treat TMZ-resistant tumours.

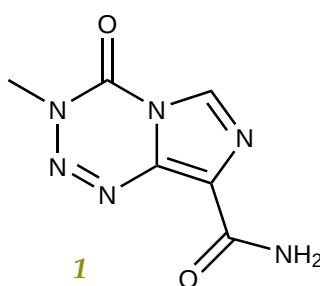


Figure 1.1 | Structure of temozolomide (1)

1.1.2 Targets for therapy

The Cancer Genome Atlas Research Network have identified three main signalling pathways involved in GBM.¹³ They are the retinoblastoma, p53 and the receptor tyrosine kinase (RTK)-Ras-phosphoinositide 3-kinase (PI3K) pathways. Various drugs have been synthesised which target receptors and proteins found in these signalling pathways (Table 1.1).

Table 1.1 | Overexpressed or mutated receptors present in GBM and targeted drug inhibitors¹¹

Target	Function of Target	Drug
EphA3	Regulation of cell-cell adhesion and cell migration	GLPG1790, IIIA4-USAN
EGFR	Regulation of cell growth and survival	Erlotinib, Gefitinib
VEGF	Promotion of angiogenesis	Bevacizumab, Tivozanib, Pazopanib
PDGFR	Regulation of cell growth and survival	Imatinib, Sunitinib
PI3K Pathway	Regulation of cell growth and survival	Buparlisib, Sonolisib
HGFR	Tumour growth and angiogenesis	SGX532, Crizotinib

1.1.2.1 Ephrin Type-A Receptor 3

The Ephrin Type-A Receptor 3 (EphA3) has been shown to be present in 40-60% of GBM.¹⁴ It helps the tumours maintain their differentiated stem-cell like states and their tumourigenic potential in the GBM. As such, EphA3 is a target for GBM therapy.¹⁵ GLPG1790 is molecular inhibitor of EphA3 that shows cytotoxic effect on U87MG and U251MG (immortalised GBM cell lines) after radiotherapy treatment. It does this by inhibiting the tyrosine phosphorylation in EphA3, leading to downregulation of the signalling system. Unfortunately compared to TMZ, GLPG1790 is not as effective.¹⁶ Work has also been done on with IIIA4-USAN, an EphA3 binding monoclonal antibody (IIIA4) conjugated using a succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) linker to maytansine (Figure 1.2); a cytotoxic agent that targets microtubules. It was shown to be effective against primary GBM cell lines *in vitro* and in orthotopic models *in vivo*.¹⁷

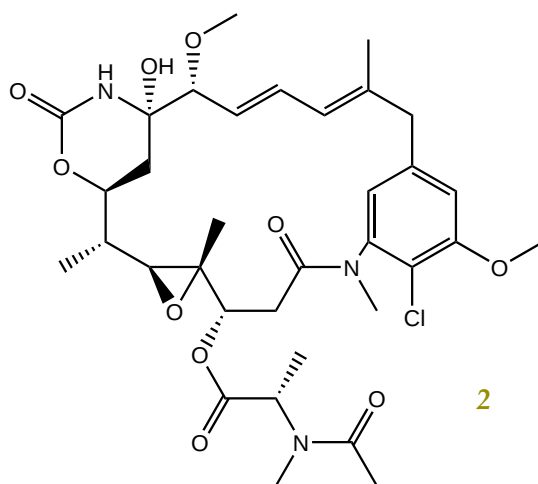


Figure 1.2 | Structure of maytansine (2)

1.1.2.2 Epidermal Growth Factor Receptor

As with EphA3, 40-60% of GBM show amplification of the Epidermal Growth Factor Receptor (EGFR).¹⁸ EGFR is one of the four members of the ErbB family which are related to receptor tyrosine kinases (RTKs). Activation of the EGFR tyrosine kinase domain is involved in downstream cell signalling pathways. As such, over expression of EGFR has been heavily involved in interfering with cell apoptosis and enhancing proliferation and angiogenesis.¹⁹

Erlotinib (3)(Figure 1.3) is one of the RTK inhibitor drugs that work in an EGFR dependent manner. It kills tumour initiating cells which are normally resistant to radiotherapy and was shown to reduce tumour burden in xenograft models.^{20,21} Unfortunately, erlotinib requires phosphatase and tensin homolog (PTEN) to be expressed. PTEN is involved as a tumour suppressor gene where its phosphatase prevents rapid growth and cell division. 34% of GBM have downregulated levels of PTEN which decreases the effectiveness of erlotinib.²² Gefitinib (4)(Figure 1.3) on the other hand does not depend on the expression of EGFR but provides inhibition of cell migration and decreased proliferation.²³ Phase II trials with gefitinib showed good activity after radiotherapy but no obvious OS increase.²⁴

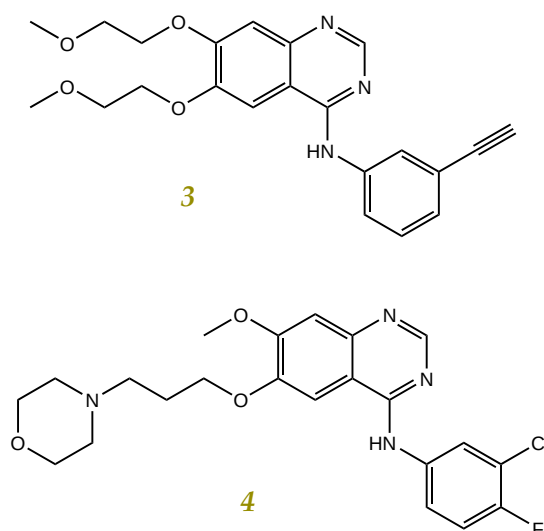


Figure 1.3 | Structures of erlotinib (3) and gefitinib (4)

1.1.2.3 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) is a protein that is highly expressed in GBM that helps mediate proliferation and angiogenesis hence increasing the malignancy in GBM.²⁵ Bevacizumab, a monoclonal antibody, is one of the drugs that is used against VEGF. It is able to inhibit the function of the VEGF in the lumen of blood vessels in the brain and therefore does not require to cross the BBB.²⁶ It has been used to treat recurrent GBM, especially when combined with radiotherapy. However, due to severe side effects, such as loss in neurocognitive ability and no OS improvement, concerns were raised for using it as treatment.²⁷ Other drugs, such as tivozanib (5) and pazopanib (6) (Figure 1.4), have shown minimal activity in phase II clinical trials.^{28 29}

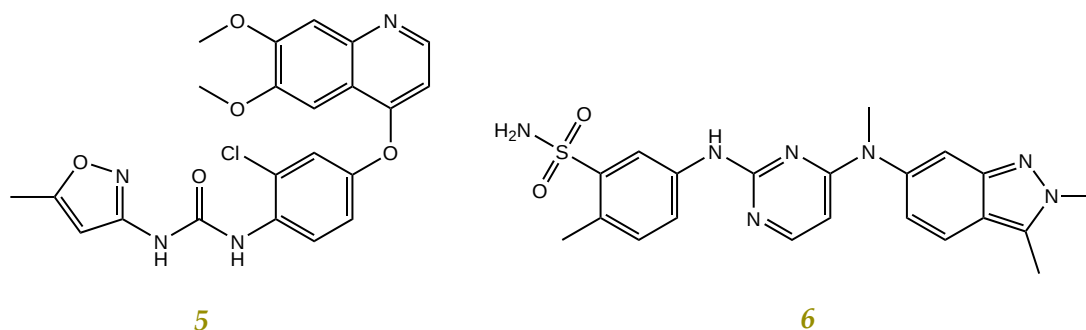


Figure 1.4 | Structures of tivozanib (5) and pazopanib (6)

1.1.2.4 Platelet Derived Growth Factor Receptor

Platelet Derived Growth Factor Receptor (PDGFR) is a protein that is co-expressed with EGFR in 37% cancers and regulates cell proliferation, growth and development.³⁰ Imatinib (7) (Figure 1.5) is a drug that inhibits the two PDGFR subunits (PDGFRA and PDGFRB). Unfortunately, imatinib showed low anti-tumour activity during clinical trials³¹ and, instead, showed an increase in GBM migration and invasion causing it to be discarded as a potential drug for GBM.³² Sunitinib (8) (Figure 1.5) is another PDGFR inhibitor that has cytotoxicity in GBM by causing the cell to undergo apoptosis. Mice models showed encouraging positive results with increased survival.³³ A phase II trial, however, showed minimal activity in recurrent GBM.³⁴

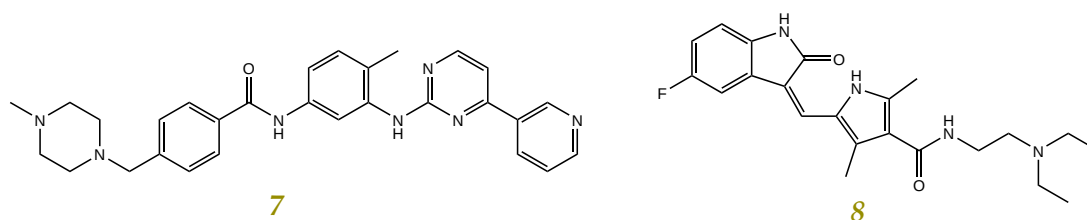


Figure 1.5 | Structures of imatinib (7) and sunitinib (8)

1.1.2.5 PI3K pathway

The PI3K/AKT/mTOR pathway is one of the main signalling pathways that regulate cell growth. Phosphoinositide 3-kinases (PI3K) activation leads to the phosphorylation/activation of protein kinase B (AKT) which then leads to various downstream signalling events, such as activating of the mammalian target of rapamycin (mTOR) and cAMP response element-binding protein (CREB). All these different proteins play key roles in cell growth and thus are heavily overexpressed in GBM leading to proliferation.¹¹ Buparlisib (9) (Figure 1.6) is a PK inhibitor involved in cell growth reduction and has been shown to work well in GBM in clinical trials. However, the effect of the drug shown to be low in recurrent GBM. This is due to the fact that buparlisib does not completely inhibit the PI3K pathway, thus allowing for tumour re-growth.³⁵ Sonolisib (10) (Figure 1.6) is another drug that inhibits angiogenesis and invasion of the cancers but again it exhibited low activity in recurrent GBM trials.³⁶

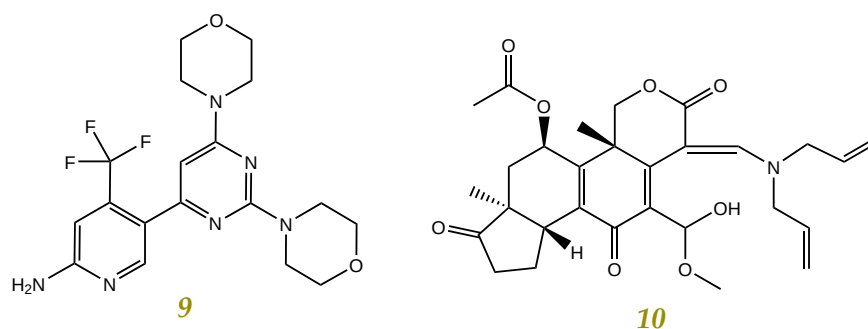


Figure 1.6 | Structures of buparlisib (9) and sonolisib (10)

1.1.2.6 Hepatocyte Growth Factor Receptor

Hepatocyte Growth Factor Receptor (HGFR) also known as c-Met is a tyrosine kinase essential for cell growth and overexpression of MET in 4-6% of GBM has led to angiogenesis in tumours.¹⁸ SGX-523 (11) (Figure 1.7) is an inhibitor of HGFR, angiogenesis and metastasis of glioma cells in xenograft models.³⁷ Crizotinib (12) (Figure 1.7) is another inhibitor of tumour cell growth via MET kinase activity and has shown promising activity in U87MG xenograft models. Unfortunately, erlotinib was shown to be needed with crizotinib to be active in other GBM cell models such as Mayo39 and Mayo69.²¹

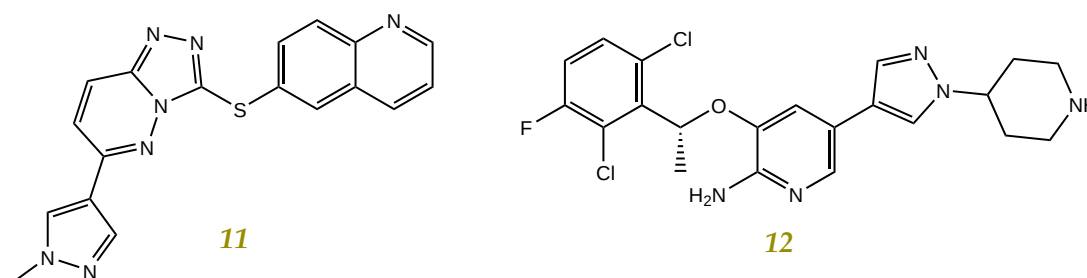


Figure 1.7 | Structures of SGX-523 (11) and crizotinib (12)

Even with so many different types of drugs available for GBM treatment, the cancer still persists. Therefore, research has been carried out to use drug conjugates for target and treatment of GBM. These are built by attaching a small molecule anticancer drug to a non-cytotoxic but GBM targeting molecule via a labile or permanent linker. One of these GBM targeting molecules is chlorotoxin (ClTx).³⁸

1.2 Chlorotoxin

CITx is a 36-amino acid protein (amino acid sequence: [MCMPCFTTDHQMARCDD-CCGGKGRGKCYGPQCLCR])³⁹ found in the venom of the deathstalker scorpion (*Leiurus quinquestriatus*) that binds to glioma cancer cells (Figure 1.8). It contains 8 cysteine residues that form disulfide bonds in a unique knottin domain conferring greater stability (Figure 1.9). The 3D solution structure which was determined in 1995 by nuclear magnetic resonance (NMR) revealed that CITx had an α -helix between residues 11-21 and three β -sheets between residues 1-4, 26-29 and 32-36.⁴⁰

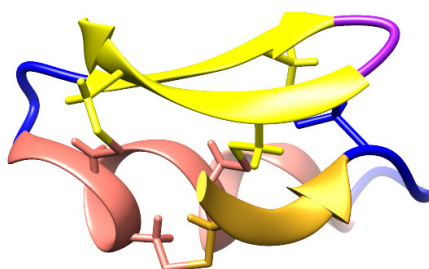


Figure 1.8 | Structure of chlorotoxin. (1CHL)⁴⁰

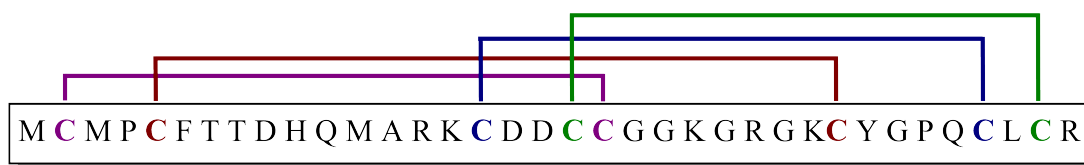


Figure 1.9 | Disulfide bridges in CITx highlighting the knottin domain

CITx causes paralysis in small insects and invertebrates, but has no toxic effects in vertebrates.⁴¹ Interest in CITx emerged after it was suggested that it had either the ability to pass through the BBB or blood brain tumour barrier (BBTB) (TransMolecular, Inc., Cambridge, MA, USA; unpublished data). Further studies found that CITx did this by diffusing into the tumour while other targeting drugs such as antibodies, could not manage to cross the barrier.^{42 43} The studies did not fully identify which of the two barriers CITx crossed and also if this movement has an effect on the integrity of the barrier. Instead it is believed that the disturbance of the barrier depends on the type of tumour and state of cancer progression.^{41 44}

1.2.1 Biological activities of chlorotoxin

Interest in CITx increased after it was discovered to have chemotherapeutic properties. *In vivo* studies using ¹²⁵Iodine-labelled CITx in tumour mice models showed that CITx accumulates at the tumour.³⁸ This study found that the ¹²⁵I-CITx specifically bound to glioma cells and not to normal astrocytes (glial) cells.³⁸ CITx also binds to melanomas, neuroblastomas, lung carcinomas and tumours originating from the neuroectodermal dysplasia (tumour cells originating from either the central or peripheral nervous system) but not to normal skin, kidney, brain or lung cells.⁴⁵ Interestingly, CITx also bound to neurological tumours in the presence of non-tumourigenic neurological diseases like Alzheimer's and Parkinson's.⁴⁵

CITx's tumour binding ability also resulted in anti-metastatic activity during *in vitro* studies using a Transwell migration assay.⁴⁶ The Transwell migration assay is an analysis of endothelial cell migration in response to angiogenic inducers and inhibitors. CITx inhibited invasion and migration of U-251 MG glioma cells.⁴⁷ Migration inhibition of normal human umbilical vein endothelial cells (HUVEC) in the presence of CITx was also shown, but this was only when the HUVEC cells were stimulated with growth factors, such as the vascular endothelial growth factor (VEGF) or the basic fibroblast growth factor (bFGF).⁴⁷ The effect of chlorotoxin on growth factors was analysed using a chick chorioallantoic membrane (CAM) assay.⁴⁸ The CAM assay is a widely used analytical technique to study angiogenesis and invasion of brain tumours.⁴⁸ Using the CAM assay, CITx was found to inhibit vessel formation in a dose-dependent manner when pre-induced by stimuli such as VEGF, TNF α , EGF and bFGF.⁴⁹ Additionally, CITx was found to be non-toxic as it inhibited invasion and migration without causing apoptosis in cancer cells.⁴⁹

1.2.2 Protein targets of chlorotoxin

CITx was first identified as a chloride channel targeting protein causing inhibition of chloride channels when added to the intracellular side. Inhibitory effects on small conductance chloride ion channels in embryonic rat brains and later in human brain cancer cells were shown in the presence of CITx. However, such inhibitory effects were not detected in AMP-activated chloride channels in human and monkey cells.⁵⁰ This

suggested that the CITx is not a general chloride channel inhibitor but only targets specific chloride channels. Studies carried out with the ^{125}I -CITx identified a 72 kDa target protein and CITx was found to bind glioma-specific chloride channels instead of the glial chloride channels.⁵¹

1.2.2.1 Matrix Metalloproteinase-2 (MMP-2)

Matrix Metalloproteinase-2 (MMP-2) is a 72 kDa type IV collagenase enzyme which degrades proteins found in the extracellular matrix (ECM).⁴⁶

Ramachandran *et al.* showed an increase in MMP2 expression and activity from diffuse astrocytomas to anaplastic astrocytomas and to glioblastomas, the latter exhibiting the highest level of MMP2 expression. This study also showed that most MMP2 expression was present inside the cytoplasm but expression was also present in the membrane and surrounding ECM.⁵²

Co-purification of HT-CITx with MMP-2 via affinity purification by Deshane *et al.* in glioma cells but not in normal glial cells highlighting MMP-2 as a possible target for CITx. Furthermore, CITx inhibited the enzymatic activity of MMP-2 and decreased expression of the MMP-2 enzyme. Interestingly, CITx only targeted the MMP-2 enzyme and no other MMP enzymes, such as MMP-1, MMP-3 and MMP-9.⁴⁶ CITx targets the collagen binding domain of the MMP-2 protein, mainly via hydrophobic interactions and Van der Waals forces.⁵³ In particular, the CITx N-terminal phenylalanine and C-terminal residues play an important role in the binding of CITx to MMP-2.⁵³

1.2.2.2 Annexin A2

Another target for CITx is annexin A2. Annexin A2 is a calcium dependent phospholipid binding protein that is involved in various cell signalling pathways and in cellular growth.⁵⁴ siRNA knock-down of annexin A2 expression in the pancreatic tumour cells, Panc-1 resulted in inhibition of CITx binding on the cell surface.⁵⁵ This suggests that annexin A2 is a target for CITx.

1.2.3 Uses of chlorotoxin

CITx has been used as a "tumour paint". This was achieved by conjugating the CITx to Cy5.5 (Figure 1.10).⁵⁶ Cy5.5 is a fluorescent molecule that emits light in the near infra-red region (710 nm). This allows it to be used as an optical intraoperative imaging due to low absorption of photons at this wavelength by water and haemoglobin. CITx: Cy5.5 was able to pass through the BBB and was used to distinguish tumour medulloblastoma cells from non-tumorous cells in ND2: Smo1 mouse models.⁵⁶ Furthermore, CITx: Cy5.5 was found to be non-toxic and can be used as an optical imaging drug.⁵⁶ CITx: Cy5.5 binding to human prostate cancer and lung metastases was also observed, all which are MMP-2 positive tumours, which suggested that conjugation of Cy5.5 to CITx did not affect the MMP-2 binding activity of CITx.⁵⁶

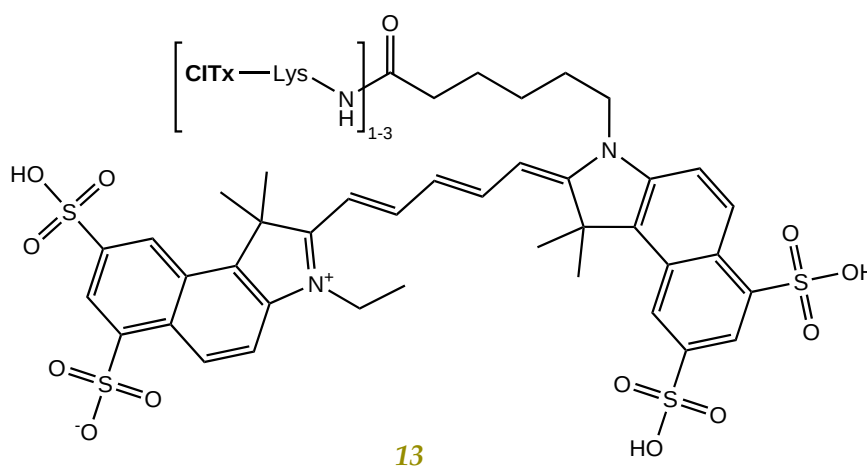


Figure 1.10 | Structure of CITx conjugated to Cy5.5 (13)

CITx was used in clinical trials under the name, TM-601, a synthetic version of CITx which is radio-labelled with ¹³¹Iodine (¹³¹I).⁵⁷ It was used for detecting (via magnetic resonance imaging (MRI) and single-photon emission computed tomography (SPECT)) and inhibiting glioma tumour migration.⁵⁷ The drug has successfully passed clinical phase I/II with a single dose of 10 mCi of ¹³¹I-TM-601 received well and a dose of 0.25 to 1.0 mg TM-601 exhibiting anti-tumoural effects.^{41, 57, 58}

CITx has also been conjugated to nano-particles (NP),⁵⁹ onconase⁶⁰ and gemcitabine⁶¹ all shown to successfully create a cytotoxic version of CITx which can be used to treat glioblastomas.^{59–61} This research attempts to further this work in CITx

1.3 Perillyl alcohol

Perillyl alcohol (POH) **15** is a natural monoterpene derived from the mevalonate pathway (Figure 1.12).⁶⁵ It is the hydroxylated version of d-limonene (p-mentha-1,8-diene) **14** which is formed via condensation of two isoprene molecules (Figure 1.12).⁶⁵ POH is found in small concentrations in essential oils such as spearmint, peppermint, lemon grass and has been shown to target cancers such as breast, liver and pancreatic cancers.⁶⁵

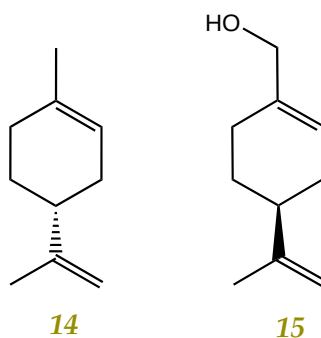


Figure 1.12 | Structures of d-limonene (**14**) and perillyl alcohol (**15**)

1.3.1 Synthesis of perillyl alcohol

POH as a natural monoterpene that can be obtained from plants. It is extracted using methanol and purified by reversed-phase high-performance liquid chromatography.⁶⁶ d-limonene can be used to biologically synthesise POH via hydroxylation of the d-limonene using the cytochrome P450 enzyme.⁶⁷

1.3.2 Biological activities of perillyl alcohol

POH was first identified to cause pancreatic cancer cells to undergo apoptosis by increasing the expression of a pro-apoptotic protein, Bak.⁶⁸ Inhibition and apoptosis of azoxymethane (AOM) induced adenoma in rat colons was observed in the presence of POH at 2000 ppm.⁶⁵ Adenomas are transformations of colorectal epithelium via the inhibition of apoptosis.⁶⁵ As POH induces apoptosis, it prevents this transformation and therefore can be used to treat patients with colorectal cancers. POH was found to have protective properties against colon cancer by helping the liver with the

detoxification of carcinogens.⁶⁹ Limonene, the POH precursor increases the urinary excretion of a carcinogen known as 7, 12-dimethylbenz-(a)-anthracene (DMBA).⁶⁹ Like CITx, POH may also be able to inhibit metastasis as d-limonene was found to prevent seeding of murine colon tumour cells to the liver.⁷⁰ POH reduces skin cancer formation by reducing the erythema and dryness that is caused due to UV radiation.⁷¹ Moreover, POH decreases adenoma tumour formation in the presence of nicotine-derived nitrosamine ketone (NNK), a carcinogen normally found in tobacco suggesting that it may help in the prevention of tobacco-induced lung cancer.⁷² Studies using HepG2 liver cancer cell lines showed an IC₅₀ of 2.7 mM.⁷³ POH also showed cytotoxicity to GBM with an IC₅₀ of 0.6 mM in wild-type GBM and 1.2 mM in TMZ-resistant GBM.⁷⁴

1.3.3 Molecular targets of POH

The exact targets for POH apoptosis inhibition are still unknown. POH increases the expression of mannose-6-phosphate (M6P) and insulin like growth factor II (IGFII) receptors and decreases p21ras protein formation and nuclear lamin B replication; all key factors involved in the cell growth cycle.⁷⁵ IGFII has mitogenic effects on mammary glands when bound to IGFI receptors. The increased M6P/IGFII receptor binds to the IGFII and internalizes the growth factor causing its degradation.⁷⁵ This prevents mammary tumour formation. POH also increased the mRNA levels for TGF-beta type I, II and II receptors in liver tumours.⁷⁶ TGF-beta is a protein that is secreted in its latent form by human breast cancer cells. Activation of TGF-beta by the M6P receptor at low concentrations leads to G₁ cell cycle arrest or apoptosis at higher concentrations.⁷⁶ Additionally, POH has been shown to cause a decrease of G₁ cyclin D1 levels in mammary cancer cells. This protein allows cancer cells to replicate by entering the S phase of the cell cycle.⁶⁸

Fas-receptor (Fas-R) and Fas-ligand (Fas-L) are a ligand receptor pair involved in cell death and therefore, faulty in most tumours. POH acts as a chemo-sensitizer by activating the Fas-receptor (Fas-R)/Fas-ligand (Fas-L) signalling cascade in glioma tumours causing the cells to undergo apoptosis.⁷⁷

1.3.4 Clinical uses of perillyl alcohol

POH was used in phase I clinical trials in gelatine capsules that were orally administered into patients. The capsules were found to cause severe gastrointestinal (GI) side effects with many patients complaining of nausea, fatigue and vomiting.⁷⁸ This led to the termination of the clinical trials. Later clinical trials in Brazil found that intra-nasal delivery eliminated the side effects resulting from oral administration of POH.⁷⁸ The intra-nasal trials also showed that long-term daily chemotherapy using the POH drug was effective with patients showed no sign of toxicity when receiving daily dosage of 55 mg of POH over a year. Furthermore, POH was able to increase PFS and decrease the size of the tumour.⁷⁸ Follow-up studies showed that 19% of patients remained in clinical remission, but with continuous dosing deemed necessary with patients who discontinued treatment were prone to relapse.⁷⁹

1.4 Temozolomide

TMZ (1) is an alkylating agent that has been used in the treatment of glioblastoma since 2005. It has structural similarity to decarbazine (16) which has been used to treat melanoma and Hodgkin's lymphoma since 1975 (Figure 1.13).²

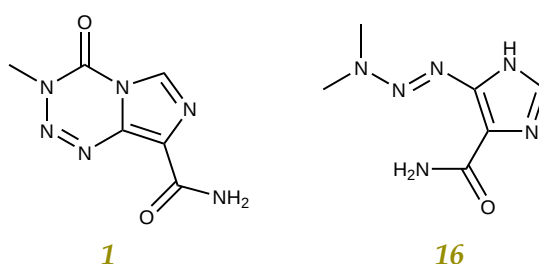


Figure 1.13 | Structures of temozolomide (1) and decarbazine (16)

TMZ causes methylation at either the O^6 or N^7 position of guanine or the N^3 position of adenine (Figure 1.14). These methylated bases then alter the base-pairing abilities of the bases interfering with normal Watson-Crick base-pairing and adversely affecting DNA replication. TMZ behaves like a prodrug, as it is stable at physiological pH but breaks down at pH >7.4 such as in the case of the acidic GBM tumour environment. It has a plasma half-life of 1.8 hr at pH 7.4.⁸⁰

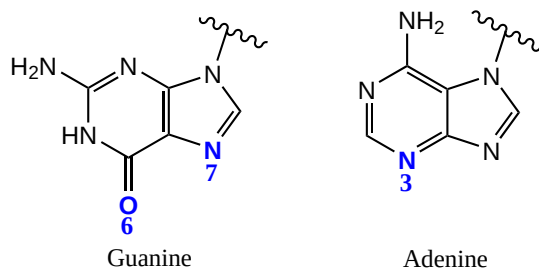
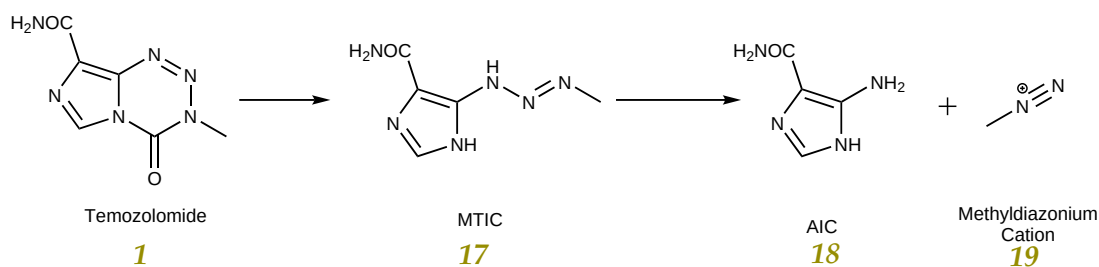


Figure 1.14 | Methylation positions of guanine and adenine

1.4.1 Mechanism of TMZ action

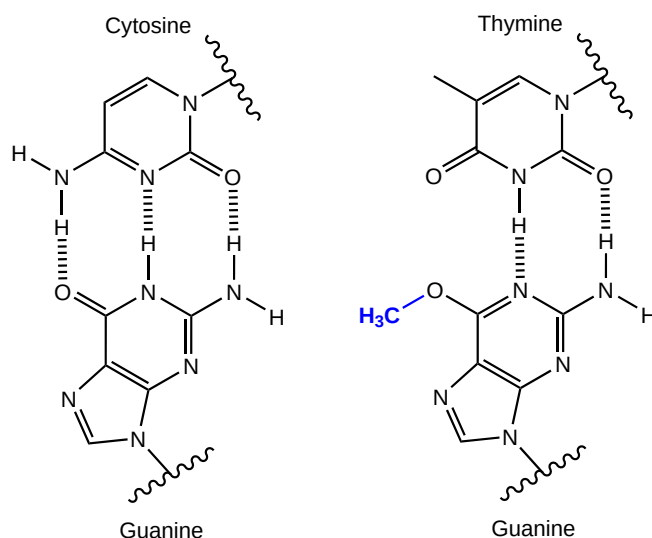
The main active component of TMZ is the methyldiazonium cation that is formed during the breakdown of TMZ. TMZ first undergoes spontaneous breakdown to monomethyl triazene 5-(3-methyltriazene-1-yl)-imidazole-4-carboxamide (MTIC) (17) above pH 7.4. The MTIC then reacts with water to form 5-aminoimidazole-4-carboxamide (AIC) (18) and the methyldiazonium cation (19) (Scheme 1.1).⁸¹



Scheme 1.1 | Breakdown of TMZ

The methyldiazonium cation is then involved in the methylation of DNA. It mainly targets the N^7 position of guanine (N^7 -MeG) (70%) but also targets the N^3 of adenine (N^3 -MeA) (9%) and the O^6 of guanine (O^6 -MeG) (6%).

Out of these three methylations, the O^6 -MeG mainly causes the cell death.⁸² Unrepaired O^6 -MeG causes the guanine to mis-pair with thymine. The thymine mismatch can be repaired by the DNA mismatch repair system (MMR) but failed MMR leads to apoptosis (Scheme 1.2).⁸²

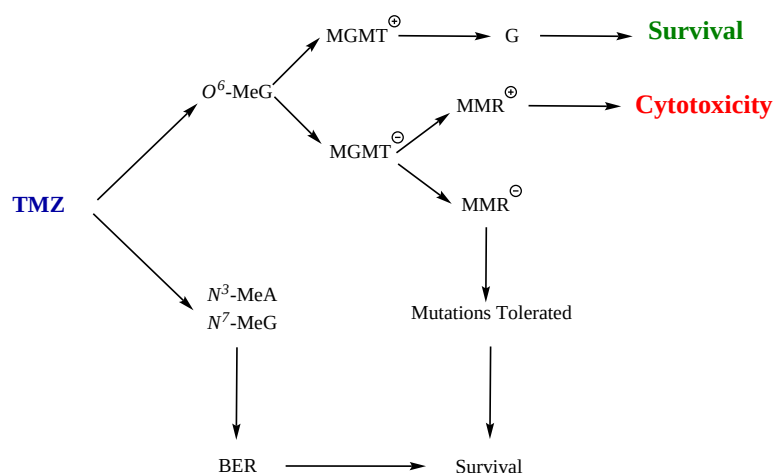


Scheme 1.2 | Base pairing of guanine compared to O⁶-methylguanine.

The N⁷-MeG does not cause cytotoxic effects, but the N³-MeA is cytotoxic if not repaired.⁸³

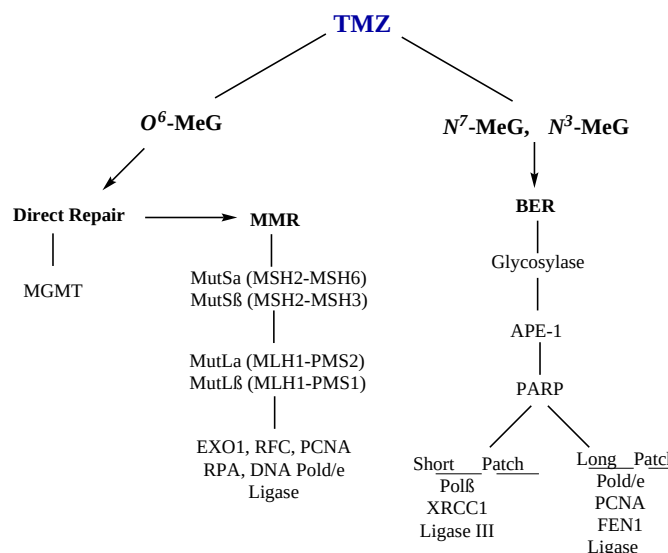
1.4.2 Resistance to TMZ

Over time different pathways have emerged showing resistance to TMZ. The main DNA repair pathways are due to either the O⁶-methylguanine-DNA methyltransferase (MGMT) system, the MMR system, or the base excision repair (BER) system.⁸⁴



Scheme 1.3 | DNA repair mechanism of mutations caused by TMZ.⁸⁴

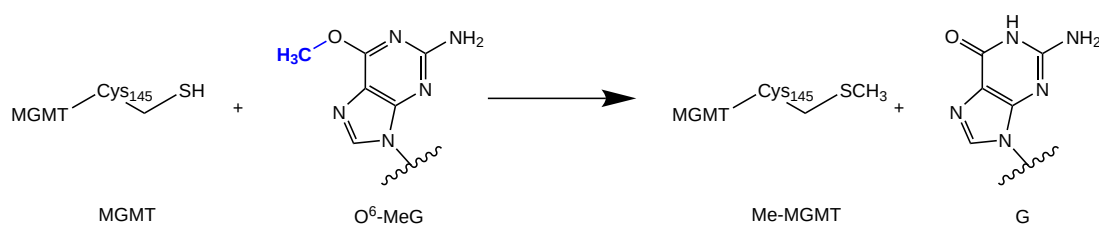
All three DNA repair mechanisms are involved in maintaining a healthy genome in cells. Proteins involved in these pathways are found to have mutations and gene defects in many cancers.



Scheme 1.4 | Key proteins involved in TMZ DNA repair mechanisms.⁸⁴ **Direct Repair:** MGMT repairs the O^6 -MeG. **MMR:** MutS α or MutS β recognise the repair site, followed by MutL α and MutL β being recruited. These proteins are then followed by proteins such as EXO1, RPA, DNA pol and ligase to repair the mismatch. **BER:** The repair begins with glycosylase removing the damaged base, followed by APE-1 creating a DNA nick. A short patch repair is for a single nucleotide gap or a long patch involves a nucleotide gap of 2-10 nucleotides.

1.4.2.1 Direct repair

Repair of the O^6 -MeG is mediated by O^6 -methylguanine-DNA methyltransferase. MGMT is a small protein involved in directly repairing the O^6 -MeG.⁸⁵ It does this by using an active site Cys145 to covalently bind to the O^6 -MeG.⁸⁶ Binding of MGMT to the mis-paired site causes the C to flip out of the double helix into the active site of MGMT thus allowing Cys145 to bind covalently to the O^6 -alkyl group and remove it from the DNA (Scheme 1.5). Binding of the alkyl group results in the inactivation of MGMT (suicide enzyme) leading to its degradation via the ubiquitin proteasome pathway.⁸⁷



Scheme 1.5 | Demethylation of Guanine via MGMT

As a MGMT is a suicide protein requiring one molecule of MGMT to remove one O⁶-MeG, excess DNA adducts can lead to depletion of the intracellular MGMT. As such, higher concentration of TMZ was still shown to have an effect even in GBM with high intracellular levels of MGMT. The intracellular concentration of MGMT can vary quite significantly in different tumours. High levels were shown to be present in breast and lung cancers while the lowest levels were found in gliomas and malignant melanomas.⁸⁸ A loss of the MGMT gene in chromosome 10 was observed in 60-80% of gliomas with phosphorylations and mutations in MGMT were also shown to be present.⁸⁹ Due to low levels of MGMT in gliomas, TMZ is highly effective in the treatment of GBM and clinical trials are in place for TMZ treatment of metastatic malignant melanomas.⁹⁰

1.4.2.2 DNA mismatch repair (MMR) system

DNA mismatch repair (MMR) is the method in which the cells recognize and repair mis-paired bases, insertions, deletions during DNA synthesis. Unlike the MGMT system, MMR is a multiple step process involving several proteins in the repair process.

The first step is the recognition of the mismatch by MutS α or MutS β protein. MutS α is involved in repairing bases mismatches and insertions/deletions while the MutS β is involved in repairing loops. These are heterodimeric proteins (MSH2-MSH6 for MutS α , MSH2-MSH3 for MutS β). Following recognition of the site to be repaired, MutL α (comprising of MLH1 and PMS2) then is recruited to this site through its interaction with MutS α . MutL α plays a key role in 3' nicked directed MMR as it further recruits the proliferating cellular nuclear antigen (PCNA)/ replication factor C (RFC) complex which in turn coordinates the recruitment of the exonuclease 1 (EXO1) which is involved in the 3' nick excision. The single strand DNA (ssDNA) binding replication protein A (RPA) is also involved in various stages during MMR, including binding

before MutS α and MutL α thus enhancing mismatch excision, protecting the ssDNA gap after excision and facilitating resynthesis with the help of DNA polymerase δ .⁹¹

As MMR only repairs the newly formed strand, this leaves the *O*⁶-MeG in the parental strand causing further mismatches during multiple rounds of replication and multiple cycles of MMR repair finally lead to cell cycle arrest and apoptosis.⁸⁴

Loss of MMR in tumour cells ultimately result in resistance to TMZ. Mutations in MSH2, MLH1, PMS2, MSH6 have been detected in tumours, with MSH6 mutations identified in recurrent GBM.⁹² MMR deficient cancer cells cannot recognize and repair the *O*⁶-MeG thymine mismatch and continue to repeat the cell cycle allowing the cancer cells to avoid apoptosis and keep replicating albeit with significant mutations.⁹³

1.4.2.3 Base Excision Repair (BER)

BER is the major pathway for removal of damaged nucleotides. It is also the pathway in which the *N*⁷ and *N*³ methylations are removed post TMZ activity.⁸⁴

The first step in BER involves a lesion specific DNA glycosylase that removes the damaged base by cleaving the *N*-glycosidic bond via hydrolysis, leaving an abasic site (AP-site). This is then followed by an AP endonuclease (APE-1) that cleaves the phosphodiester backbone of the DNA, creating a DNA nick with a 5' deoxyribose phosphate (dRp) end and a 3' hydroxyl end. Following the DNA break, the nucleotide at the 5' dRp end is then removed leaving a nucleotide gap which can then be repaired in one of two ways. A short patch repair for a single nucleotide gap as in the case of TMZ methylation or a long patch involving a nucleotide gap of 2-10 nucleotides.⁹⁴

In the short patch repair, the gap is filled using DNA polymerase β and then the nick sealed using DNA ligase III and X-ray repair cross-complementing 1 (XRCC1) complex. In the long patch repair, the DNA strand forms a flap that is cleaved by the flap endonuclease-1 (FEN-1) and sealed using DNA ligase I.⁹⁵

1.5 Aims and Objectives

The aims of this research can be summarized into six stages.

1. Stage one is the cloning of CITx- constructs into the expression vectors, transformation into *E. coli* expression strains cells, large scale expression of these cells and purification of the CITx protein constructs.
2. Stage two involves testing the activity of the purified CITx protein constructs *in vivo* in GBM using cell lines as the U87 immortalised cells. This can be done via invasion assays and fluorescence microscopy.
3. Stage three is the synthesis of cytotoxic ligands, such as POH and TMZ, to create linkers which are both fixed or environmentally cleavable.
4. Stage four is the conjugation of these newly synthesised ligands to amine and carboxylic acids groups of CITx, mainly via the EDC/NHS coupling method.
5. Stage five is the *in vitro* activity analysis of the CITx-POH/TMZ conjugates via cell cytotoxic assays such as the Presto Blue viability assay.
6. The sixth and final stage will be the *in vivo* analyses of the effects of conjugated CITx-POH/TMZ via mouse models with induced gliomas.

2

Methods

2.1 Instruments

Unless otherwise stated, chemical reagents were obtained from commercial sources and used without further purification.

The oligonucleotide primers were chemically synthesised by Sigma Aldrich

All centrifugation was carried out using either a Beckman Avanti[®] J-25I High Speed Centrifuge with either a JA-20 rotor (for samples < 50 mL, max speed 48,400 × g) or a JLA-10.500 rotor (for samples < 500 ML, max speed 18600 × g) or a Beckman Coulter Avanti[®] J-26XP with either a JA-25.50 rotor (for samples < 50 mL, max speed 75,600 × g) or a JA-10 Rotor (for samples < 500 ML, max speed 17,700 × g). The MSE Soniprep 150 plus ultrasonic disintegrator was used for *E coli* sonicating cell cultures for protein purification.

All FPLC purifications were carried out with an ÄKTA prime equipped with a UV detector at 280 nm and an automated fraction collector. Analytical FPLC was carried out using an ÄKTA purifier.

TLC analysis of reaction mixtures was carried out using Merck Kieselgel 60 F254 plates; visualization was carried out by irradiation with a UV lamp (254 nm) and staining with potassium permanganate. Compounds were purified via flash column chromatography on Sigma-Aldrich silica gel technical grade, pore size 60 Å, 230-400 mesh particle size, 40-63 nm ¹H and ¹³C NMR spectra were recorded at ambient temperature with Bruker AV (III) 400 HD spectrometer and were referenced to residual ¹H and ¹³C signals of the

deuterated solvents respectively (δ H 7.26, δ C 77.00 for chloroform).

High resolution mass spectra (HRMS) were recorded on a Bruker microTOF with ESI source. Values of mass to charge ratio (m/z) are given to four decimal places. Protein mass spectra were recorded on a Thermo LTQ FT-Ultra. The HPLC used in combination with the LTQ FT ultra was a dionex Ultimate 3000 equipped with a pepmap C18 column. Peptide mass fingerprinting was analysed on Water[®] SYNAPT G2-Si High Definition Mass Spectrometry.

2.2 General Methods

2.2.1 Plasmid DNA miniprep method

Plasmid DNA miniprep is the method in which plasmid DNA is extracted from *E. coli* cells. This was performed using the Monarch[®] Plasmid DNA Miniprep Kit. The first step involves growing an overnight culture (5 mL) in the presence of an antibiotic. The culture was centrifuged, and the pellet was redissolved in the plasmid re-suspension buffer (200 μ L) followed by the addition of Monarch[®] lysis buffer (200 μ L). The tubes were gently inverted until the solution went from cloudy to clear and incubated at room temperature for 1 min. This was then followed by the addition of the Monarch[®] neutralisation buffer (400 μ L) where precipitation occurred. After further incubating for 2 min, the sample was centrifuged at 16,000 $\times$ g for 10 min. The supernatant was then transferred onto a spin column and centrifuged for another minute at 16,000 $\times$ g. The flow-through was discarded and the column washed with Monarch[®] wash buffer 1 (200 μ L) which removed any RNA, protein or endotoxins bound onto the column. After another minute of centrifugation, the column was washed with the Monarch[®] wash buffer 2 (400 μ L) and the spin column was transferred into a clean tube. Monarch[®] DNA elution buffer (50 μ L) was added to the column and left to incubate for a minute and then centrifuged to elute the plasmid DNA into the tube. After determining the concentration via NanoDrop (A_{280}), the plasmid DNA was stored at -20 °C until further use.

2.2.2 Gel electrophoresis

2.2.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis is the technique used for the separation of DNA for analysis and purification. This technique was used for PCR product analyses, PCR gene/vector extraction, restriction digestion extraction and transformation analyses. A 1.2% w/v agarose gel (with ethidium bromide, 0.5 µg/mL) was used for DNA experiments with TAE running buffer (40 mM Tris, 10 mM EDTA and 19 mM acetic acid). All samples were analysed via electrophoresis using the New England Biolabs® Gel loading dye, purple (6x) to load the sample on the gel and the New England Biolabs® Quick-Load® Purple 2-Log DNA Ladder as molecular weight markers. Gels were run at 100 V for 30 min (gel dimensions: 7 x 10 x 0.75 cm) unless stated otherwise. The DNA in the gel was visualised under ultra-violet light using a Geneflow GBOX and gel images were obtained using the GeneSnap software.

2.2.2.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used for the analysis of proteins. SDS-PAGE comprises of three main parts: the stacking gel, the resolving gel and finally the SDS-running buffer. The stacking gel contained 4% w/v acrylamide (with 125 mM Tris-HCl pH 6.8 and 0.1% w/v SDS) while the resolving gel (375 mM Tris-HCl pH 8.8 and 0.1% w/v SDS) either had 12%, 15% or 20% acrylamide. Electrophoresis was run at a constant voltage of 180 V for 60 min (gel dimensions: 8.6 x 6.7 x 0.1 cm) in the SDS-PAGE running buffer (25 mM Tris, 192 mM Glycine, 3.5 mM SDS). SDS loading dye (50 mM Tris-HCl pH 6.8, 0.05% w/v Bromophenol blue, 12.5 mM EDTA, 100 mM DTT, 2% w/v SDS and 10% w/v glycerol) was added to all samples prior to heating them to 95 °C for 3 min and then loading them onto the gel. PageRuler™ Unstained Protein Ladder was used as a MW marker. All SDS-PAGE gels were stained with GelCode™ Blue Safe protein stain, imaged and analysed using a Geneflow GBOX and GeneSnap software.

2.2.3 Cloning methods

2.2.3.1 Polymerase chain reaction

PCR is a technique used for the exponential amplification of DNA and consists of three stages; denaturation of the double stranded DNA, annealing of the primers and elongation of the primers to form the new DNA strands. The reagents needed to carry out the PCR reactions are given below.

Table 2.1 | PCR Reagents

Reagent	Concentration
Template DNA	10-100 ng/μL
Q5® High-Fidelity DNA Polymerase	1 μL per 50 μL
dNTPs	200 μM
Forward Primer	0.2μM
Reverse Primer	0.2 μM
5x Q5 Reaction Buffer	1x

This is then followed by the running PCR cycles which involves heating and cooling the sample so that the DNA can be amplified.

Table 2.2 | PCR Cycling Conditions

Stage	Cycles	Temp	Time
Denaturation	25	98 °C	10 sec
Annealing	25	50-72 °C	10-30 sec
Elongation	25	72 °C	20-30 sec/kb
Final Elongation	1	72 °C	2 min
Final Hold	1	4 °C	30 sec

Initial Denaturation is required for polymerases that require heat activation such as Q5 Hot start.

2.2.3.2 DNA gel extraction

DNA gel extraction is the method of purifying DNA that has been run through an agarose gel. This was achieved using the Thermo Scientific GeneJET Gel Extraction Kit. First, the required band was excised from the gel and excess agarose was trimmed off. The gel slice was then dissolved in binding buffer at a 1:1 ratio of agarose to buffer (100 mg agarose = 100 μL buffer) and incubated at 50 °C until completely dissolved before being transferred onto a GeneJET purification column. The column was then centrifuged at 16,000 x g for 1 min and the flow-through discarded. The purification

column was then washed with the addition of wash buffer followed by centrifugation. After an additional wash centrifugation step, the column was transferred to a clean tube and was incubated with the elution buffer for 2 min. A final centrifugation eluted the purified DNA from the column which was then be stored at -20 °C, until further use.

2.2.3.3 Restriction enzyme digestion

Restriction digestion is the process in which restriction enzymes cleave the DNA at specific sequences. Each enzyme recognizes a different specific sequence. A sample is prepared as shown in Table 2.3 below and then incubated at 37 °C for 30 min. The DNA fragment of interest can be purified by running agarose gel electrophoresis and extracted using the DNA gel extraction kit (Monarch® DNA Gel Extraction Kit).

Table 2.3 | Restriction Double Digestion

Reagent	Concentration
Template DNA	1 µg
10X CutSmart Buffer	1x
Restriction Enzyme 1	10 Units
Restriction Enzyme 2	10 Units

2.2.3.4 T4 DNA ligation

DNA ligation is the process by which two DNA strands can be ligated together. This is done using T4 DNA ligase. For cloning work, the vector and insert DNA are digested with the same restriction enzymes, purified and then a ligation reaction is set up as shown below in Table 2.4.1. The sample is then incubated overnight at 16 °C and the ligated DNA product is confirmed by agarose gel electrophoresis. The ligated DNA is then transformed into competent *E. coli* cells.

Table 2.4 | T4 DNA Ligation

Reagent	Concentration
Vector DNA	50 ng (1x)
Insert DNA	50 ng (1-10x excess)
10X T4 DNA Ligase Buffer	1x
T4 DNA Ligase (20000 units)	1 µL/20 µL

2.2.3.5 Q5 site-directed mutagenesis

Q5 Site-Directed Mutagenesis is the method of introducing insertions, deletions or substitutions into a plasmid DNA. This was done using the New England Biolabs^{Q5®} Site-Directed Mutagenesis kit. All primers necessary for the mutagenesis were designed using NEB's on-line primer design software, NEBaseChanger[™].

A Polymerase Chain Reaction (PCR) reaction was prepared by mixing Q5 Hot-start High-Fidelity 2x Master Mix, Primers (Forward and Reverse Primer, 0.5 µM) and template DNA (1-25 ng/µL). The PCR reaction was carried out using the following cycling conditions:

Table 2.5 | PCR Cycling Conditions

Stage	Cycles	Temp	Time
Initial Denaturation	1	98 °C	30 sec
Denaturation	25	98 °C	10 sec
Annealing	25	50-72 °C	10-30 sec
Elongation	25	72 °C	20-30 sec/kb
Final Elongation	1	72 °C	2 min
Final Hold	1	4 °C	30 sec

Initial Denaturation is required for polymerases that require heat activation such as Q5 Hot start.

After the PCR reaction the mixture was prepared for the Kinase, Ligase and DpnI (KLD) treatment in which the PCR mixture, KLD buffer and the KLD enzyme mix were added together and incubated for 10 min at room temperature and then 1 hr at 4 °C. Finally, the whole reaction mixture was added to NEB 5-alpha competent *E. coli* cells, incubated on ice for another 30 min, heat-shocked for 30 s at 42 °C and then placed back on ice for another 5 min. The *E. coli* cells were then suspended into super optimal broth with catabolite repression (SOC) medium, left to incubate at 37 °C for 1 hr, plated on an antibiotic selection plate and incubated overnight at 37 °C. Colonies were then selected and sent for DNA sequencing to confirm mutagenesis.

2.2.4 Competent cell preparation and transformation

2.2.4.1 Chemically competent cells

E. coli cells were grown in an overnight culture (5 mL) at 37 °C, diluted (1 in 1,000) into fresh LB (5 mL) and grown until the OD reached 0.3. The cells were then centrifuged (3,000 $\times$ g for 10 min), suspended and incubated for 1 hr in ice-cold 50 mM CaCl₂. The cells were then centrifuged again and suspended in 50 mM CaCl₂ (800 μ L) before being aliquoted into 50 μ L aliquots which were then stored at -80 °C until further use.

Aliquots were thawed for transformation, plasmid DNA (2 μ L) was added to the cells and incubated at 4 °C for 30 min. The sample was then heat shocked in 42 °C for 45 sec before placed on ice for a further 5 min. LB was then added, and the cells were grown at 37 °C for 1 hr. The transformed culture was plated on an antibiotic selective agar plate (100 μ L) and colonies were left to grow overnight at 37 °C.

2.2.4.2 Electrocompetent cells

E. coli cells were grown in an overnight culture (10 mL) at 37 °C. The cells were then diluted (1 in 1,000) into fresh LB (5 mL) and left to grow until the culture reached an OD₅₉₅ of 0.7. The culture was then incubated at 4 °C for 30 min, followed by centrifugation at 3,500 $\times$ g for 20 min at 4 °C. The cell pellet was then washed four times with ice cold sterile water (2 $\times$ 200 mL 1 $\times$ 100 mL and 1 $\times$ 20 mL) with centrifugation between each wash (6,000 $\times$ g for 15 min at 4 °C). Finally, the cell pellet was suspended in ice cold LB + 50% v/v glycerol (4 mL for storage strains and 6 mL for expression strains). The cells were then aliquoted, snapped frozen in liquid N₂ and stored at -80 °C until further use.

When required, the cells were thawed for transformation, where plasmid DNA (2 μ L) was added into the competent cell aliquot and incubated at 4 °C for 1 min. It was then transferred into an electro-cuvette and subjected to electroporation followed by the immediate addition of LB (1 mL). The transformed cells were left to grow at 37 °C for 1 hr and then plated on an antibiotic selective agar plate (100 μ L) which was incubated overnight at 37 °C.

2.2.5 Glycerol stocks

Plasmids were constructed and transformed into storage strains (Look Materials section in Appendix) before plated onto Agar plates and incubated overnight at 37 °C. The following day a single colony of the *E. coli* cells (5 mL) was grown in LB overnight at 37 °C. The cells were then pelleted and resuspended into storage solution (200 µL, LB + 50% v/v glycerol) before snapped frozen in liquid N₂ and stored at -80 °C, until further use. The same procedure was also followed for expression strains containing plasmids but the maximum lifetime storage of such glycerol stocks at -80 °C was 6 months.

2.2.6 Fast protein liquid chromatography (FPLC)

Fast protein liquid chromatography (FPLC) is a technique used for the purification of proteins. FPLC was carried out using a GE Healthcare AKTA purification system that has a UV module (280 nm) for detection of proteins that are purified with appropriate purification columns. The functions of each of the columns are highlighted below.

2.2.6.1 HisTrap column

A GE Healthcare HisTrap 5 mL column is used for proteins that have 6 histidine residues (6xHis) tagged either at the N- or C-termini of their protein sequences. This column works by utilizing specific binding of the 6xHis tag to Ni²⁺ which are present in the column. An imidazole concentration gradient is then applied to the column to remove the bound proteins as the imidazole out-competes the histidines for binding to Ni²⁺ ions.

In order to load the HisTrap column with Ni²⁺, it was first washed with water followed by 100 mM EDTA. This strips any residual metal attached to the column and any contaminants from previous purifications. After a second wash with water, the column was loaded with 100 mM NiSO₄, washed again with water and then equilibrated with the appropriate protein binding buffer. After the protein purification was completed the column was washed with water and stored long-term in 20% v/v ethanol.

2.2.6.2 Gel filtration

The GE Healthcare HiLoad™ 16/600 Superdex 75 pg column was used for gel filtration in this work. The matrix of this column contains beads with holes of varying sizes that delay smaller proteins as they pass through the column. Therefore, this column is useful for separating proteins based upon size and shape. The column is prepared by first washing it in water followed by equilibration in the appropriate protein buffer. Following purification, the column was washed with water and stored in 20% v/v ethanol.

2.2.6.3 Buffer desalting

Protein solutions were desalted using a GE Healthcare HiTrap Desalting column. This process is similar to gel filtration, in the sense that the column matrix has beads of varying sizes that exchange the protein into a new buffer. The column was prepared by washing in water followed by equilibration in the appropriate buffer. Following buffer exchange and desalting, the column was washed with water and stored in 20% v/v ethanol.

2.2.7 Electron spray ionisation ZipTip® protocol for denatured protein mass spectrometry

All samples analysed via denatured protein mass spectrometry need to be buffer exchanged before analysis. This was done using ZipTip®Pipette Tips with C₄ resin.

First, to the protein sample (20 µM, 10 µL), trifluoroacetic acid (TFA) was added to a final concentration of 0.1%. Then the protein sample was loaded onto the ZipTip which was pre-equilibrated with wash buffer (5% Methanol v/v and 0.1% v/v TFA). This was done by aspirating the solution onto the ZipTip. The ZipTip was then washed for at least 25 aspirations with the wash buffer before the protein sample was eluted (10 aspirations) into a tube containing the elution buffer (80% v/v acetonitrile, 0.1% v/v formic acid).

2.2.8 Cell culture: Splitting adherent glioma cells

A T75 flask containing glioma cells in Dulbecco's Modified Eagle's medium (DMEM) was first analysed under a microscope to check for 70% confluency of the cells. The DMEM media was then removed from the flask and the flask was washed with 4 mL of Hanks' Balanced Salt Solution (HBSS, pre-warmed to 37 °C) followed by the addition of 3 mL trypsin solution (0.05 % w/v porcine trypsin and 0.02% w/v EDTA dissolved in HBSS, pre-warmed to 37 °C) and incubation at 37 °C for 5 min. The flasks were then checked under a microscope for detachment of cells from the surface of the flask, followed by addition of DMEM to inhibit the trypsin. The cells were then collected, centrifuged (200 x g, 5 min) and suspended in DMEM (5 mL). 1 mL of the suspended cells were then added to 11 mL of DMEM and all the cells were added into a new pre-labelled (date, passage number, cell line and dilution) T75 flask and incubated at 37 °C for 3 days.

2.2.9 Cell counting

Centrifuged cells after trypsinisation were suspended in 5 mL of DMEM media. The cells (50 µL) were then diluted with trypan blue (50 µL). Using a Haemocytometer, cells were added under the coverslip and examined under a microscope. Viable cells appeared white while non-viable cells were blue due to uptake of trypan blue. The number of cells in the four outer grids of the two quadrants were counted and the average number of cells per grid was calculated. This number was then multiplied by 2 (trypan dilution factor) and 10^4 (haemocytometer value) to obtain the final concentration of cells/mL.

2.2.10 Long-term storage of glioma cells

Centrifuged glioma cells after trypsinisation were suspended in 1 mL of DMEM + DMSO medium (9:1). The cells were transferred into cryovials before immediately put in a Mr. Frosty™ Freezing Container and frozen overnight at -80 °C. The Mr. Frosty achieves a rate of cooling of -1 °C per min which is optimal for cell preservation. The vial is then transferred into liquid nitrogen for long-term storage.

2.3 Chlorotoxin purification and analysis

2.3.1 Cloning of CITx constructs

The CITx constructs used in the research was designed by ATUM (formally known as DNA2.0) and was based off the CITx sequence found in the deathstalker scorpion. Sequences for the constructs can be found in **Chapter 3 Table 3.1**.

The coding region for the required construct was amplified by PCR from the template plasmid (extracted using the Monarch® Plasmid DNA Miniprep Kit) using an overnight growth (Ampicillin 100 µg/mL). The amplified DNA fragment was digested by the required restriction enzymes and subsequently ligated into a vector plasmid, pre-treated with the same restriction enzymes. (**Table 2.6**) Sub-cloning for all gene constructs was carried out in the same way. The ligated plasmid was transformed into XL-1 Blue or Neb5ff competent *E. coli* cells. The sequence of the construct was confirmed by DNA sequencing.

Table 2.6 | Template and vector plasmids for different protein constructs during sub-cloning.

Target Construct	Template	Vector	Restriction sites
CITx-TEV-HT-cometGFP	pD441-CC	pET22b	<i>NdeI</i> , <i>XhoI</i>
CITx-HT	pJ411	pET22b	<i>NdeI</i> , <i>XhoI</i>
HT-cometGFP	pD441-CC	pET22b	<i>NdeI</i> , <i>XhoI</i>

All CITx constructs were amplified from a template plasmid and ligated into the vector plasmid. The pD441-CC/CITx-TEV-HT-cometGFP template plasmid was used for the amplification of HT-cometGFP which also introduced the *NdeI* restriction site N-terminal to the HT-cometGFP

Table 2.7 | Primers used for template amplification.

Primer	Sequence	Tm
CITx-TEV-HT-cometGFP Forward	CGCATATGTGCAACCCGTGCTT	56.7 °C
CITx-TEV-HT-cometGFP Reverse	GAATGGCACCAACTGAGCTCCT	56.7 °C
CITx-CT-HT Forward	CGCATATGTGCAACCCGTGCTT	56.7 °C
CITx-CT-HT Reverse	GTGGTGGTGATTGAGCTCGCAT	56.7 °C
HT-cometGFP Forward	CGCATATGCATCATCATCACC- ACCACGGTATGA	71 °C
HT-cometGFP Reverse	GACCTTTGAATGGCACCAACT- GAGCTCCT	71 °C

2.3.2 Transformation and expression test analysis of CITx Constructs

The sub-cloned constructs were transformed into *E. coli* Rosetta™ (DE3) or Origami™ 2 (DE3) strains. The transformed cells were cultured in 2xYT liquid broth (2xYT LB) medium from Sigma Aldrich, supplemented with 50 µg/mL carbenicillin, to an OD₅₉₅ of ~0.7 at 37 °C, with vigorous shaking (250 rpm). Protein expression was induced for either 3-4 hr at 30 °C or overnight induction at 16 °C with 500 µM IPTG and finally cells were harvested by centrifugation (3,000 × g, 30 min).

2.3.3 Solubility test for CITx constructs

A sample of uninduced and induced Rosetta™ (DE3) or Origami™ 2 (DE3) cells containing the CITx protein constructs were suspended in sonication buffer (50 mM Tris-HCl pH 7.5 and 0.5 M NaCl) and sonicated for 30 s using a MSE Soniprep 150. The soluble and insoluble fractions from the sonicated cultures were then clarified by centrifugation and the soluble fraction was analysed by SDS-PAGE using 15% or 20% w/v acrylamide gels, depending on the size of the proteins analysed.

2.3.4 Purification of CITx-TEV-HT-cometGFP

Rosetta™ (DE3) *E. coli* carrying the CITx-TEV-HT-cometGFP construct were suspended in sonication buffer (50 mM Tris-HCl, pH 7.5 and 0.5 M NaCl) and sonicated using the MSE Soniprep 150 (8 × 30 sec). Following the sonication, 1-2 drops of Benzonase® Nuclease (degrades DNA and RNA contaminants) was added to the sample which was then centrifuged at 35,000 × g for 30 min. The soluble fraction was separated and 40% w/v ammonium sulfate was added to precipitate the proteins. Precipitated proteins were pelleted by centrifugation (35,000 × g for 30 min) and the pellet was suspended in HisTrap buffer (50 mM Tris-HCl pH 7.5 and 0.5 M NaCl).

The protein sample was loaded onto a GE Healthcare Life Sciences 5mL HisTrap™ HP column that was pre-equilibrated with the wash buffer (50 mM Tris- HCl pH 8.0, 0.5 M NaCl). The CITx-TEV-HT-cometGFP protein was then eluted from the column by a step-wise increment of increasing imidazole concentration in the wash buffer. The eluted CITx-TEV-HT-cometGFP protein fractions were pooled, and the protein was concentrated by ammonium sulphate precipitation into 5-8 mL of gel filtration buffer

(50 mM Tris-HCl pH 7.5 and 200 mM NaCl) before loaded onto a GE Healthcare Life Sciences HiLoad™ 16/600 Superdex 75 pg column. Fractions containing the CITx-TEV-HT-cometGFP protein were pooled, the purity of the protein was verified by SDS-PAGE (180 V for 60 min, gel dimensions: 8.6 x 6.7 x 0.1 cm; with a 15% w/v acrylamide gel, aliquoted, snapped frozen in liquid N₂ and stored at -80 °C with 10% v/v glycerol. Protein quantification was carried out spectrophotometrically using a Thermo Scientific NanoDrop 1000 and the extinction coefficient of 33640 M⁻¹ cm⁻¹ (calculated using ExPASy ProtParam Tool).

2.3.5 Purification of HT-cometGFP

Rosetta™ (DE3) *E. coli* carrying the HT-cometGFP construct were suspended in sonication buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl) and sonicated using the MSE Soniprep 150 (8 x 30 sec). Following sonication, 1-2 drops of Benzonase® Nuclease was added and the soluble and insoluble fractions were clarified by centrifugation at 35,000 x g for 30 min. Proteins in the soluble fraction were precipitated by the addition of 40% (w/v) ammonium sulfate and pelleted by centrifugation (35,000 x g for 30 min). The protein pellet was re-suspended in HisTrap buffer (50 mM Tris-HCl pH 7.5, 0.5 M NaCl) and loaded onto a GE Healthcare Life Sciences 5mL HisTrap™ HP column that was pre-equilibrated with the washing buffer (50 mM Tris-HCl pH 8.0, 0.5 M NaCl). The HT-cometGFP protein was then eluted from the column by a step-wise increment of increasing imidazole concentration in the wash buffer. The eluted HT-cometGFP protein fractions were pooled, concentrated by ammonium sulphate precipitation, re-suspended in gel filtration buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl) and then loaded onto a GE Healthcare Life Sciences HiLoad™ 16/600 Superdex 75 pg column. Fractions containing the HT-cometGFP protein were pooled, aliquoted, snapped frozen in liquid N₂ and stored at -80 °C with 10% v/v glycerol. The purity of the protein was assessed by SDS-PAGE (180 V for 60 min; gel dimensions: 8.6 x 6.7 x 0.1 cm) through a 15% w/v acrylamide gel. Protein quantification was carried out spectrophotometrically using a Thermo Scientific NanoDrop 1000 and the extinction coefficient of 30160 M⁻¹ cm⁻¹ (calculated using ExPASy ProtParam Tool).

2.3.6 Removal of contaminants from CITx-TEV-HT-cometGFP

2.3.6.1 Immobilised metal affinity chromatography using 3 M urea

The column was first washed with the HisTrap loading buffer (50 mM Tris-HCl pH 7.5 and 0.5 M NaCl) and then with an increasing gradient from 0% to 100% of HisTrap urea buffer (3 M Urea, 50 mM Tris-HCl pH 7.5 and 0.5 M NaCl). Following the urea wash, the CITx-TEV-HT-cometGFP protein was eluted by stepwise increments of imidazole concentration (up to 1 M) in the HisTrap wash buffer. Fractions containing CITx-TEV-HT-cometGFP were analysed with SDS-PAGE through a 15% w/v acrylamide gel (gel dimensions: 8.6 x 6.7 x 0.1 cm) run at 180 V for 60 min.

2.3.6.2 Immobilised metal affinity purification using 3 M guanidinium hydrochloride

Following the two-step purification of CITx-TEV-HT-cometGFP, a sample of the impure CITx-TEV-HT-cometGFP was loaded on a GE Healthcare Life Sciences 5 mL HisTrap™ HP column to repeat the IMAC purification using guanidinium hydrochloride (GuHCl) and remove the remaining impurities.

The column was first washed with the HisTrap loading buffer (50 mM Tris-HCl pH 7.5 and 0.5 M NaCl) and then with an increasing gradient from 0% to 100% of HisTrap GuHCl buffer (3 M GuHCl, 50 mM Tris-HCl pH 7.5 and 0.5 M NaCl). Following the GuHCl wash, the CITx-TEV-HT-cometGFP protein was eluted by running another gradient with imidazole (1 M, 0% to 100%) Fractions were analysed with SDS-PAGE through a 15% w/v acrylamide gel (gel dimensions: 8.6 x 6.7 x 0.1 cm) run at 180 V for 60 min.

2.3.6.3 DTT analysis

Varying concentrations of DTT (0/400 mM/467 mM/500 mM) was added into the samples containing the CITx-TEV-HT-cometGFP after the Superdex 75 gel filtration column and left at 4 °C for 1 hr. SDS-loading buffer was added to the solution and the sample was loaded on a 15% SDS-PAGE gel (gel dimensions: 8.6 x 6.7 x 0.1 cm) and run at 180 V for 60 min.

2.3.6.4 β -mercaptoethanol analysis

Varying concentrations of β -mercaptoethanol (0/76 mM/156 mM/313 mM/625 mM/1.25 M/2.5 M/5 M) was added to the CITx-TEV-HT-cometGFP protein after the Superdex 75 gel filtration column and left at 4 °C for 1 hr. Samples were then analysed with SDS-PAGE through a 15% w/v acrylamide gel run (gel dimensions: 8.6 x 6.7 x 0.1 cm) at 180 V for 60 min.

2.3.6.5 S-Sulfonation analysis

The CITx-TEV-HT-cometGFP protein was S-sulfonated by the addition of solid sodium sulfite (200 mM) and sodium tetrathionate (150 mM). This reaction was left to gently shake at 4 °C for 3 hr and the effect of sulfonation on the CITx-TEV-HT-cometGFP protein was assessed by SDS-PAGE using a 15% w/v acrylamide gel (gel dimensions: 8.6 x 6.7 x 0.1 cm) run at 180 V for 60 min.

2.3.6.6 Analytical gel filtration

The Superdex 75 gel filtration column showed a slight but distinct separation of remaining impurities from the CITx-TEV-HT-cometGFP protein. Therefore, analytical gel filtration was carried out using a GE Healthcare Life Sciences Superdex 200 10/300 GL column with the CITx-TEV-HT-cometGFP protein to remove remaining contaminants. Fractions were then analysed with SDS-PAGE through a 15% w/v acrylamide gel run (gel dimensions: 8.6 x 6.7 x 0.1 cm) at 180 V for 60 min.

2.3.7 Purification of CITx-HT

RosettaTM (DE3) /OrigamiTM 2 (DE3) *E. coli* CITx-HT cells were suspended in sonication buffer (50 mM Tris-HCl, pH 7.5 and 0.5M NaCl) and sonicated using the MSE Soniprep 150 (8 x 30 sec). Following the sonication, 1-2 drops of Benzonase[®] Nuclease was added to the sample before clarifying the soluble and insoluble fractions with centrifugation at 35,000 $\times g$ for 30 min. The soluble fraction containing the CITx-HT protein was loaded onto a GE Healthcare Life Sciences 5 mL HisTrapTM HP column that was pre-equilibrated with the washing buffer (50 mM Tris-HCl pH 8.0 and 0.5M NaCl). The CITx-HT was eluted by running a gradient with imidazole (1 M, 0% to 100%).

The fractions were analysed with SDS-PAGE through a 20% w/v acrylamide gel (gel dimensions: 8.6 x 6.7 x 0.1 cm) at 180 V for 60 min.

2.3.8 Presto blue assay of CITx-TEV-HT-cometGFP

Before analysing the activity of CITx-TEV-HT-cometGFP, the toxicity of the protein was assessed. U87 cells (Children's Brain Tumour Research Centre, University of Nottingham, United Kingdom) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (1% w/v Penicillin/Streptomycin Solution) and 10% v/v fetal bovine serum. The cells were detached via trypsin digestion, suspended again in DMEM (with 10% v/v FBS) and seeded onto a Corning™ Costar® 96-Well clear bottom cell culture plate (5×10^4 cells/well). The cells were cultured for 24 hr at 37 °C, prior to the addition of CITx-TEV-HT-cometGFP at the indicated concentrations with fresh DMEM medium. The cells were further cultivated for 2 hr at 37 °C. Following this, the medium in each well was removed, the wells were washed with HBSS and Presto blue was added into each well (1:10 dilution in HBSS). The cells were then incubated with presto blue for 1 hr at 37 °C. The solution from each well wash was transferred into a Corning™ 96-Well Solid Black plate and the absorbance at 570 nm was measured using a plate reader.

2.3.9 Activity analysis of CITx-TEV-HT-cometGFP

The activity of CITx-TEV-HT-cometGFP was analysed via fluorescence microscopy. U87 cells (Children's Brain Tumour Research Centre, University of Nottingham, United Kingdom) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (1% w/v Penicillin/Streptomycin Solution) and 10% v/v fetal bovine serum. The cells were detached via trypsin digestion, suspended again in DMEM (with 10% v/v FBS) and seeded into chamber slides. (5×10^4 cells/chamber, Thermo Scientific™ Nunc™ Lab-Tek™ II, 8 wells.) The cells were cultured for 24 hr at 37 °C, prior to CITx-TEV-HT-cometGFP added at the indicated concentrations with fresh DMEM medium. The cells were further cultured for 2, 6 and 24 hr at 37 °C and then fixed with 4% v/v paraformaldehyde for 20 min, following by washing with Phosphate Buffered Saline (PBS). The cells were then stained with 4',6-Diamidino-2-Phenylindole,

Dihydrochloride (DAPI) (VECTASHIELD® Antifade Mounting Medium) and cover slipped. The fluorescence was monitored using a fluorescence microscope under UV light using different wavelength filters [DAPI - Excitation (358 nm)/Emission (461 nm), cometGFP - Excitation (403 nm)/ Emission (511 nm)].

2.3.10 Cleavage of CITx from HT-cometGFP using the TEV protease

TEV protease was added to pure CITx-TEV-HT-cometGFP sample (Molar ratio 1:20, TEV:CITx) which and left to cleave the tag off the CITx at either 4 °C or at 37 °C for 4 hr. Samples were analysed with SDS-PAGE through a 15% w/v acrylamide gel (gel dimensions: 8.6 x 6.7 x 0.1 cm) run at 180 V for 60 min and with TOF-ESI+ mass spectrometry.

2.3.11 Purification of CITx

CITx-TEV-HT-cometGFP Rosetta™ (DE3) *E. coli* cells were suspended in sonication buffer (50 mM Tris-HCl, pH 7.5 and 0.5 M NaCl) and sonicated using the MSE Soniprep 150 (8 x 30 secs). Following sonication, 1-2 drops of Benzonase® Nuclease was added to the sample and the soluble, insoluble fractions were clarified by centrifugation at 3,000 $\times$ g for 30 min. The CITx-TEV-HT-cometGFP protein was loaded onto a GE Healthcare Life Sciences 5 mL HisTrap™ HP column that was pre-equilibrated with the wash buffer (50 mM Tris-HCl pH 7.5, 0.5 M NaCl and 20 mM imidazole). The CITx-TEV-HT-cometGFP protein was eluted from the column by gradient of increasing imidazole concentrations (1 M, 0 to 100%) in wash buffer. The eluted CITx-TEV-HT-cometGFP fractions were pooled and the protein was concentrated by ammonium sulphate precipitation (60% w/v). The protein was then dissolved in cleavage buffer (50 mM Tris-HCl pH 7.5 and 0.5 M NaCl) and TEV protein was added at a molar ratio of 1:20 TEV : CITx-TEV-HT-cometGFP and left to cleave at 4 °C overnight. Following TEV cleavage, the CITx/HT-cometGFP sample was loaded onto HisTrap™ HP column that was pre-equilibrated with the wash buffer. The flow-through was collected and the CITx protein concentrated by ammonium sulphate precipitation. The CITx protein was suspended into gel filtration buffer (200 mM ammonium acetate pH 7.5) before being loaded onto a GE Healthcare Life Sciences HiLoad™ 16/600 Superdex 75 pg column,

pre-equilibrated in the 200 mM ammonium acetate pH 7.5. Fractions containing the CITx protein were analysed with SDS-PAGE through a 20% w/v acrylamide gel (180 V for 60 min, gel dimensions: 8.6 x 6.7 x 0.1 cm) and the purest fractions were pooled together and desalted using a GE Healthcare Life Sciences HiTrap™ 5x 5 mL desalting column (200 mM ammonium acetate pH 7.5). The CITx protein was finally lyophilised, quantified by weighing, dissolved in 50 mM potassium phosphate pH 8.0 buffer at a concentration of 100 µM and stored at -80 °C. Protein concentration was measured spectrophotometrically using a Thermo Scientific NanoDrop 1000 and the extinction coefficient of 3480 M⁻¹ cm⁻¹ (calculated using ExPASy ProtParam Tool).

2.3.12 Activity analysis of CITx

The activity of CITx was analysed via the Transwell Invasion assay. Corning Matrigel Basement Membrane Matrix Growth Factor was thawed to 4 °C, diluted with serum-free DMEM to 2.5 mg/ml 50 µL of the dilute matrigel and was then added into ThinCert™ cell culture inserts. The inserts were then placed into a 24 well-plate and left to solidify for 1 hr at 37 °C. This created a two-chamber system which was separated by a cell-permeable membrane. U87 cells (Children's Brain Tumour Research Centre, University of Nottingham, United Kingdom) cultured in Dulbecco's modified Eagle's medium (DMEM) and containing antibiotics (1% w/v Penicillin/Streptomycin Solution) and 10% v/v fetal bovine serum were detached via trypsin digestion, washed with HBSS, re-suspended in DMEM (with 1% v/v FBS) and seeded into the top chamber of the ThinCert™ cell culture inserts. DMEM (with 10% v/v FBS) was added into the bottom chamber. CITx (10 µM) was added immediately to both chambers of the Transwell assay and the cells were further cultured for 24 hr at 37 °C. The insert was moved into a fresh well followed by removal of media from the top chamber of the insert. Cells were fixed on the bottom end of the ThinCert™ insert with ice-cold methanol for 1 hr and stained with 0.5% w/v crystal violet for 30 min. The inserts were finally washed, air-dried overnight and the cells that were adhered to the bottom of the insert were imaged using a microscope.

2.3.13 Conjugation of CITx to fluorescein

CITx was conjugated to 5,6-carboxyfluorescein succinimidyl ester, commonly known as NHS-Fluorescein (NHS-FLR) which can be used to visualise CITx binding to glioma cells under fluorescence. NHS-FLR (10 mM) dissolved in DMSO was added to CITx (100 μ M) dissolved in PBS. The solution was then left to react for 2 hr with gentle mixing. The excess NHS-FLR was then removed from the CITx-FLR conjugate via dialysis (1000 MWCO dialysis tubing) into PBS buffer. The dialysis buffer volume was 100x excess and 2 buffer changes (4 °C, one for 4 hrs, other overnight) needed to be done to remove excess NHS-FLR. Completion of the reaction was confirmed via mass spectrometry.

2.3.14 Fluorescence microscopy of CITx-FLR

U87 cells (Children's Brain Tumour Research Centre, University of Nottingham, United Kingdom) which were cultured in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (1% w/v Penicillin/Streptomycin Solution) and 10% v/v fetal bovine serum were detached via trypsin digestion, washed with HBSS and re-suspended in DMEM and seeded onto chamber slides (5x10⁴ cells/chamber, Thermo Scientific™ Nunc™ Lab-Tek™ II, 8 wells). The cells were cultured for 24 hr at 37 °C, prior to adding CITx-FLR (10 μ M) with fresh DMEM medium. The cells were further cultured for 20 min intervals or for 24 hr at 37 °C. The cells were fixed with 4% w/v paraformaldehyde for 20 min and washed with PBS. The cells were then stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (VECTASHIELD® Antifade Mounting Medium) and cover slipped. The fluorescence was monitored using a fluorescence microscope under UV light using different wavelength filters [DAPI - Excitation (358 nm)/Emission (461 nm), cometGFP - Excitation (403 nm)/ Emission (511 nm)] or using the Zeiss LSM 710 confocal microscopy in the School of Life Sciences Imaging (SLIM) (University of Nottingham).

2.3.15 Site-directed mutagenesis of CITx

CITx has three lysine residues (K15, K23 and K27) and therefore, including the N-terminal amine, there are four possible sites of conjugation for NHS ester-based ligands. All three lysine do not play a direct role in CITx activity while K15 provides the most

exposed amine group, allowing for easiest conjugation. To reduce the conjugation sites of NHS esters to multiple lysines on the surface of ClTx, K23 and K27 were mutated to arginine.

This was achieved using the Q5® Site-Directed Mutagenesis by New England Biolabs® (NEB). The mutagenic primers (Table 2.8) were designed using NEB's online primer design software, NEBaseChanger™. The sequences of all constructs were confirmed by DNA sequencing to ensure the correct residues were mutagenised without the occurrence of aberrant mutations.

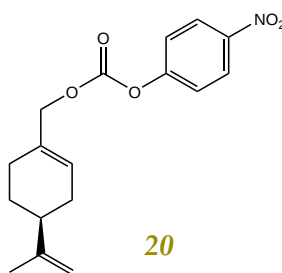
Table 2.8 | Primers used for Q5 Site-Directed Mutagenesis.

Primer	Sequence	Tm
Forward	TGGTAGATGCTACGGTCCGCAGTGC	71 °C
Reverse	CGACCTCTACCACCGCAGCAGTCGTC	71 °C

2.4 Synthesis of ligands

2.4.1 Perillyl alcohol ligands

2.4.1.1 Synthesis of 4-nitrophenyl perillyl carbonate (4NPC)

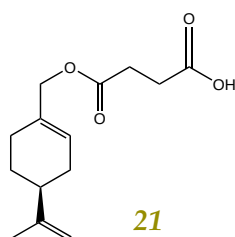


(S)-(-)-Perillyl alcohol (0.50 g, 0.52 mL, 3.3 mmol) was dissolved in dichloromethane (DCM, 20 mL). 4-nitrophenyl chloroformate (1.25 eq, 0.83 g, and 4.13 mmol) was added stepwise while stirring. This was followed by the addition of pyridine (2.5 eq, 0.65 g, 0.68 mL, 8.25 mmol) and an increase in temperature was observed. The reaction was left stirring at room temperature and followed via TLC (1:8 ethyl acetate (AcOEt)/petrol ether). After 4 hr, 4-nitrophenyl chloroformate (0.6 eq, 0.415 g, 2.06 mmol) and pyridine (2.5 eq, 0.68 mL, 8.44 mmol) were added and the reaction was left

to progress at room temperature for 16 hr. Upon completion, the reaction mixture was washed with ammonium chloride (20 mL) and with water (3 x 20 mL). The organic solution was dried over anhydrous sodium sulfate and concentrated *in vacuo* to give 4-nitrophenyl perillyl carbonate (4NPC) as a yellow liquid (1.05 g, 3.31 mmol, 99%).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 8.29 (dd, $J = 9.2, 1.2$ Hz, 2H), 7.40 (dd, $J = 8.6$ Hz, 2H), 5.94 – 5.88 (m, 1H), 4.78 – 4.73 (m, 2H), 4.68 (s, 2H), 2.20 (dhept, $J = 7.9, 3.6$ Hz, 4H), 2.10 – 1.99 (m, 1H), 1.96 – 1.85 (m, 1H), 1.76 (s, 3H), 1.63 – 1.46 (m, 1H). $^{13}\text{C-NMR}$ (100 MHz; CDCl_3): δ 155.5, 152.3, 149.2, 145.3, 131.2, 128.0, 125.2, 121.8, 109.0, 73.4, 40.5, 30.4, 27.1, 26.2, 20.6. HRMS (ESI): $\text{C}_{17}\text{H}_{19}\text{NNaO}_5$ $[\text{M}+\text{Na}]^+$ calculated 340.1155; found 340.1155.

2.4.1.2 Synthesis of perillyl 3-carboxypropanoate (P3C)

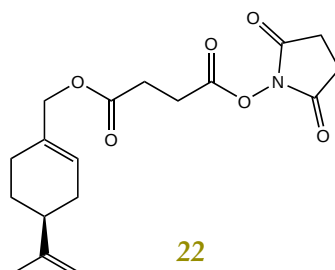


(S)-(-)-Perillyl alcohol (0.50 g, 0.52 mL, 3.3 mmol) was dissolved in dichloromethane (DCM, 10 mL). Succinic anhydride (2 eq, 0.68 g, and 6.6 mmol) was added stepwise while stirring, followed by the addition of triethylamine (TEA) (2.6 eq, 0.86 g, 1.20 mL, 8.5 mmol): the solution became purple. The reaction was followed via TLC (1:8 ethyl acetate/petrol ether) and left to progress while stirred for 16 hr at room temperature, giving a dark purple reaction mixture. The reaction mixture was washed with water (80 mL) and taken up with DCM (20 mL). The organic layer was washed with brine (10 mL) and aqueous HCl (1 M, 6 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo* to give the perillyl 3-carboxypropanoate (P3C) as a white solid (0.531 g, 2.10 mmol 67%).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 5.77 (ddt, $J = 5.3, 2.4, 1.3$ Hz, 1H), 4.75 – 4.71 (m, 2H), 4.51 (d, $J = 1.7$ Hz, 2H), 2.74 – 2.62 (m, 5H), 2.23 – 2.18 (m, 1H), 2.18 – 2.13 (m, 1H), 2.12 – 2.05 (m, 1H), 2.05 – 1.94 (m, 1H), 1.93 – 1.79 (m, 1H), 1.75 (s, 3H), 1.50 (ddt, $J = 12.7, 11.3, 8.6$ Hz, 1H). $^{13}\text{C-NMR}$ (100 MHz; CDCl_3): δ 178.0, 172.2, 149.6, 132.4, 126.0,

108.8, 68.9, 40.8, 30.5, 28.9, 27.3, 26.3, 21.1, 20.7. HRMS (ESI): $C_{14}H_{20}NaO_4$ $[M+Na]^+$ calculated 275.1254; found 275.1250.

2.4.1.3 Synthesis of perillyl 3-carboxypropanoate NHS ester (NHS-P3C)

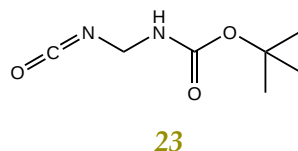


Perillyl 3-carboxypropanoate (468 mg, 1.86 mmol) was dissolved in anhydrous dimethylformamide (DMF, 10 mL). *N*-Hydroxysuccinimide (NHS) (323 mg, 2.80 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (560 mg, 2.92 mmol) was added to the solution with triethylamine (TEA) (266 mg, 2.62 mmol, 367 μ L) as the base and 4-dimethylaminopyridine (DMAP) (38 mg, 0.311 mmol) as the catalyst. The reaction was followed by TLC (1:1 ethyl acetate/hexane) and left to progress while stirred for 16 hr at room temperature under nitrogen. Following completion, the solution was concentrated under reduced pressure and the crude product was purified using silica column chromatography. (30% v/v EtOAc/Hex) (0.188 g, 0.538 mmol 29%).

^1H -NMR (400 MHz, $CDCl_3$): δ 5.79 – 5.75 (m, 1H), 4.77 – 4.70 (m, 2H), 4.57 – 4.46 (m, 2H), 3.01 – 2.94 (m, 2H), 2.85 (s, 4H), 2.78 (t, J = 7.1 Hz, 2H), 2.67 (td, J = 3.8, 1.1 Hz, 0H), 2.23 – 2.12 (m, 2H), 2.11 – 2.05 (m, 2H), 1.90 – 1.81 (m, 1H), 1.75 (t, J = 1.1 Hz, 3H), 1.50 (ddt, J = 12.9, 11.6, 8.7 Hz, 1H). ^{13}C -NMR (100 MHz; $CDCl_3$): δ 171.01, 169.00, 167.82, 149.67, 132.39, 126.41, 108.89, 69.24, 40.86, 30.56, 28.85, 27.38, 26.46, 26.41, 25.68, 20.86.

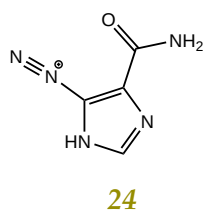
2.4.2 Temozolomide ligands

2.4.2.1 Synthesis of *tert*butyl isocyanato methylcarbamate⁹⁶



Ethyl chloroformate (2.03 g, 18.6 mmol) followed by triethylamine (4.60 g, 45.5 mmol) were added to an ice-cold (0 °C) solution of *N*-(*tert*-butoxycarbonyl) glycine (4 g, 22.8 mmol, in THF (80 µL) at 0 °C under nitrogen. The mixture was stirred (45 min) and then followed by the addition of aqueous (20 mL sodium azide (2.23 g, 34.3 mmol) which turned the colourless solution cloudy. This was left to react for a 1 hr at 0 °C followed by dilution with water (80 µL) and extraction of the crude acyl azide using toluene (4 x 40 µL). The organic solution was then further washed with saturated sodium bicarbonate (2 x 250 µL), HCl (250 µL) and water (250 µL) and finally dried using MgSO₄ at 0 °C. The mixture was then heated slowly to 63 °C and left to stir for 1.5 hr under nitrogen before heated slowly to 70 °C for 30 min. Finally, the reaction was concentrated *in vacuo* (taking care not to concentrate it to dryness) to give the *tert*-butyl isocyanato methylcarbamate as a 1:1.42 mixture with toluene. The isocyanate was used without any further purification with 5-diazoimidazole-4-carboxamide.

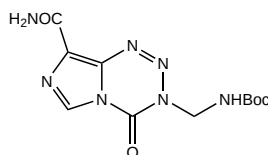
2.4.2.2 Synthesis of 5-diazoimidazole-4-carboxamide⁹⁶



A filtered solution of 5-amino-1-*H*-imidazole-4-carboxamide (4.29 g, 34.0 mmol) in 1 M HCl (40 mL) was added dropwise to an ice-cold stirred solution of sodium nitrite (2 g, 29.0 mmol) in water (40 mL) over 50 min under nitrogen. After formation of an orange precipitate, the solution was left to react for a further 5 min and the solid

was filtered, washed with cold water, ethanol, diethyl ether and dried *in vacuo* to give 5-diazoimidazole-4-carboxamide as an orange solid (3.33 g, 24.1 mmol, 84%). The carboxamide was used without any further purification with *tert*butyl isocyanato methylcarbamate.

2.4.2.3 Synthesis of *N*³-*boc* temozolomide (*N*³-Boc TMZ)⁹⁶

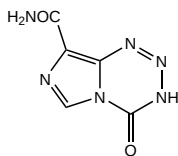


25

Crude *tert*butyl isocyanato methylcarbamate **23** (2.52 g, 14.6 mmol) was added dropwise in the dark in a solution of 5-diazoimidazole-4-carboxamide **24** (1.51 g, 11.0 mmol) in DMSO (18 mL) and left to stir for 16 hr at room temperature under nitrogen. The solution was then poured onto ice and the solid was filtered, washed with cold water, ethyl acetate and diethyl ether to give *N*³-*boc* temozolomide (*N*³-*boc* TMZ) (**25**) (2.36 g, 7.64 mmol 69%) as a pale pink solid.

¹H-NMR (400 MHz, DMSO-*d*₆): δ 8.85 (s, 1H), 8.04 (s, 1H), 7.82 (s, 1H), 7.70 (s, 1H), 5.50 (d, *J* = 6.4 Hz, 2H), 1.39 (s, 1H). ¹³C-NMR (100 MHz; DMSO-*d*₆): δ 161.91, 155.42, 139.09, 134.59, 131.44, 129.59, 79.40, 55.33, 28.52.

2.4.2.4 Synthesis of *nor*-temozolomide⁹⁶

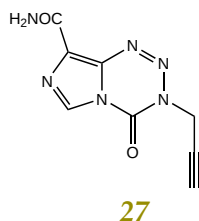


26

*N*³-*boc* TMZ **25** (2.36 g, 7.63 mmol) was dissolved in HCl (3 M, 105 mL) and left to stir for 16 hr at room temperature under nitrogen. The solution was then cooled to 4 °C for 4 hr and the precipitate was filtered, washed with cold water, ethyl acetate and diethyl ether to give *nor*-temozolomide (1.12 g, 6.22 mmol 82%) as a pale pink solid.

^1H -NMR (400 MHz, DMSO- d_6): δ 8.78 (s, 1H), 7.76 (s, 1H), 7.66 (s, 1H). ^{13}C -NMR (100 MHz; DMSO- d_6): δ 162.08, 139.53, 134.97, 130.83, 128.99. HRMS (ESI): $\text{C}_5\text{H}_5\text{N}_6\text{O}_2$ $[\text{M}+\text{H}]^+$ calculated 181.0468; found 181.0461.

2.4.2.5 Synthesis of N^3 -propargyl temozolomide (N^3P -TMZ)⁹⁶



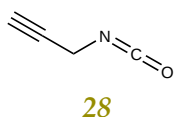
Method 1: Sodium hydride (60% v/v in mineral oil, 46.6 mg, 1.17 mmol) was added to a solution of *nor*-temozolomide **26** (140 mg, 0.777 mmol) in dry DMF (9 mL) at 0 °C. The solution was stirred for 1 hr under nitrogen, followed by the addition of propargyl bromide. The mixture was then left to stir at 0 °C for 30 min and then for 16 hr at room temperature. The mixture was concentrated to dryness under reduced pressure and the crude product was adsorbed into alumina and purified using alumina column chromatography (95% DCM/MeOH). The second fraction gave N^3P -TMZ as a brown solid (45.0 mg, 0.206 mmol 27%).

^1H -NMR (400 MHz, DMSO- d_6): δ 8.87 (s, 1H), 7.83 (s, 1H), 7.71 (s, 1H), 5.15 (d, J = 2.5 Hz, 2H), 3.53 (t, J = 2.5 Hz, 1H). ^{13}C -NMR (100 MHz; DMSO- d_6): δ 161.86, 139.03, 134.68, 131.79, 129.68, 77.81, 76.96, 38.94. HRMS (ESI): $\text{C}_8\text{H}_6\text{N}_6\text{NaO}_2^+$ $[\text{M}+\text{Na}]^+$ calculated 241.0444; found 241.0445.

Method 2: *Nor*-temozolomide **26** (112.5 mg) was dissolved in 37% v/v formaldehyde (4 mL) and left to react at room temperature for 16 hr. The crude product was filtered, washed with water ethyl acetate and diethyl ether before added into a solution of propargyl bromide in acetonitrile. DBU was then added dropwise and left to stir at room temperature overnight. The solution was then acidified with 1 M HCl and extracted with ethyl acetate. After washing the organic layer with water, the solvent was removed *in vacuo* to give a brown product that was analysed by mass spectrometry.

Method 3: A solution of propargylamine and triethyl amine in DCM was added dropwise in a solution of triphosgene dissolved in DCM under argon atmosphere. The reaction was stirred for 30 min followed by the dropwise addition of a solution of 5-diazoimidazole-4-carboxamide **24** dissolved in DMSO. The reaction was left to stir at room temperature for 16 hr before analysed by mass spectrometry.

2.4.2.6 Synthesis of propargyl isocyanate



Triphosgene (1.80 g, 6.00 mmol) was added in a solution of propargylamine (1.00 g, 18.2 mmol) in DCM (75 mL) and saturated sodium hydrogen carbonate (75 mL) at 0 °C. The mixture was stirred for 1 hr followed by extraction of the organic layer with DCM. The DCM was then removed via distillation *in vacuo* to give propargyl isocyanate as a colourless oil.

2.5 Conjugation and testing of chlorotoxin conjugates

2.5.1 Conjugation of perillyl 3-carboxypropanoate (P3C) to CITx

A stock solution of perillyl 3-carboxypropanoate (P3C) (10.1 mg, 0.4 mmol, 20 mM), EDC (27.0 mg, 0.174 mmol, 87 mM) and sulfo-NHS (86.7 mg, 0.4 mmol, 200 mM) was each made in acetonitrile (2 mL). P3C (4 mM, 200 μ L), EDC (23 μ L, 2 mM) and Sulfo-NHS (25 μ L, 5 mM) were added to 50 mM Phosphate buffer (pH 8.0, 10% v/v acetonitrile) (1 mL). The mixture was left to react for 15 min at room temperature, followed by addition of 2-mercaptoethanol (20 mM) for 10 min to quench the reaction. The reaction mixture was then added to a CITx solution (100 μ M, 200 μ L) and left to react for varying times lengths at room temperature. The completion of the reaction was confirmed by mass spectrometry.

2.5.2 Conjugation of perillyl 3-carboxypropanoate NHS ester (NHS-P3C) to CITx

A stock solution of perillyl 3-carboxypropanoate NHS-ester (NHS-P3C) (35 mg, 0.10 mmol 100 mM) was dissolved in DMSO (1 mL). NHS-P3C (10 mM, 100 μ L) was then added in a solution of CITx (100 mM) in PBS (1 mL). The solution was then gently mixed by rotation and left to react for 24 hr. After the reaction was completed, the excess NHS-P3C was removed from the CITx-POH conjugate by dialysis into PBS. Completion of the reaction was confirmed by mass spectrometry.

2.5.3 Presto blue cytotoxic assay of CITx-P3C ester

The toxicity of CITx-P3C was determined using the presto blue assay. U87 cells (Children's Brain Tumour Research Centre, University of Nottingham, United Kingdom) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (1% w/v Penicillin/Streptomycin Solution) and 10% v/v fetal bovine serum. The cells were detached via trypsin digestion, re-suspended in DMEM (with 10% v/v FBS) and seeded onto a Corning™ Costar® 96-Well clear bottom cell culture plate (5x10⁴ cells/well). The cells were cultured for 24 hr at 37 °C, prior to the addition of CITx-P3C at the indicated concentrations with fresh DMEM medium. The cells were cultured for a further 24 hr at 37 °C. Following this, the medium in each well was removed, the well was washed with HBSS and Presto blue was added into each well (1:10 dilution using HBSS). The cells were incubated with presto blue for 1 hr at 37 °C. The solution from each well was transferred onto a Corning™ 96-Well Solid Black plate and the absorbance at 570 nm was measured using a plate reader.

3

Formation and analysis of chlorotoxin

The deathstalker scorpion has low concentrations of ClTx and thus it cannot be used to purify large amounts of ClTx for further studies. Instead, other methods are employed to produce ClTx protein for treating GBM. The two main methods are chemical synthesis⁹⁷ and recombinant expression.⁹⁸

ClTx is synthesised chemically by solid-phase peptide synthesis (SPPS). First an amino acid is attached to a solid polymer resin which is followed by the addition of the other amino acids to form the peptide sequence. The primary peptide structure is cleaved and purified using reverse-phase high performance liquid chromatography (RP-HPLC). The ClTx is then folded using a refolding buffer and purified again via RP-HPLC.⁹⁹

Recombinant expression involves the expression of ClTx in *E. coli* strain BL21TM (DE3) with either an N-terminal hexa-histidine tag (HT-ClTx) or an N-terminal hexa-histidine tag followed by a glutathione transferase tag (HT-GST-ClTx).⁹⁸ The His-tag allows the purification of ClTx by IMAC, while the glutathione *S*-transferase increases the solubility of the ClTx.⁹⁸ HT-GST-ClTx was first *S*-sulfonated to allow enterokinase protease to cleave the ClTx from the glutathione *S*-transferase and then underwent oxidative refolding. ClTx purified using this method had similar activity to that of the native ClTx.⁴⁶

Recombination can lead to higher yield of protein produced and was the chosen method for this research over SPPS. To avoid the oxidative refolding step and to address the solubility issues with ClTx, tags were attached on the C-terminal rather than the N-terminal end of ClTx.

3.1 Construction of ClTx plasmids

The first aim was to construct a ClTx tagged genes for protein purification. The ClTx-TEV-HT-cometGFP (867 bp) and ClTx-HT (129 bp) genes were both constructed commercially. The ClTx-TEV-HT-cometGFP had four components; the ClTx gene followed by a C-terminal TEV (Tobacco Etch Virus) protease cleavage site, a 6x histidine (HT) tag and finally a cometGFP tag. The TEV cleavage site (ENLYFQG) is a sequence that is recognized by the TEV protease and cleaved between the glutamine (Q) and glycine (G). The cometGFP is a soluble fluorescent protein. This allows the cometGFP to be used as both a solubility tag, to increase the ClTx solubility, and a fluorescent tag for visualizing ClTx in GBM cells.

The ClTx-TEV-HT-cometGFP and ClTx-HT constructs were originally in the pD441-CC and pJ411 vectors, respectively. Therefore, the genes were extracted and re-ligated into pET22b vectors. These are T7 inducible vectors suitable for high protein expression in *E. coli* (Figure 3.1A).

A HT-cometGFP gene (741 bp) was also synthesized as a ‘negative imaging control’ for microscopy studies using ClTx-TEV-HT-cometGFP. The HT-cometGFP gene was amplified from the pD441-CC plasmid (Figure 3.1B) using suitable primers. (Methods, Section 2.2.1, Table 2.7)

Ligated plasmids were transformed into XL-1 Blue cells via electroporation which were then grown on agar plates containing LB agar with 50 µg/mL of carbenicillin. The correct sequences of the cloned constructs were confirmed by DNA sequencing. All transformations (Figure 3.1C-D) showed that the gene of interest was successfully ligated into the vector plasmid. The ClTx-TEV-HT-cometGFP, ClTx-HT and HT-cometGFP constructs were transformed into Rosetta (DE3) and Origami (DE3) *E. coli* expression strains for protein overexpression and purification.

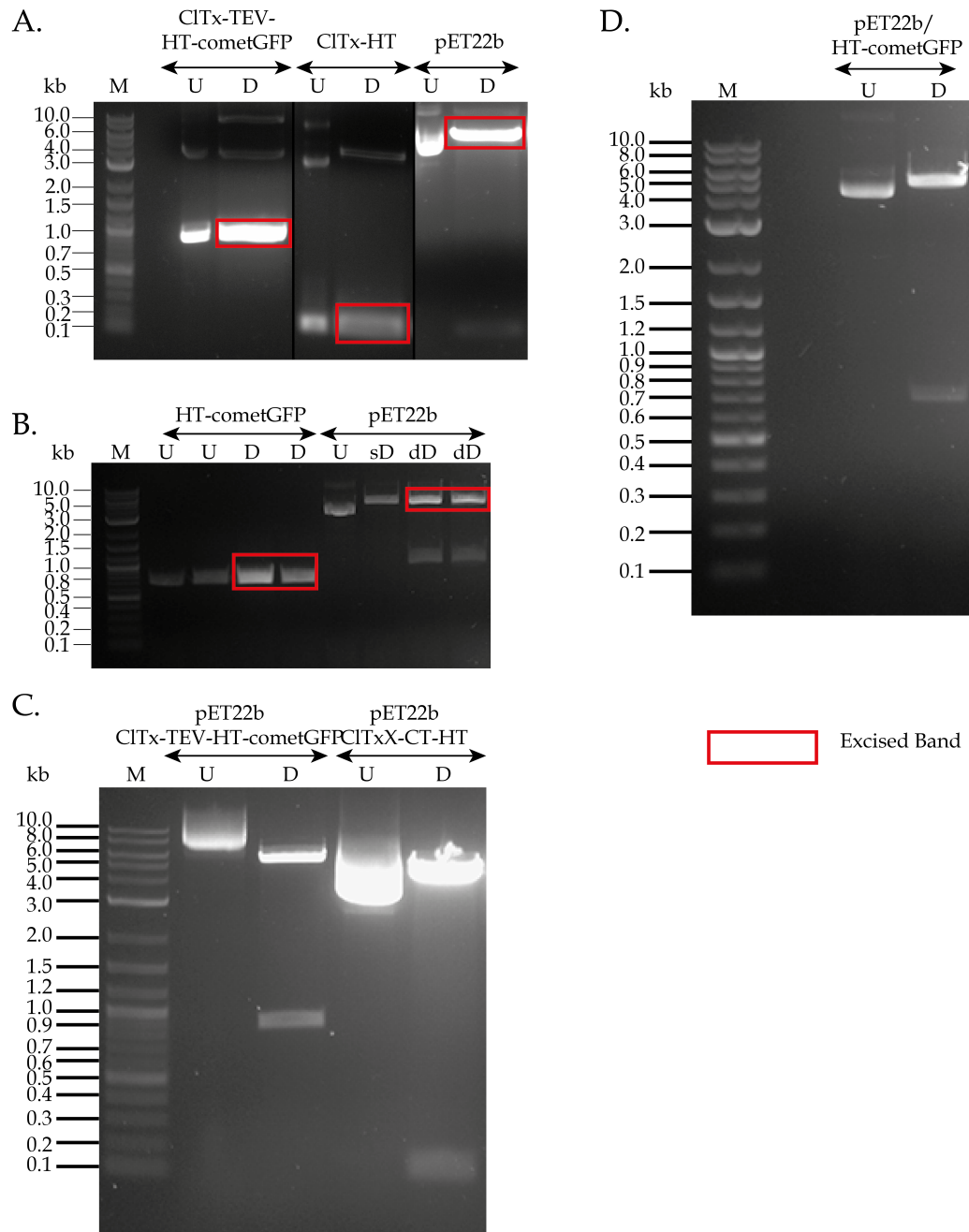


Figure 3.1 | Cloning of CITx and cometGFP constructs. **M:** DNA Size Marker, **U:** Undigested plasmid, **D:** Digested plasmid, **sD:** Single digested plasmid and **dD:** Double digested plasmid. **A.** Restriction digests of CITx-TEV-HT-cometGFP (867 bp), CITx-HT (129 bp) and pET22b plasmids using NdeI and XhoI restriction enzymes. **B.** Restriction digests of HT-cometGFP and pET22b plasmids using NdeI and XhoI restriction enzymes. **C.** Agarose gel analysis after ligation of CITx-TEV-HT-cometGFP (867 bp) and CITx-HT (129 bp) into the pET22b vector. **D.** Agarose gel analysis after ligation of HT-cometGFP (741 bp) into pET22b vector. A 1.2% w/v agarose gel was used for all plasmid DNA analyses, run at 100 V for 30 min (gel size: 7 x 10 x 0.75 cm). **U:** Undigested, **D:** Digested, **sD:** single digest using a single restriction enzyme **dD:** double digested with two restriction enzymes. Excised bands for ligation are highlighted with a red box.

Table 3.1 | Protein sequences for the different chlorotoxin constructs and the HT-cometGFP.

Protein sequence	Template
CITx-TEV-HT-cometGFP	MCNPCFTTDHQMARKCDDCCGGKGRGKCYGPQCRCRENLYFQGHHHHHHGM TALTEGAKL- FEKEIPYITELEGDVEGMKFIKGEGTGDATTGTIKAKYICTTGDVPVPWSTLVTTLTGAAQCFAK- YGPCLKDFYKSCMPEGYVQERTITFEGDGVFKTRAEVTFENGSVYNRVKLNQGQFKKDGHVLF- GKNLEFNFTPHCLCIWGDQANHGLKSAFKIMHEITGSKEDFIVADHTQMNTPIGGGPVHVPEY- HHLTVWTSFGKDPDDDETDLNIVEVIKAVDLETYRG
CITx-HT HT-cometGFP	MCNPCFTTDHQMARKCDDCCGGKGRGKCYGPQCRCRHHHHH MHHHHHGM TALTEGAKLFEKEIPYITELEGDVEGMKFIKGEGTGDATTGTIKAKYICTTGDVPV- PWSTLVTTLTGAAQCFAKYGPELKDFYKSCMPEGYVQERTITFEGDGVFKTRAEVTFENGSVY- NRVKLNQGQFKKDGHVLFKGNLEFNFTPHCLCIWGDQANHGLKSAFKIMHEITGSKEDFIVADH- TQMNTPIGGGPVHVPEYHHLTVWTSFGKDPDDDETDLNIVEVIKAVDLETYRG
CITx (Cleaved)	MCNPCFTTDHQMARKCDDCCGGKGRGKCYGPQCRCRENLYF

3.2 CITx and cometGFP expression and solubility analyses

Following the cloning of CITx and cometGFP, the next step was to test protein expression. This was done by using Origami and Rosetta (DE3) *E. coli* strains containing the CITx or cometGFP constructs in a 2xYT broth (with 10 ng/μL of carbenicillin). The cells were incubated at 37 °C to grow until the culture reached an OD₅₉₅ of 0.7. IPTG (500 μM) was added and the culture was left for a further 4 hr or overnight at either 30 °C or 16 °C, respectively, before harvesting the cells by centrifugation. A sample of the cells were sonicated and centrifuged to clarify the soluble and insoluble fractions which were analysed by SDS-PAGE in order to optimize the optimal expression conditions for a larger expression/purification experiment.

SDS-PAGE of Rosetta and Origami cell samples expressing the CITx-TEV-HT-cometGFP protein revealed a band at 32.1 kDa in the soluble induced fraction which corresponds to the CITx-TEV-HT-cometGFP protein. (Figure 3.2A-B) This confirmed that the cometGFP protein, being a soluble tag protein,¹⁰⁰ increased the solubility of the CITx peptide. Furthermore, due to the fluorescent nature of the cometGFP, when samples were illuminated with UV light the CITx-TEV-HT-cometGFP protein exhibited a bright green fluorescent colour (Figure 3.3). This allows easier tracking of the protein during purification and activity testing. Compared to the expression of soluble CITx-TEV-HT-cometGFP in the Rosetta cells, the Origami cells showed expression of CITx-TEV-HT-cometGFP in the insoluble fraction and thus the Rosetta cells were used for all the subsequent expression and purification experiments.

The Rosetta (DE3) and Origami 2 (DE3) cells containing the 4.8 kDa CITx-HT constructs did not exhibit any expression of the correct proteins (Figure 3.2C-D). This could be due to the small size of the CITx-HT peptide as the resolution of the gel might not have been sufficient to clearly resolve the small CITx-HT peptide. As such, it was decided that a single step purification to remove the majority of the contaminants will give a clearer picture on the expression and solubility of the CITx-HT peptide.

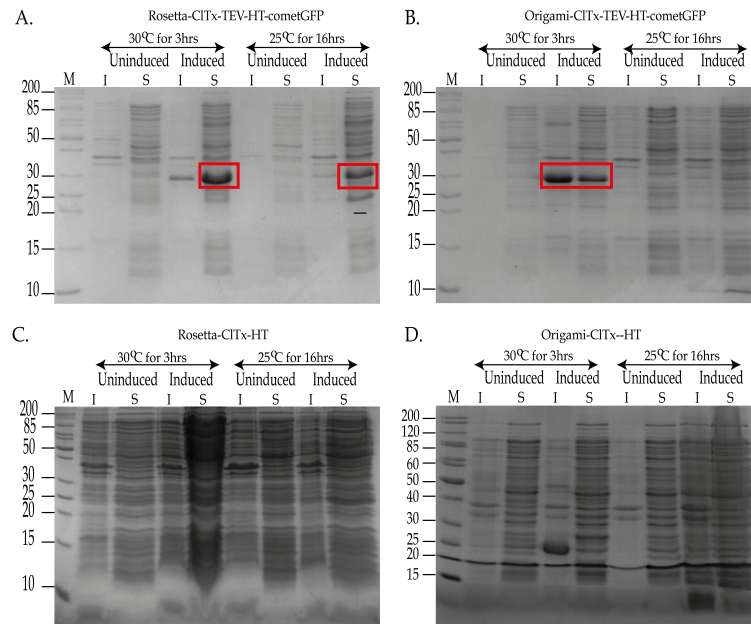


Figure 3.2 | Solubility analysis of CITx protein constructs by SDS-PAGE. SDS-PAGE (15% w/v acrylamide) analyses. **A.** Rosetta (DE3): CITx-TEV-HT-cometGFP protein showing as a 32.1 kDa band present in the induced soluble fraction. **B.** Origami (DE3): CITx-TEV-HT-cometGFP protein showing as a 32.1 kDa band present in the induced insoluble and soluble fractions. **C.** Rosetta (DE3): CITx-HT protein was not clearly resolved as a 4.8 kDa band and therefore the solubility of the CITx-HT could not be confirmed. **D.** Origami (DE3): CITx-HT protein was not clearly resolved as a 4.8 kDa band and therefore the solubility of the CITx-HT could not be confirmed. M: Protein Ladder used as a marker showing molecular weights in kDa. I: Insoluble proteins from pellet and S: Soluble proteins from supernatant following sonication and centrifugation of cells.

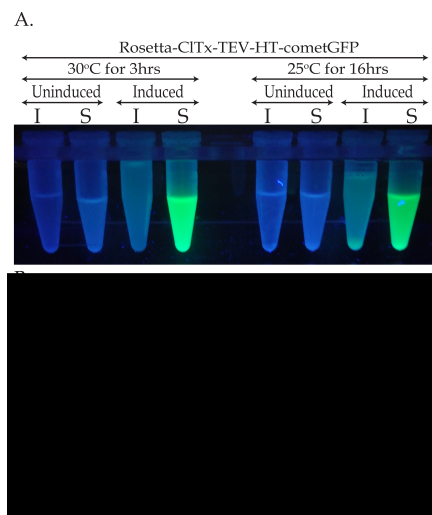


Figure 3.3 | **A.** Rosetta (DE3): CITx-TEV-HT-cometGFP protein showing the fluorescence in the induced soluble fraction. **B.** Origami (DE3): CITx-TEV-HT-cometGFP protein showing the fluorescence in the induced soluble fraction.

3.3 Purification of CITx-TEV-HT-cometGFP

CITx-TEV-HT-cometGFP was expressed in Rosetta cells carrying the CITx-TEV-HT-cometGFP construct. The cells were sonicated and the soluble/insoluble fractions were clarified by centrifugation. This was followed by precipitation of the protein in the soluble fraction using 40% w/v ammonium sulfate (**Figure 3.4B**). Ammonium sulfate precipitation can be used as both a purification step and a protein extraction step as some protein impurities remain in solution while the CITx-TEV-HT-cometGFP protein precipitates and is pelleted following centrifugation.

The pellet was dissolved in HisTrap wash buffer and loaded onto a GE Healthcare Life Sciences 5 mL HisTrap™ HP column pre-equilibrated with the HisTrap wash buffer. After a column wash, 5% HisTrap elution buffer was used to remove weakly, non-specific bound proteins from the column. The CITx-TEV-HT-cometGFP was eluted with a gradient of increasing concentration of imidazole (50 mM to 400 mM). CITx-TEV-HT-cometGFP started to elute at around 100 mM imidazole (**Figure 3.4A**). The purity of the CITx-TEV-HT-cometGFP protein after elution was analysed by SDS-PAGE (**Figure 3.4B**).

The CITx-TEV-HT-cometGFP protein was further purified by gel filtration using a GE Healthcare Life Sciences HiLoad™ 16/600 Superdex 75 pg column. Eluted fractions were analysed with SDS-PAGE and showed the presence of some contaminants (**Figure 3.5B**). There were two main contaminants present with molecular weights of 70 kDa and 160 kDa, respectively. Therefore, further experiments were carried out to establish the optimal conditions necessary for the purification of the CITx-GFP protein.

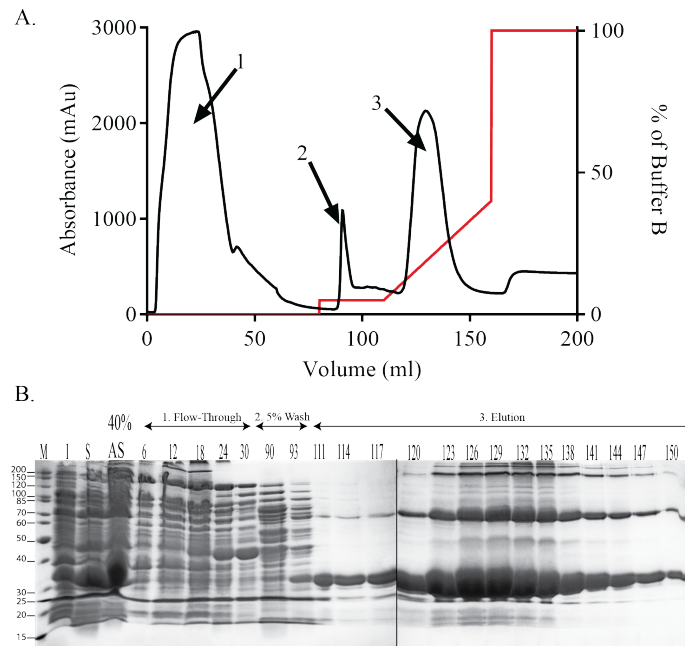


Figure 3.4 | IMAC of CITx-TEV-HT-cometGFP. HisTrap wash buffer: 50 mM Tris pH 7.5, 500 mM NaCl; HisTrap elution buffer: 50 mM Tris pH 7.5, 500 mM NaCl and 1 M Imidazole. **A.** Chromatogram showing the UV absorbance of proteins eluting out from the HisTrap™ column. 1: Flow-through (Non-binding proteins), 2: 5% Imidazole wash (non-specific bound proteins). 3: Elution of CITx-TEV-HT-cometGFP. **B.** SDS-PAGE (15% w/v acrylamide) analysis of eluted fractions; the numbers above the lanes indicate the elution volumes of the specific fractions. M: Protein Ladder used as a marker showing molecular weights in kDa, I: Insoluble fraction after sonication/centrifugation (Pellet), S: Soluble fraction after sonication/centrifugation (Supernatant), AS: 40% w/v Ammonium Sulfate precipitation.

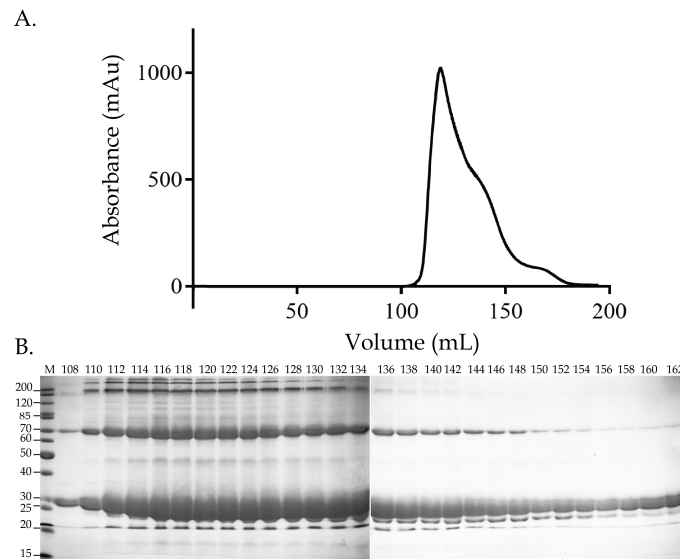


Figure 3.5 | Size-exclusion chromatography of CITx-TEV-HT-cometGFP. Buffer: 50 mM Tris pH 7.5, 200 mM NaCl and 10% v/v glycerol. **A.** Chromatogram showing the elution profile. **B.** SDS-PAGE (15% w/v acrylamide) analysis of eluted fractions; the numbers above the lanes indicate the elution volumes of the specific fractions. Results showed contaminants still present with the CITx-TEV-HT-cometGFP protein (32.1 kDa). M: Protein Ladder used as a marker showing molecular weights in kDa.

CITx has 8 cysteine residues which form 4 disulfide bridges known as the knottin domain: a "disulfide through disulfide knot". These tend to contribute greatly to the stability of the protein but may mis-form and result in aberrant disulfide bridges between monomers to form unwanted dimers/multimers during expression/purification. The size exclusion chromatography shows the presence of a band at approximately 65 kDa (**Figure 3.5B**). However, mass spectrometry only showed mass corresponding to the CITx-TEV-HT-cometGFP suggesting that the contaminant is a possible dimer of the CITx-TEV-HT-cometGFP protein. Further experiments were carried out to identify the contaminant (**Figure 3.6**). Assuming it was a dimer, urea and guanidinium hydrochloride were used to disrupt non-polar and electrostatic bonds between the dimer. DTT, β -mercaptoethanol and S-sulfonation were also used to remove any mis-formed disulfide bonds which may have contributed to the persistence of a dimer during purification.

Peptide mass fingerprinting (PMF) was used to analyse the contaminant. PMF is a technique that is used to identify unknown protein samples by first cleaving them into smaller peptides using proteolytic enzymes, such as trypsin or chymotrypsin, and then carrying out mass identification of the smaller peptides with ESI-TOF or MALDI-TOF mass spectrometry. The molecular weights of the peptide fragments are compared to protein databases online against proteins that were treated with the same proteolytic enzymes *in silico* to find statistical matches for the unknown peptides. The 65 kDa contaminant band was excised from an SDS-PAGE gel and analysed via PMF. The results showed that the 65 kDa contaminant matched the sequence for the TEV cleavage site, the 6x His-Tag and parts of the cometGFP protein (**Appendix**), confirming the purification of the CITx-TEV-HT-cometGFP protein. In conclusion, the contaminants are aberrant dimers of mis-formed multimers, but it was not possible to remove the aberrant disulfide bridges.

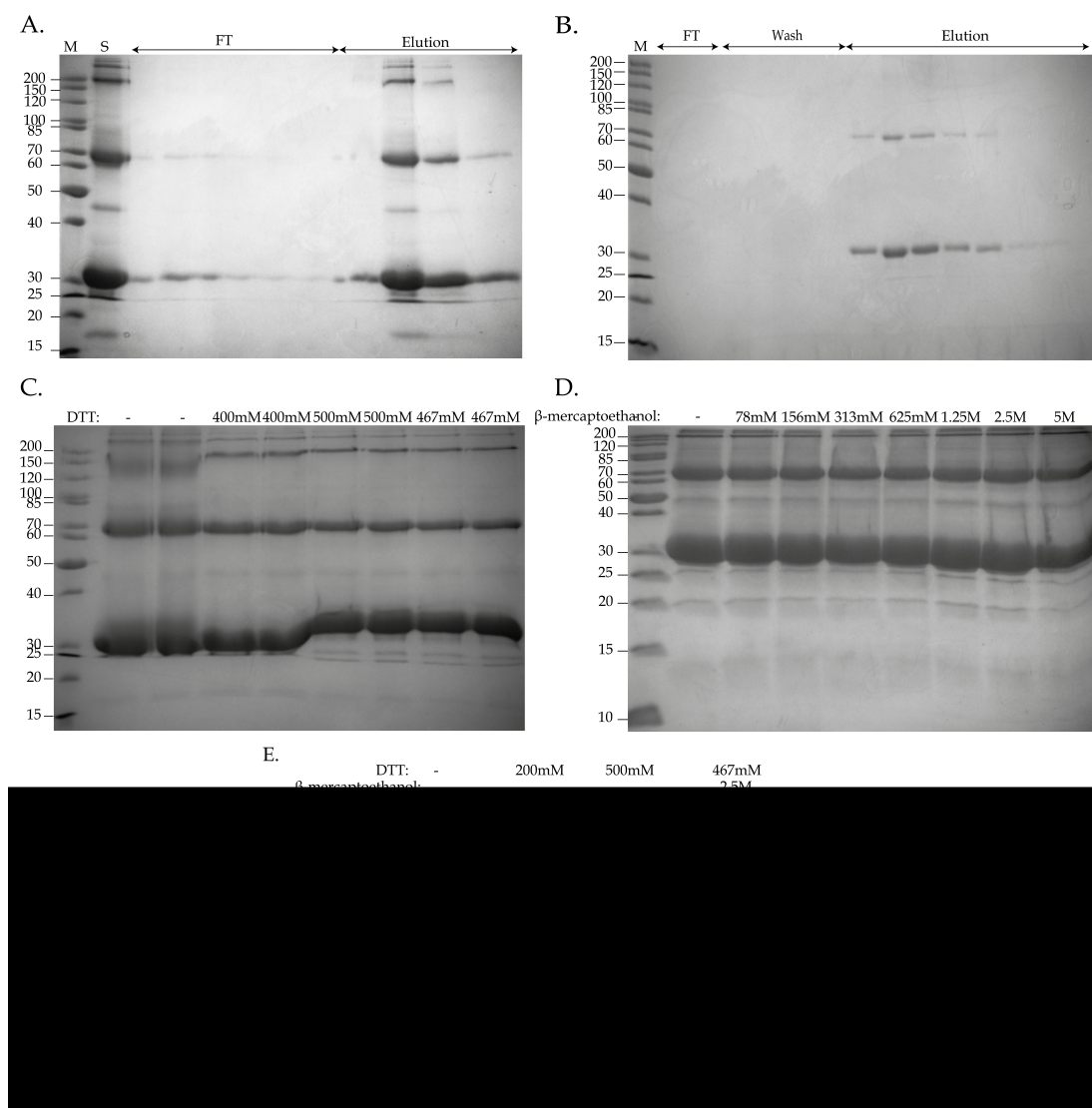


Figure 3.6 | Analysis of the contaminant 65 kDa band using reducing reagents and SDS-PAGE. **A.** Two-step IMAC purification of the CITx-TEV-HT-cometGFP protein using increasing concentrations of urea (0 to 4 M) followed by standard elution using imidazole, S: Sample before purification. **B.** Two-step IMAC purification using increasing concentrations of guanidinium hydrochloride (0 to 4 M) followed by standard elution using imidazole. **C.** The effect of adding increasing concentrations of DTT. **D.** The effect of adding increasing concentrations of β -mercaptoethanol. **E.** The effect of S-sulfonation with the presence of DTT and β -mercaptoethanol. M: Protein Ladder used as a marker showing molecular weights in kDa.

3.4 Purification of HT-cometGFP

Fluorescence microscopy experiments with ClTx-TEV-HT-cometGFP requires a negative cometGFP control to show that ClTx is the protein that is binding to the glioma cells and not the cometGFP. Therefore, the HT-cometGFP protein was purified using the same protocol as that used to purify the ClTx-TEV-HT-cometGFP.

Rosetta (DE3) *E. coli* cells were induced to express the HT-cometGFP protein, the cells were then harvested by centrifugation (35,000 g for 30 min), re-suspended in sonication buffer and disrupted by sonication. The cell extract was clarified by centrifugation to separate the soluble and insoluble fractions and the HT-cometGFP protein was purified from the soluble fraction. The same two-step purification method (IMAC and size exclusion chromatography) was used to purify the HT-cometGFP protein.

The soluble fraction of the bacterial extract was loaded onto a GE Healthcare Life Sciences 5 mL HisTrap™ HP column pre-equilibrated with the HisTrap wash buffer. The column was then washed with HisTrap wash buffer, followed by a second wash with 5% HisTrap elution buffer to remove non-specific bound proteins from the column. The HT-cometGFP protein was eluted by running a gradient of increasing concentration of imidazole (50 mM to 400 mM). The chromatogram shows that the HT-cometGFP protein starts eluting at approximately 100 mM imidazole (peak 2 in **Figure 3.7A**). The purity of the HT-cometGFP protein was assessed by SDS-PAGE as shown in **Figure 3.7B**.

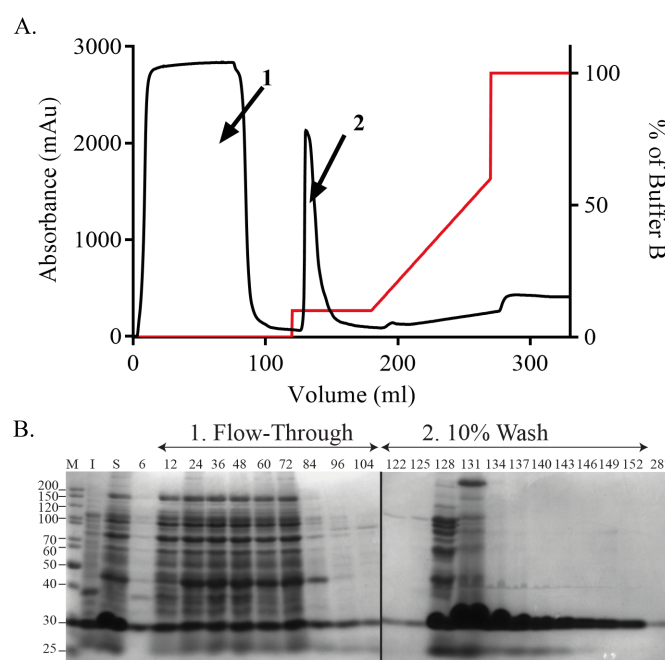


Figure 3.7 | IMAC of HT-cometGFP. HisTrap wash buffer A: 50 mM Tris pH 7.5, 500 mM NaCl; HisTrap elution buffer B: 50 mM Tris pH 7.5, 500 mM NaCl and 1 M Imidazole. **A.** Chromatogram showing the elution profile of proteins from the HisTrap™ column (black line) and the buffer B gradient applied (red line). **B:** SDS-PAGE (15% w/v acrylamide) of eluted fractions (the numbers above the lanes indicate the elution volumes at which the samples were taken for analysis). M: Protein Ladder used as a marker showing molecular weights in kDa. 1: Flow-through containing unbound proteins when passed through the column. 2: Bound protein eluted from column using Imidazole.

The second step of the HT-cometGFP purification was size exclusion chromatography using a GE Healthcare Life Sciences HiLoad™ 16/600 Superdex 75 pg column. Fractions from the IMAC step containing the HT-cometGFP protein were pooled and loaded onto the HiLoad™ 16/600 Superdex 75 pg column pre-equilibrated in 50 mM Tris pH 7.5, 200 mM NaCl and 10% v/v glycerol. Eluted fractions were analysed with SDS-PAGE as shown below in **Figure 3.8**. Higher molecular weight contaminants were present in the final HT-cometGFP protein preparation but can be ignored as they are minor as seen in the gel.

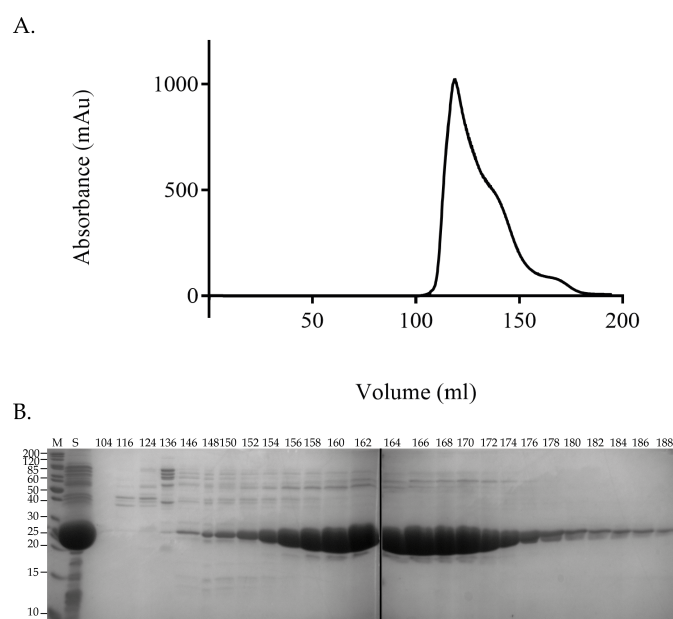


Figure 3.8 | Size-exclusion chromatography of HT-cometGFP. Buffer: 50 mM Tris pH 7.5, 200 mM NaCl and 10% v/v glycerol. **A.** Chromatogram from the size-exclusion chromatography of the HT-cometGFP preparation. **B.** SDS-PAGE (15% w/v acrylamide) of the eluted fractions (the numbers above the lanes indicate the elution volumes at which the samples were taken for analysis). S: Soluble fraction sample that was loaded onto the column. M: Protein Ladder used as a marker showing molecular weights in kDa.

3.5 Purification of CITx-HT

Following over-expression of the CITx-HT protein in Rosetta CITx-HT *E. coli* cells, the cells were harvested by centrifugation, suspended in sonication buffer, sonicated, and the bacterial extract was clarified by centrifugation to separate the soluble and insoluble fractions. The soluble fraction was loaded onto a GE Healthcare Life Sciences 5 mL HisTrap™ HP column pre-equilibrated with the HisTrap wash buffer. The column was washed with HisTrap wash buffer, followed by a second wash with 5% HisTrap elution buffer to remove non-specific bound proteins from the column. Protein was eluted from the column by running a gradient of increasing concentration of imidazole (50 mM to 400 mM). Eluted fractions were assessed by SDS-PAGE (20% w/v acrylamide), as shown in **Figure 3.9**. Unfortunately, the small 4.8 kDa CITx-CT-HT protein could not be detected. Since the CITx-TEV-HT-cometGFP purification showed good yield of relatively pure protein which can be tracked due to the fluorescent nature of the cometGFP tag, the purification of the CITx-CT-HT protein was abandoned and instead

the CITx-TEV-HT-cometGFP protein was used for further analyses of CITx activity and POH conjugation to the CITx.

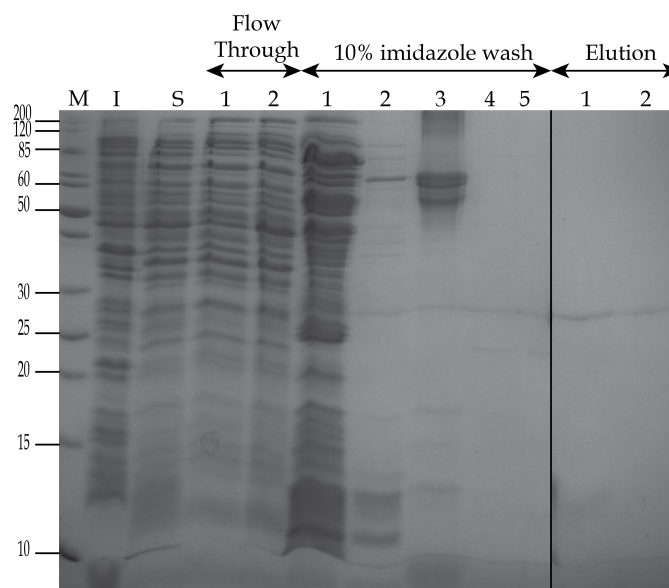


Figure 3.9 | IMAC of CITx-CT-HT. HisTrap wash buffer A: 50 mM Tris pH 7.5 and 500 mM NaCl, HisTrap elution buffer B: 50 mM Tris pH 7.5, 500 mM NaCl and 1 M Imidazole. Fractions were assessed by SDS-PAGE (20% w/v acrylamide). M: Protein Ladder used as a marker showing molecular weights in kDa. I: Insoluble fraction after sonication/centrifugation (Pellet), S: Soluble fraction after sonication/centrifugation (Supernatant).

3.6 Toxicity and activity of CITx-TEV-HT-cometGFP

Following confirmation of CITx purification the next stage was to test the activity of the CITx-TEV-HT-cometGFP protein. As explained previously, CITx has been shown to interact with the metalloproteinase-2 (MMP-2) which is present on the surface and cytoplasm of the glioblastoma cells and CITx protein decrease metastasis of these cells (see **Chapter 1.2.1**). In order to assess whether CITx-TEV-HT-cometGFP protein is able to bind to glioblastoma cells, microscopy experiments were carried out whereby chamber slides containing adhered U87 glioblastoma cells in the presence or absence of CITx-TEV-HT-cometGFP were visualized. U87 cells are known to have high levels of MMP-2,¹⁰¹ making this cell line an ideal *in vivo* model to test the binding ability of CITx-TEV- HT-cometGFP protein to glioblastoma tumours.

The toxicity of CITx-TEV-HT-cometGFP was also assessed. This was done using the presto blue assay. U87 cells were seeded onto a cell culture 96 well-plate (5×10^4 cells/well) and cultured for 24 hr at 37 °C, followed by the addition of CITx-TEV-HT-

cometGFP at varying concentrations dissolved in DMEM. The cells were then cultured for a further 24 hr at 37 °C. The medium in each well was then removed, the wells were washed with HBSS and presto blue was added into each well (1:10 dilution in HBSS). The cells were incubated with presto blue for 1 hr at 37 °C then the solution from each well was transferred onto a black 96-well plate and the absorbance at 570 nm was measured using a plate reader. The data were plotted as percentage of cell survived compared to the control (Y-axis) against the concentration of the CITx-TEV-HT-cometGFP protein. (Figure 3.10)

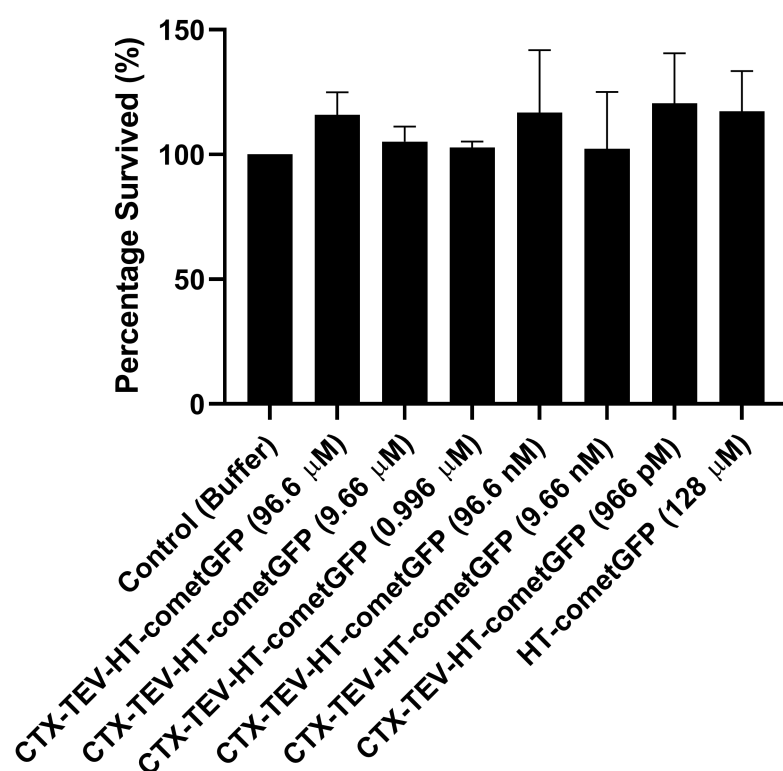


Figure 3.10 | Presto blue assay for CITx-TEV-HT-cometGFP. Cell viability at varying concentrations of CITx-TEV-HT-cometGFP and HT-cometGFP (as a negative control) was assessed in duplicates and plotted as a graph of percentage cell survived in relation to the control at 570 nm against protein concentration. The data showed no change in toxicity at these concentrations of CITx-TEV-HT-cometGFP and HT-cometGFP.

The Presto Blue assay data showed that the CITx-TEV-HT-cometGFP is not toxic and can be used for microscopy imaging to assess binding to U87 cells. U87 cells were seeded onto chamber slides (5×10^4 cells/chamber) and cultured for 24 hr at 37 °C. CITx-TEV-HT-cometGFP was added to the cells and the cells were cultured for a further for 2, 6 or 24 hr at 37 °C. The cells were then fixed with 0.4% v/v paraformaldehyde,

washed with PBS, stained with DAPI and cover slipped. The fluorescence was assessed using a fluorescence microscope under UV light with different wavelength filters.

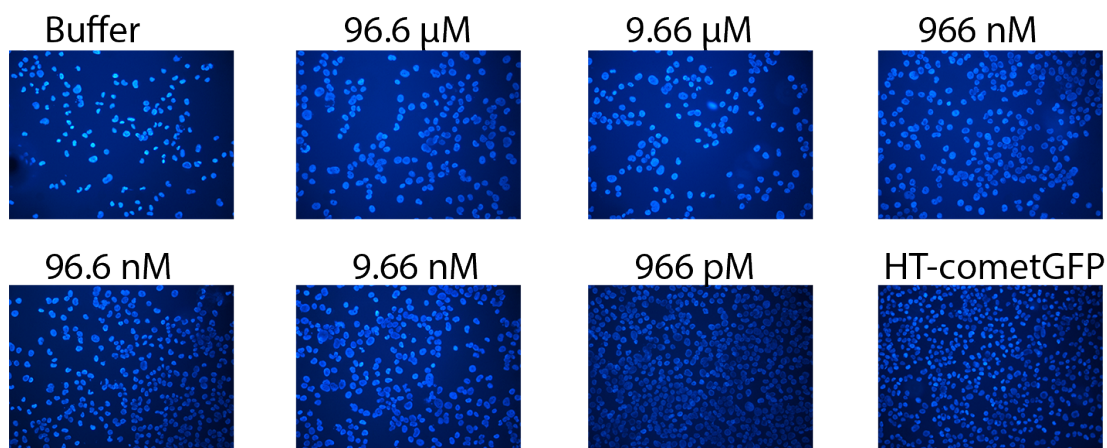


Figure 3.11 | Fluorescence UV microscopy analysis of CITx-TEV-HT-cometGFP. U87 cells incubated for 24 hr in the presence of buffer, HT-cometGFP or CITx-TEV-HT-cometGFP at different concentrations, as indicated. The imaging showed that DAPI has successfully stained the cells but no green fluorescence (CITx-TEV-HT-cometGFP) was observed.

Unfortunately, the imaging from the 24 hr CITx-TEV-HT-cometGFP incubation experiment was inconclusive. As shown in **Figure 3.11**, DAPI has successfully stained the U87 cell nucleus but the green halo, caused by the binding of CITx-TEV-HT-cometGFP to the MMP-2 protein on the cell membrane, was not visible. The same result was obtained with both the 2 hr and the 6-hr protein incubation. This could be due the large size of the cometGFP protein (27.3 kDa) relative to the CITx (4.8 kDa) which may sterically prevent the CITx from interacting with the MMP-2 protein on the surface of the cells. Therefore, it was decided instead to use non-tagged CITx to assess if it interacts with the U87 cells.

3.7 Cleavage of CITx from cometGFP using TEV protease

The non-tagged CITx protein can potentially be obtained from the CITx-TEV-HT-cometGFP protein utilizing the TEV protease to cleave the HT - cometGFP tag off. A sample of CITx-TEV-HT- cometGFP was subjected to TEV protease treatment. TEV protease was added to a CITx-TEV-HT-cometGFP samples and left to cleave off the HT – GFP tag at either 4 °C or 37 °C for 4 hr. The reaction mixtures were analysed by SDS-PAGE (**Figure 3.12**) and the results showed that at both temperatures there was an extra band at 27.3 kDa. Also, comparing the TEV cleavage reactions at 4 °C and 37 °C, it was apparent that better cleavage was observed at 37 °C. The 27.3 kDa size protein was smaller than the CITx-TEV-HT-cometGFP (32.1 kDa) protein, suggesting that the small CITx was being cleaved off its tag. However, the limited resolution of the gel was not sufficient to reveal the cleaved CITx band.

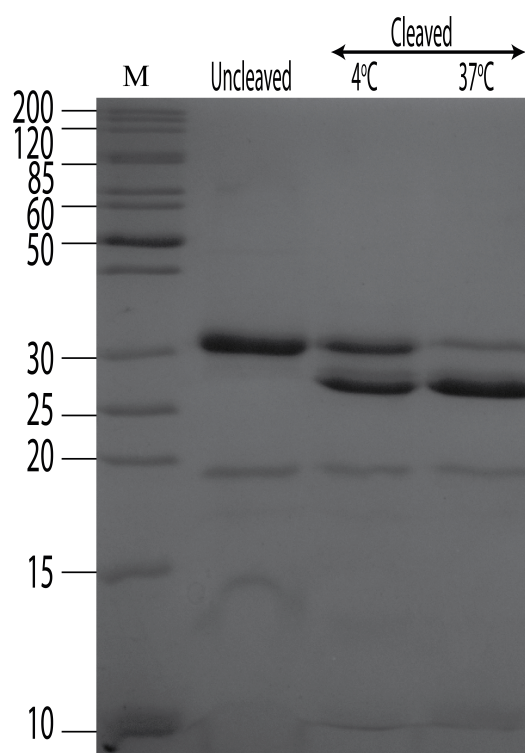


Figure 3.12 | SDS-PAGE analysis of the cleavage products from cometGFP using TEV protease. SDS-PAGE (15% w/v acrylamide) showing the products of TEV cleavage of CITx-TEV-HT-cometGFP at 4 °C or 37 °C for 4 hr, as indicated. TEV cleavage is somewhat better at 37 °C . The intact CITx-TEV-HT-cometGFP (32.1 kDa) is shown as control while the cleaved HT-cometGFP (27.3 kDa) tag is clearly visible. The small CITx (4.8 kDa) could not be seen because of the limited resolution of this gel. M: Protein Ladder used as a marker showing molecular weights in kDa.

As the ClTx (4.8 kDa) band could not be resolved clearly, a sample of the ClTx-TEV-HT-cometGFP after TEV protease treatment was analysed by Electron-spray-ionization time-of-flight (ESI-TOF+) mass spectrometry (**Figure 3.13**). The mass spectrometry data revealed 27334 Da and 4687 Da peak. These peaks correspond accurately to the molecular weights of HT-cometGFP and ClTx, respectively confirming that the TEV cleavage was successful and that ClTx can be purified as non-tagged protein.

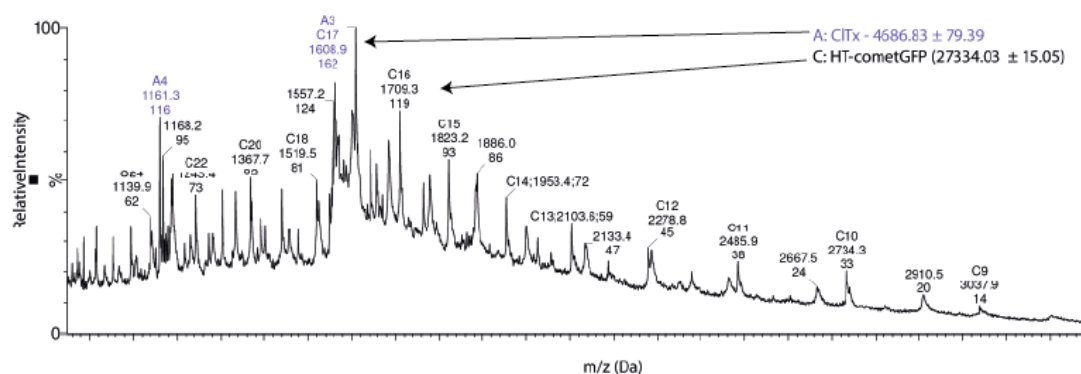


Figure 3.13 | Time-of-flight electron spray ionisation positive mass spectrum of the ClTx-TEV-HT-cometGFP sample after proteolysis with the TEV protease. The spectra show the mass to charge ratio of two proteins at 27.3 kDa and 4.8 kDa corresponding to the molecular weights of HT-cometGFP and ClTx, respectively. The number after the letter corresponds to the charge state of the protein at that particular m/z value. (e.g: A4 = 4686/1161 = 4)

3.8 Purification of ClTx

ClTx-TEV-HT-cometGFP expressing cells were cultured, harvested and suspended in sonication buffer, as described before. The bacterial extract was clarified by centrifugation to separate the insoluble and soluble fractions. The soluble fraction was loaded onto a GE Healthcare Life Sciences 5 mL HisTrap™ HP column pre-equilibrated with the HisTrap wash buffer and the column was then washed with HisTrap wash buffer. The ClTx-TEV-HT-cometGFP was eluted by running a gradient of increasing concentration of imidazole (50 mM to 400 mM). The chromatogram and SDS-PAGE (**Figure 3.14**) showed that the ClTx-TEV-HT-cometGFP started to elute at approximately 100 mM imidazole. The purity of ClTx-TEV-HT-cometGFP was assessed by SDS-PAGE, as shown in **Figure 3.14B**.

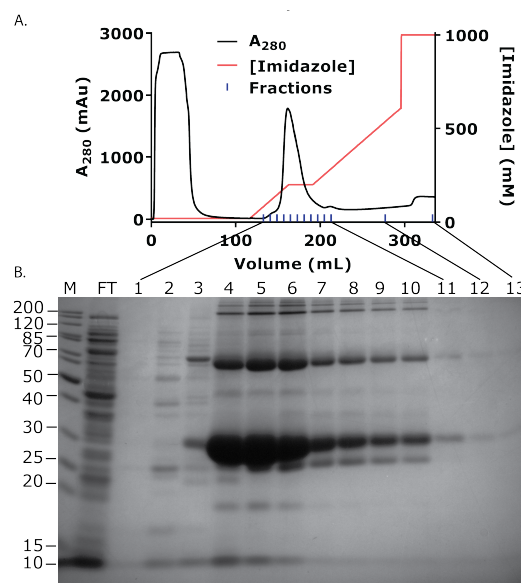


Figure 3.14 | IMAC of CITx-TEV-HT-cometGFP. HisTrap wash buffer A: 50 mM Tris pH 7.5, 500 mM NaCl and 20 mM Imidazole; HisTrap elution buffer B: 50 mM Tris pH 7.5, 500 mM NaCl and 1 M Imidazole. **A.** Chromatogram showing the protein elution profile from the HisTrapTM column. **B.** SDS-PAGE (15% w/v acrylamide) of eluted fractions (the numbers above the lanes correspond to the elution volumes at which the samples were taken for analysis) M: Protein Ladder used as a marker showing molecular weights in kDa FT: Flow-through containing unbound proteins when passed through the column.

The CITx-TEV-HT-cometGFP fractions were pooled and concentrated using 60% w/v ammonium sulphate precipitation followed by re-suspension in the HisTrap wash buffer. The concentrated CITx-TEV-HT-cometGFP protein was subjected to TEV cleavage at 4 °C overnight. Following TEV cleavage, the IMAC purification step was repeated to remove the HT-cometGFP tag (bound to the Ni²⁺ on the column) while the untagged CITx eluted in the flow-through (as shown in **Figure 3.15** Lane AC). Compared to the control sample before cleavage (**Figure 3.15** Lane BC), there is a clear decrease in the size of the major protein band from 32.1 kDa to the 27.3 kDa consistent with cleavage of the HT-cometGFP tag. Furthermore, in the flow-through a lower major band can be seen which corresponds to the 4.8 kDa untagged CITx protein, although the resolution of the gel is not high enough to accurately estimate its correct molecular weight when compared to the lower molecular weight marker at 10 kDa.

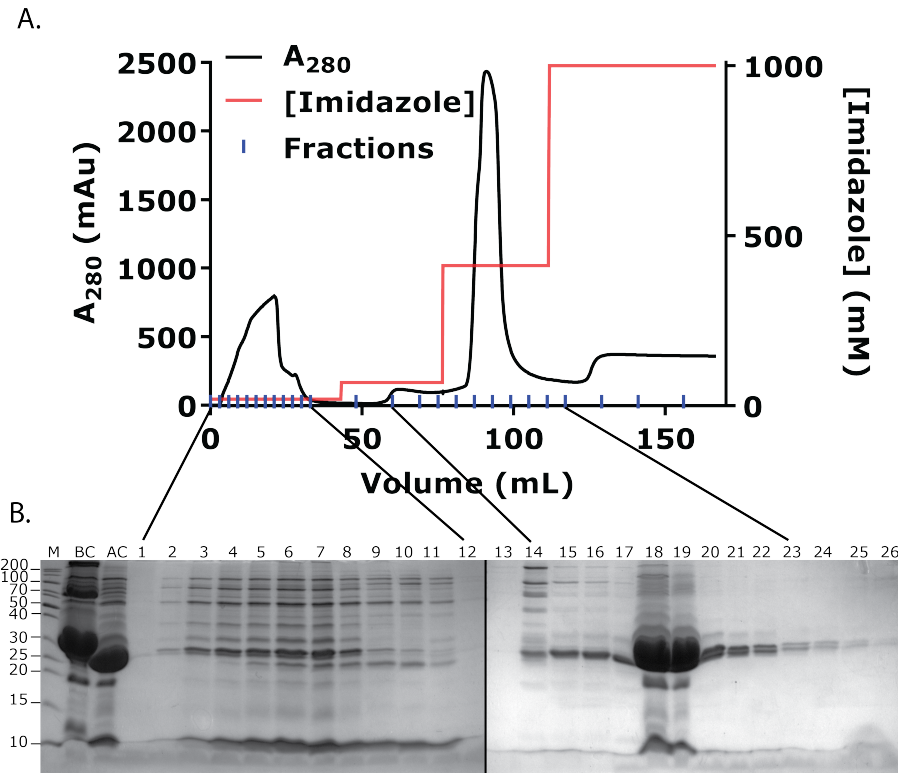


Figure 3.15 | Reverse IMAC to purify untagged CITx. HisTrap wash buffer A: 50 mM Tris pH 7.5, 500 mM NaCl and 20 mM Imidazole; HisTrap elution buffer B: 50 mM Tris pH 7.5, 500 mM NaCl and 1 M Imidazole. **A.** Chromatogram showing the protein elution profile from the HisTrap™ column. **B.** SDS-PAGE (15% w/v acrylamide) of various fractions, as indicated (the numbers above the lanes correspond to the elution volume at which each sample was taken for analysis) M: Protein Ladder used as a marker showing molecular weights in kDa BC: CITx sample before cleaved using TEV protease. AC: CITx sample after cleaved using TEV protease.

The flow-through was collected and the remaining contaminants were separated from the untagged CITx protein by gel filtration using a GE Healthcare Life Sciences HiLoad™ 16/600 Superdex 75 pg column. The eluted fractions from gel filtration were assessed by SDS- PAGE, as shown in **Figure 3.16**. Gel filtration separated higher molecular weight contaminants from the CITx leaving only small amounts of trace contaminants in the final untagged CITx preparation.

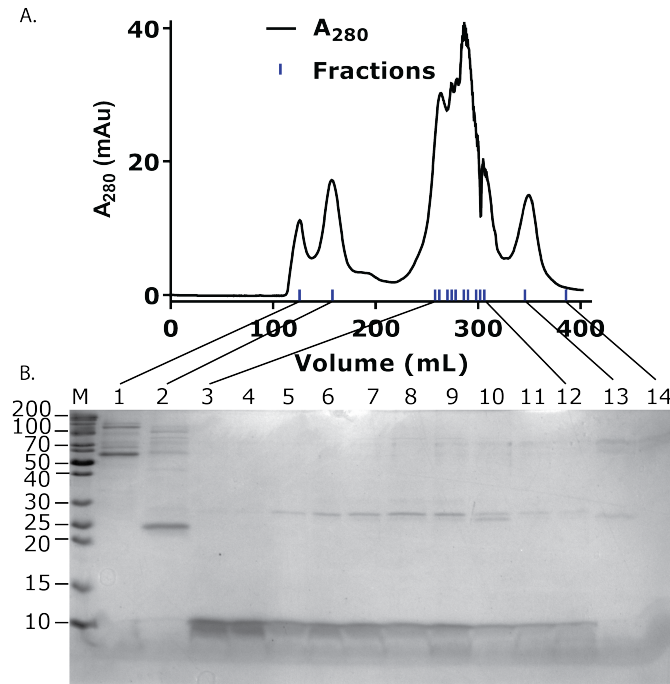


Figure 3.16 | Size-exclusion chromatography of CITx. Buffer: 200 mM ammonium acetate pH 7.5. **A.** Chromatogram showing the elution profile from the size-exclusion chromatography purification step of untagged CITx. **B.** SDS-PAGE (15% w/v acrylamide) of eluted fractions (the numbers above the lanes correspond to the elution volume at which each sample was taken for analysis). M: Protein Ladder used as a marker showing molecular weights in kDa.

The untagged CITx protein fractions were pooled and desalted through a desalting column, before the CITx protein was lyophilized for storage **Figure 3.17**. Lyophilized CITx can be reconstituted in the buffer required for microscopy analysis or POH conjugation. Mass spectrometry further confirmed the purification of the untagged CITx protein **Figure 3.18**.

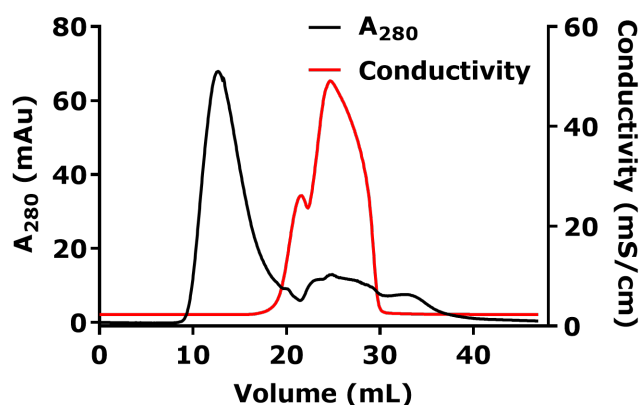


Figure 3.17 | Desalting of CITx. Untagged CITx was desalted using a GE Healthcare Life Sciences HiTrap™ 5x 5 mL desalting column. The desalted CITx fractions were pooled and the CITx protein was lyophilized for safe storage. The graph showed impurities (second peak) being separated from the CITx (major peak)

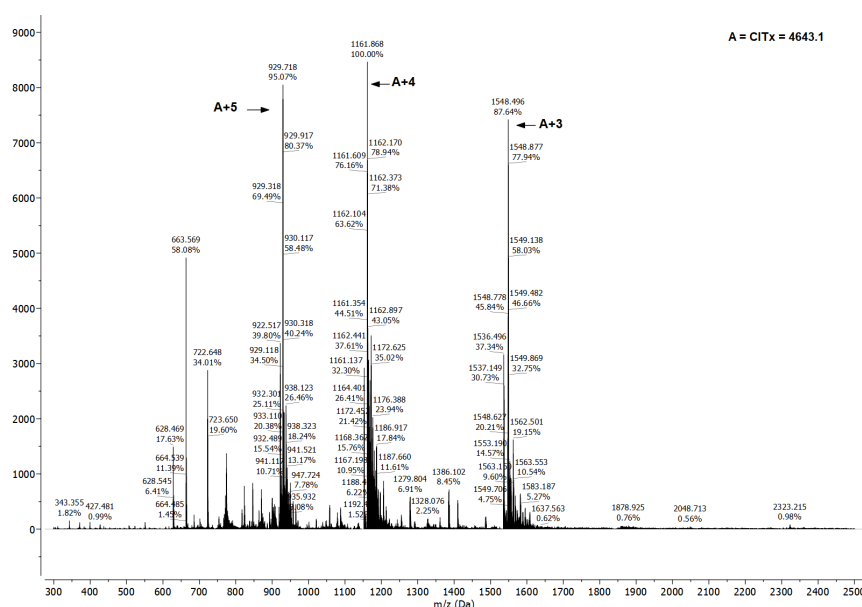


Figure 3.18 | Time-of-flight electron spray positive mass spectrum of CITx. The identity of the untagged CITx was confirmed with mass spectrometry. The spectrum revealed a protein at 4643 Da corresponding to the CITx with 4 disulfide bonds and the N-terminal methionine cleaved off. The number after the letter corresponds to the charge state of the protein at that particular m/z value. (e.g: A+4 = 4643/1162 = 4)

3.9 Activity analysis of CITx

The Transwell™ invasion assay was used to determine activity of CITx. The Transwell invasion assay is a quantitative *in vitro* assay that measures the invasive capabilities of cancer cells. The target for CITx is the MMP-2 which is involved in the breakdown

of the extracellular matrix and is vital for metastasis in glioblastoma tumours. It was hypothesized that addition of CITx should significantly inhibit the invasion capability of U87 cancer cells.

The Corning Matrigel Basement Membrane Matrix Growth Factor was diluted with serum-free DMEM to 2.5 mg/mL, followed by the addition of 50 μ L into each ThinCert™ cell culture inserts to create a two-chamber system with the two chambers separated by a cell-permeable membrane. U87 cells were seeded onto the top chamber of the ThinCert™ cell culture inserts. DMEM (with 10% v/v FBS) was added into bottom chamber. FBS acts as a chemo-attractant and encourages the migration of the U87 cells from the top to the lower chamber. In order to test whether CITx inhibits this migration, CITx was added to both chambers of the Transwell assay and the cells were cultured for 24 hr at 37 °C. Ice cold methanol was used to dissolve the Matrigel and fix the U87 cancer cells that had migrated through the Matrigel membrane and adhered to the bottom end of the ThinCert™ insert. The cells were stained with 0.5% w/v crystal violet and visualized using a fluorescence microscope (**Figure 3.19**).

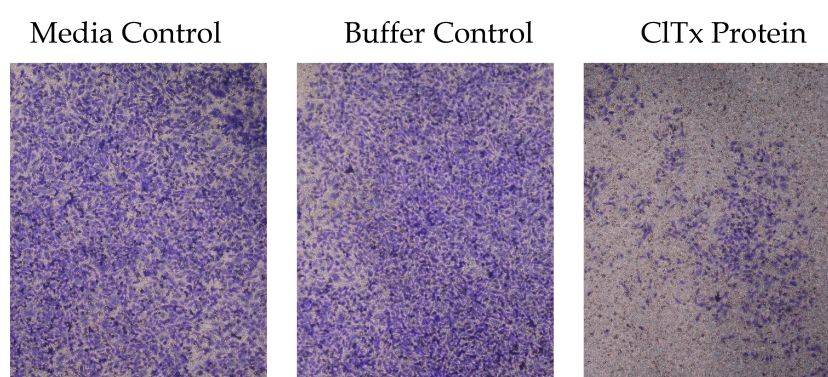


Figure 3.19 | Transwell invasion assay using CITx. U87 cells were imaged using a fluorescence microscope at 4x magnification. The media control has just DMEM media (with 1% v/v FBS) while the buffer control has 50 mM potassium phosphate buffer, pH 8.0 diluted with the DMEM (1% v/v FBS). The ThinCert™ insert containing the CITx (10 μ M) was dissolved in 50 mM potassium phosphate buffer, pH 8.0 and DMEM (1% v/v FBS).

As shown in **Figure 3.19**, there is a clear difference in the number of cells (stained in purple) present in the controls compared to the cells in the presence of the CITx protein. This suggests that the CITx is active, inhibiting the MMP-2 protein from breaking down the Matrigel membrane thus inhibiting the migration of the cancer cells through the membrane. This allows us to move onto the next stage and start conjugating POH to CITx to make a cytotoxic protein molecule that can both target and kill glioma cells.

3.10 Florescence analysis of CITx

Having established the ability of CITx to inhibit migration of U87 cancer cells, the binding of CITx to these cells using fluorescence microscopy was investigated. In order to do this, CITx was conjugated to Fluorescein.

NHS-Fluorescein (5/6-carboxyfluorescein Succinimidyl ester, NHS-FLR) contains the N-Hydroxysuccinimide (NHS) which can react with amines to form amides. CITx contains three lysines which can be targeted for NHS-FLR conjugation.

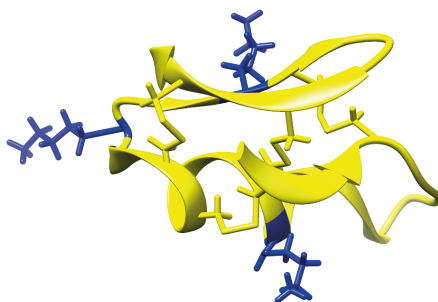
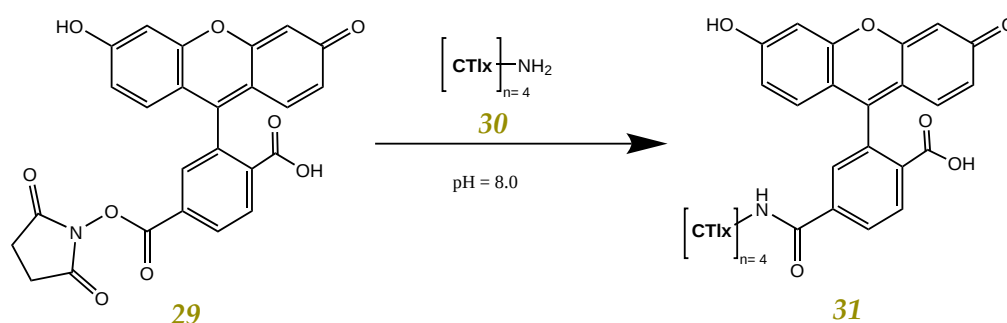


Figure 3.20 | Structure of CITx highlighting the lysine residues (blue) (1CHL)⁴⁰. There are 3 lysine (K) residues in CITx, K15, K23 and K27. Including the N-terminal amine there is 4 possible conjugation sites and currently there is no control which of these amines will be conjugated and therefore can potentially end up with any combination of these being conjugated to the NHS-FLR



Scheme 3.1 | Conjugation of NHS-FLR to CITx

NHS-FLR was added to CITx at a molar ratio of 1:10 CITx:FLR and left to react at room temperature for 2 hr. Excess, unreacted NHS-FLR was then removed by dialysis and the CITx was assessed by SDS-PAGE and visualization of the gel under UV (**Figure 3.21**) and mass spectrometry (**Figure 3.22**).

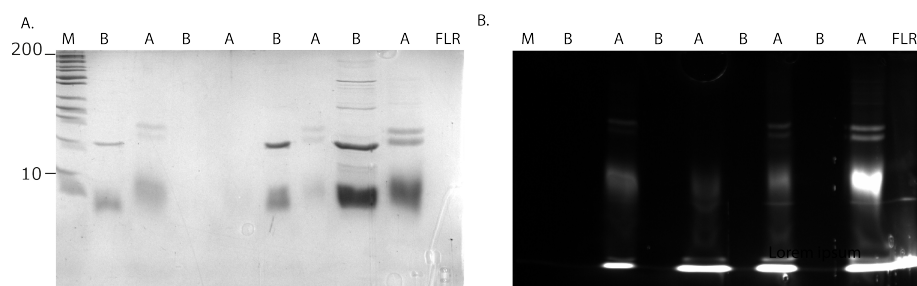


Figure 3.21 | Conjugation of FLR to CITx. **A.** SDS-PAGE and visualization of the gel under normal visible light showing the CITx protein before (lanes B) and after (lanes A) conjugation with an increase in concentration for each pair from left to right. **B.** The same gel as in panel A but visualized under UV light, showing the FLR conjugated to the CITx protein (compare lanes A exhibiting fluorescence with lanes B with no fluorescence). Lanes M and FLR show molecular weight markers (kDa) and FLR alone, respectively. The strong fluorescent band at the bottom of the gel that is visible only in lanes A in panel B is free FLR.

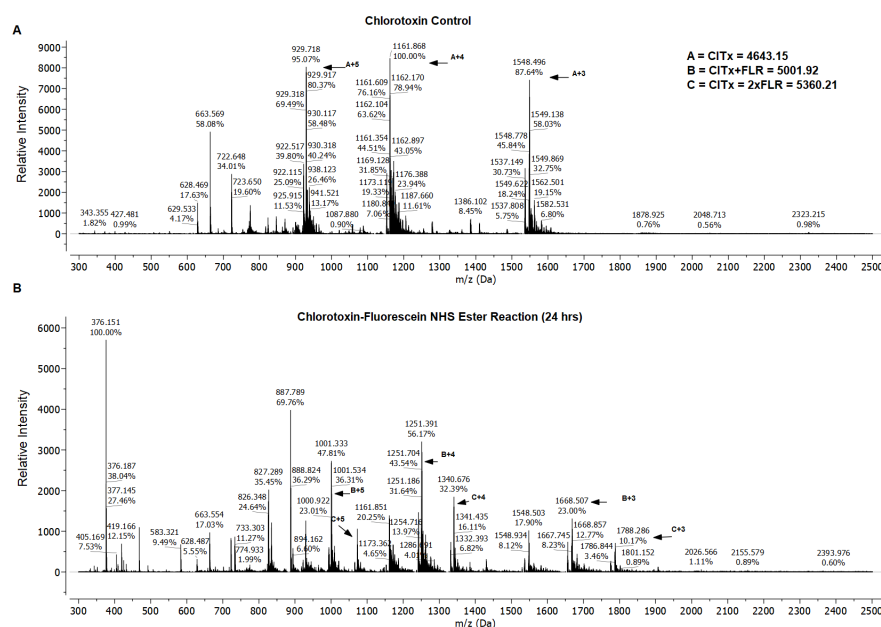


Figure 3.22 | Mass spectrum showing conjugation of fluorescein (FLR) to CITx. The spectrum was obtained using an electro spray ionization mass spectrometer **A.** Spectrum from the CITx control before conjugation. **B.** Spectrum from the CITx-FLR conjugation after 24 hr conjugation of CITx with FLR. This spectrum shows that the CITx was conjugated with FLR at either one or two of the three available lysines. The number after the letter corresponds to the charge state of the protein at that particular m/z value. (e.g: $A+4 = 4643/1162 = 4$)

In order to visualize CITx binding to U87 glioma cells, CITx-FLR (10 μ M) was added into U87 glioma cell cultures seeded onto eight-well chamber slides. The slides were incubated at 37 $^{\circ}$ C for 24 hr, washed, fixed, stained with DAPI and cover slipped. Finally, the slides were visualized with a fluorescence microscope.

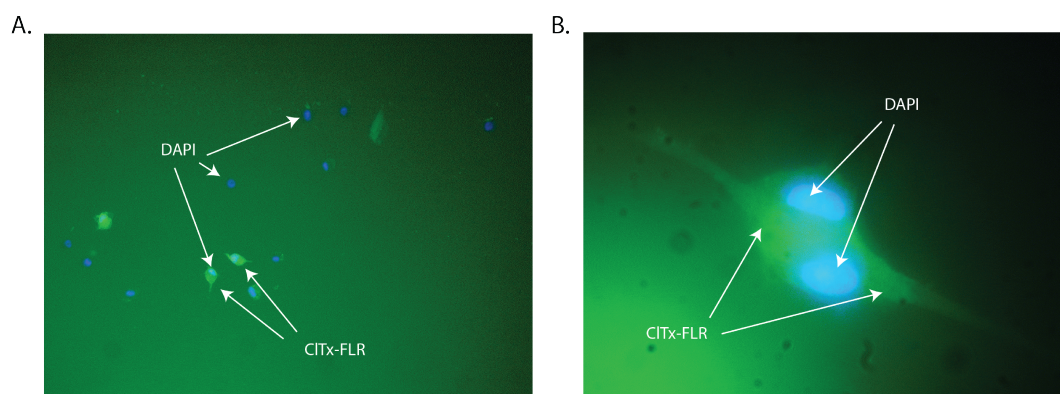


Figure 3.23 | Fluorescence microscopy of CITx-FLR binding to U87 glioma using plastic slides. U87 glioma cells were incubated for 24 hr in the presence of CITx-FLR (10 μ M (Green)). The cells were then fixed, stained with DAPI (Blue) and cover slipped. **A.** Lower magnification field view showing CITx-FLR staining of U87 cells and DAPI staining of cell nuclei, as indicated by arrows. **B.** Higher magnification showing CITx-FLR and DAPI staining in a dividing U87 cell, as indicated by the arrows

Figure 3.23 shows clearly the presence of green halos surrounding the U87 glioma cells and DAPI staining of cell nuclei. However, there was heavy background fluorescence which could not be eliminated with changing conditions (e.g. CITx concentration and incubation times). Further investigations revealed that the problem of background fluorescence was caused by FLR adsorbing onto the plastic surfaces of the slides and could not be removed in the wash stages of the slide cover-slipping process. Background fluorescence was eliminated by switching to glass slides (**Figure 3.24**).

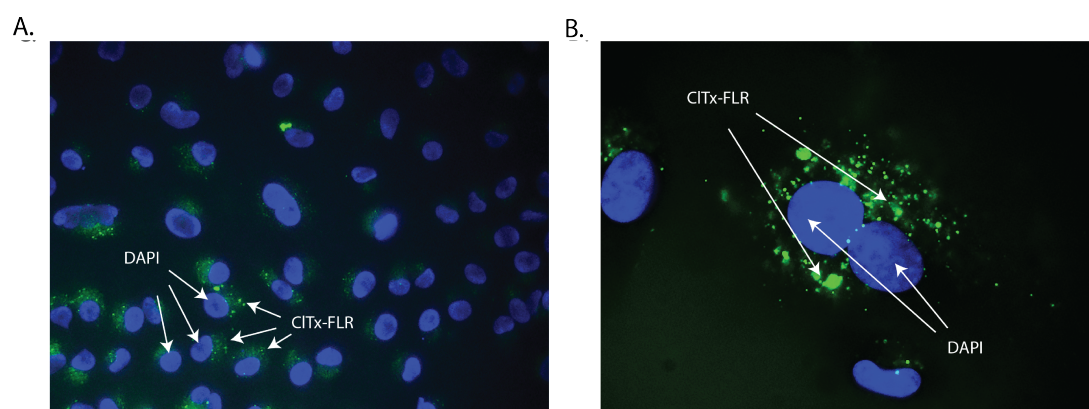


Figure 3.24 | Fluorescence microscopy of CITx-FLR binding to U87 glioma cells using glass slides. U87 glioma cells incubated for 24 hr in the presence of CITx-FLR (10 μ M (Green)). The cells were then fixed, stained with DAPI (Blue) and cover slipped. **A.** Low magnification wide view of CITx-FLR staining of U87 glioma cells and DAPI staining of cell nuclei, as indicated by the arrows. **B.** High magnification view of individual cells stained with CITx-FLR and DAPI, as indicated by the arrows.

As shown in **Figure 3.24**, using glass instead of plastic slides eliminates the problem of background fluorescence. These images also revealed that CITx-FLR was being internalized by the U87 glioma cells and was visible inside the cytoplasm of the cells.

Cellular internalization of CITx-FLR could be due to endocytosis of the CITx-MMP-2 complex.⁴⁶ Next, the time required for the CITx-FLR protein to enter into the U87 glioma cells was investigated. Further experiments were carried out with the same method of seeding the U87 glioma cells onto chamber slides, incubating them with the CITx-FLR protein and visualizing them with fluorescence microscopy at 20, 40 and 60 min (**Figure 3.25**).

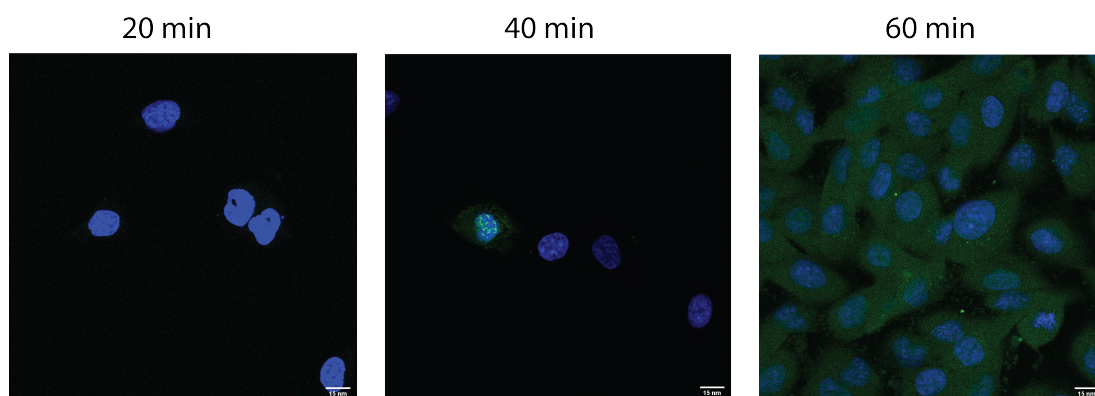


Figure 3.25 | Time interval fluorescence microscopy of CITx-FLR and DAPI staining of U87 glioma cells. U87 glioma cells were stained with CITx-FLR (10 μ M (Green)) and DAPI (blue) and visualized with fluorescence microscopy at 20, 40 and 60 min.

No CITx-FLR staining of the U87 glioma cells was visible at 20 min but some staining started to appear after 40 min with heavy staining appearing at 60 min. This indicates that CITx-FLR starts to bind to the MMP-2 on the surface of the U87 cells at approximately 40 min and after 60 min it is fully bound and internalized into the cells, under experimental conditions. These data suggest that CITx conjugates have a relatively fast response time for binding and internalizing into glioma cells, rendering CITx a viable drug delivery system to deliver cytotoxic ligands into glioma cancer cells.

In summary, Soluble CITx was purified that does not require refolding and maintains its anti-metastatic activity, as shown in the Transwell invasion assay. Furthermore, FLR-conjugated CITx binds to its target MMP-2 proteins on the surface of the U87 glioma cells and is internalized into the cytosol of the cells within 40-60

min, as shown fluorescence microscopy, rendering it a viable anti-cancer drug delivery system. In order to exploit the anti-cancer drug delivery potential of ClTx, ligands were designed with anti-cancer drug activities that can be conjugated to ClTx and delivered directly and specifically to glioblastoma cancer cells.

4

Synthesis and conjugation of CITx ligands

CITx-nano-particles (CITx-Nps) have been previously used for imaging and treatment of glioma cells. CITx conjugated to either iron oxide NPs,⁶¹ quantum dots or rare-earth NPs are used for magnetic resonance imaging and fluorescent labelling of glioma cells.⁵⁹ CITx-NPs have also been used as anticancer drug delivery systems in glioma chemotherapy.⁵⁹

CITx has been conjugated to onconase (Onc).⁶⁰ Onconase is a small cytotoxic ribonuclease that degrades RNA substrates by targeting uracil and guanine nucleotides.¹⁰² CITx was linked to onconase via a reversible disulfide bond to form CITx-Onc conjugate which had more potent cytotoxicity than a mixture of CITx and Onc (CITx + Onc), showing that CITx-Onc could be a potential anticancer drug.⁶⁰

Many non-specific small molecule anticancer drugs tend to be toxic with short half-lives.⁶¹ For example, gemcitabine, a non-specific anti-cancer drug, is used to treat stage 4 bladder and pancreatic cancers, and in combination with carboplatin is also used to treat lung cancer.¹⁰³ Gemcitabine is a nucleoside analog, with fluorines replacing the hydrogen atoms on the 2' carbon of deoxycytidine.¹⁰³ Gemcitabine acts as a replacement for nucleotides during DNA replication which results in inhibition of DNA replication and hence tumour cell growth, leading to apoptosis.¹⁰³ A CITx-gemcitabine-iron oxide NP conjugate has been reported to be a potential anti-cancer drug.⁶¹ The NPs preserve the gemcitabine potency, compared to free gemcitabine. The

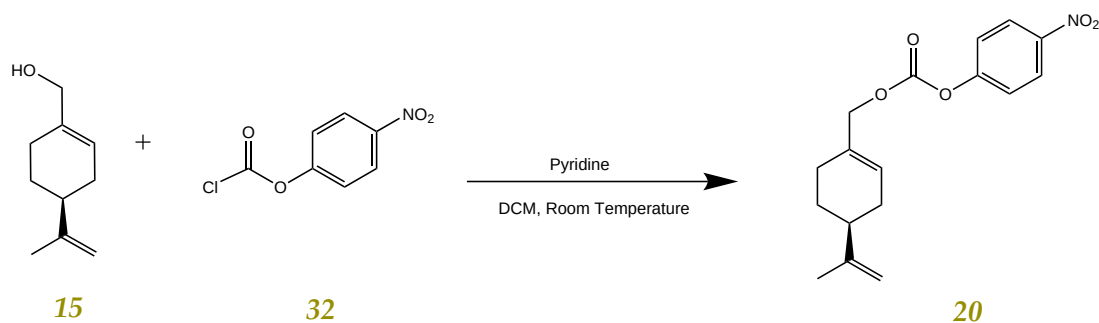
CITx-gemcitabine conjugate facilitates crossing of the BBB, specific targeting of glioma cancer cells and extends the drug's half-life in the blood.⁶¹

POH has been previously conjugated to TMZ via a carbamate bond.¹⁰⁴ TMZ, like CITx can cross the BBB and the TMZ-POH was used in chemotherapeutic treatment for glioblastoma under the name NEO212. The TMZ-POH was found to be a better anticancer drug than the TMZ on its own or a mixture of TMZ and POH.¹⁰⁴ This is due to the conjugation of POH to TMZ that increased the half-life of TMZ and allowed a greater cytotoxic effect, causing DNA damage and cell death more efficiently than unconjugated, free TMZ.¹⁰⁴ TMZ-POH also exhibited good tolerance with no side effects in mouse models, making it a promising potential anti-cancer drug.¹⁰⁴

4.0.1 4-nitrophenyl perillyl carbonate (4NPC)

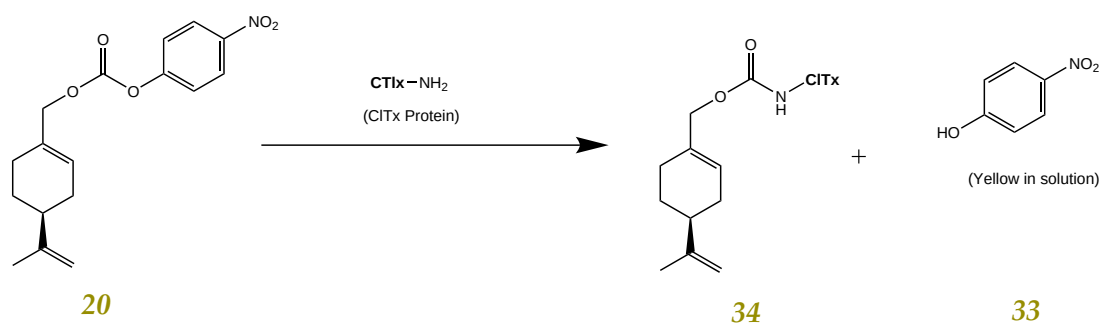
The first ligand synthesized was 4-nitrophenyl perillyl carbonate (4NPC) **20**. POH needs to be modified so that it can react with CITx. This was done by modifying the OH group of POH to a carbonate, and also by introducing a nitrophenyl group to form 4NPC (**Scheme 4.1**). The *p*-nitrophenyl moiety is a good leaving group, and the carbonate group can react with the primary amines of any of the three lysine residues or N-terminal in CITx via a carbamoylation reaction to form a carbamate linkage between the 4NPC and CITx. The carbamate linker is similar to the linker found in the NEO212 (TMZ-POH) conjugate. Carbamate is a permanent linker and should remain intact when CITx-4NPC conjugate enters into the cancer cells by endocytosis.

The first step was the addition of 4-nitrophenyl chloroformate **32** and pyridine to a solution of POH **15** dissolved in dichloromethane (DCM). After confirming the completion of the reaction via TLC analysis, 4NPC **20** was extracted and concentrated *in vacuo* to give 4NPC as a white solid crystal. The 4NPC synthesis was confirmed via mass spectrometry, ¹H NMR and ¹³C NMR.



Scheme 4.1 | Formation of 4-nitrophenyl perillyl carbonate (4NPC)

The next step was the conjugation of 4NPC to CITx (**Scheme 4.2**). Nitrophenol **33** is a by-product, with yellow colour in solution, formed during this reaction. This served as a visual marker with the colourless solution turning yellow as the 4NPC was reacting to form nitrophenol.



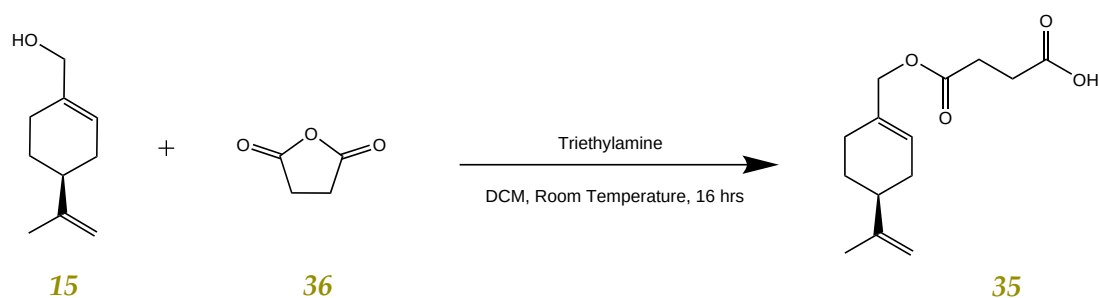
Scheme 4.2 | Conjugation of 4-nitrophenyl perillyl carbonate (4NPC) to CITx

The first stage of the reaction was the preparation of CITx. Due to the 4NPC reacting with primary amines, buffers such as Tris cannot be used. Therefore, CITx was dialyzed into potassium phosphate buffer (50 mM, pH 8.0). The solubility of 4NPC was also tested in this buffer but the solution turned yellow, suggesting nitrophenol formation even though there were no amines present. Changing the pH of the potassium phosphate buffer (pH 5.8, 6.2, 6.6, 7.0, 7.4 and 8.0) showed that the colour change was pH sensitive and that a buffer at pH 6.2 was optimal for the conjugation reaction, as there was no colour change at this pH. Unfortunately, the 4NPC was found to precipitate when diluted into this buffer. Adding high concentration of acetonitrile (up to 40% v/v), Tween 20 (up to 10% v/v) and Triton X-100 (up to 5% v/v) did not prevent the precipitation. Higher concentrations of these reagents could result in protein unfolding, hence other POH ligands were considered for conjugation to CITx.

4.0.2 Perillyl 3-carboxypropanoate (P3C)

The next ligand that was considered was perillyl 3-carboxypropanoate (P3C) **35**. This ligand modified the OH group to an ester and introduced a carboxylic acid group at the end of the ligand. Having an ester group will allow the esterases (enzymes that cleave ester bonds) found in glioma cells to cleave the P3C from CITx protein via ester hydrolysis. Furthermore, the carboxylic acid group will enable the reaction with any of the primary amines, found in the three lysine residues on the surface of the CITx protein or the N-terminal, using the EDC/NHS coupling method. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/ N-hydroxysuccinimide (NHS) coupling is the formation of a stable peptide bond between two molecules by the reaction of a primary amine group to a carboxylic acid group.

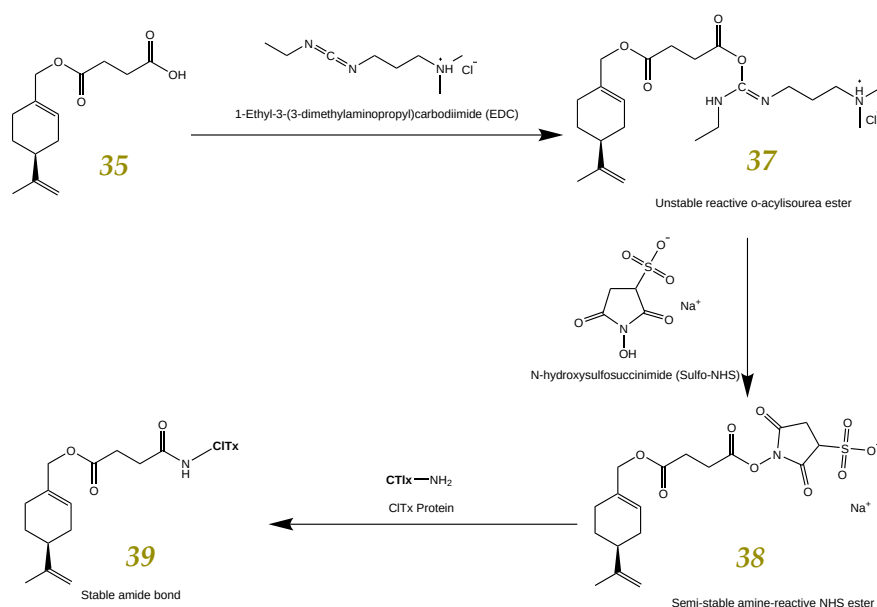
Formation of the perillyl 3-carboxypropanoate (**Scheme 4.3**) required perillyl alcohol **15** to react with succinic anhydride **36** in dichloromethane in the presence of triethylamine. The mixture was left to react for 16 hr and a dark purple colour change was noticed. The reaction was followed by TLC and upon completion of the reaction, the mixture was washed with water brine, HCl and concentrated *in vacuo* to give the perillyl 3-carboxypropanoate **35** as a white solid. The perillyl 3- carboxypropanoate was analysed by mass spectrometry, ^1H NMR and ^{13}C NMR which confirmed the successful formation of the perillyl 3-carboxypropanoate.



Scheme 4.3 | Formation of perillyl 3-carboxypropanoate

The next step was the conjugation of P3C to CITx. This was done by the EDC/NHS coupling method, using Sulfo-NHS.⁶² Sulfo-NHS is normally used for chemical labelling, crosslinking and solid-phase immobilization of amine-reactive esters from carboxylate groups. Sulfo-NHS is soluble in both organic and aqueous solvents which

is helpful as CITx is soluble in aqueous solvents while the P3C is more soluble in organic solvents. Unlike EDC, sulfo-NHS is not required for the conjugation reaction, but it enhances the coupling reaction between the carboxylate and the amine, and further increases the water solubility of the active ester.¹⁰⁵ Furthermore, using Sulfo-NHS allows the reaction to be carried out in two steps (**Scheme 4.4**). The first step of the reaction is to react the perillyl 3-carboxypropanoate **35** with EDC which forms the unstable reactive *o*-acylisourea ester **37**. This ester is reacted with Sulfo-NHS to form the semi-stable amine-reactive Sulfo-NHS ester **38**. The Sulfo-NHS ester has a half-life of 4-5 hr at pH 7, 1 hr at pH 8 and only 10 min at pH 8.6. Finally, CITx protein was added into the reaction mixture to form the CITx-P3C conjugate **39** via a peptide bond formation.



Scheme 4.4 | Conjugation of perillyl 3-carboxypropanoate to CITx

The conjugation reaction was monitored by mass spectrometry. As shown below in **Figure 4.1**, the perillyl 3-carboxypropanoate which has a *m/z* of 251.42, successfully reacted with the EDC and the Sulfo-NHS to form the perillyl Sulfo-NHS ester (*m/z*: 428.96) after 15 min **38**.

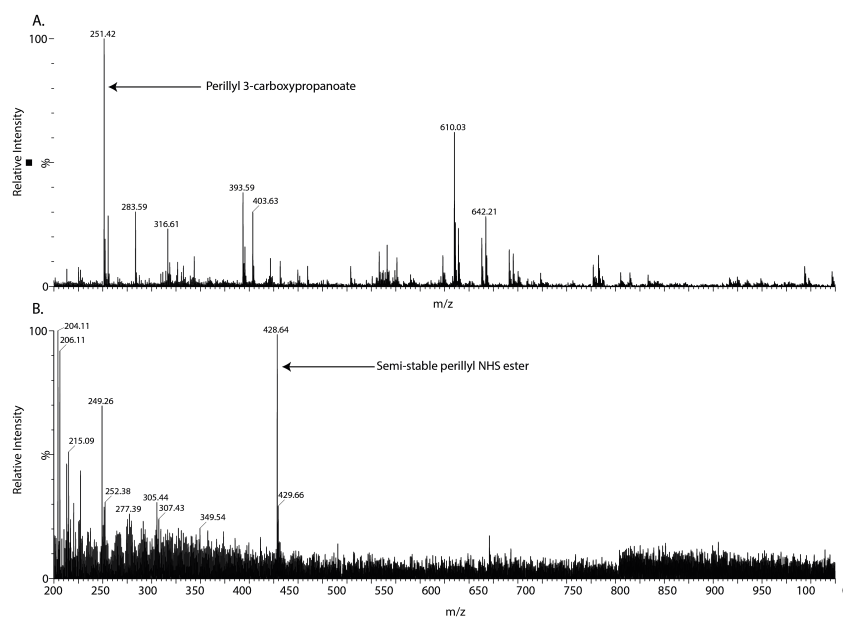


Figure 4.1 | Time-of-flight electron spray negative mass spectrum of perillyl 3-carboxypropanoate before and after EDC/Sulfo-NHS coupling. **A.** Perillyl 3-carboxypropanoate before the reaction. **B.** EDC/Sulfo-NHS reaction forming the semi-stable perillyl Sulfo-NHS ester.

This was followed by the perillyl sulfo-NHS ester conjugation with CITx (10: 1). As before, the reaction was monitored by mass spectrometry **Figure 4.2**. Unfortunately, mass spectra after both 1 hr and 2 hr into the reaction revealed no new conjugation peak and still showed the presence of the unconjugated CITx protein.

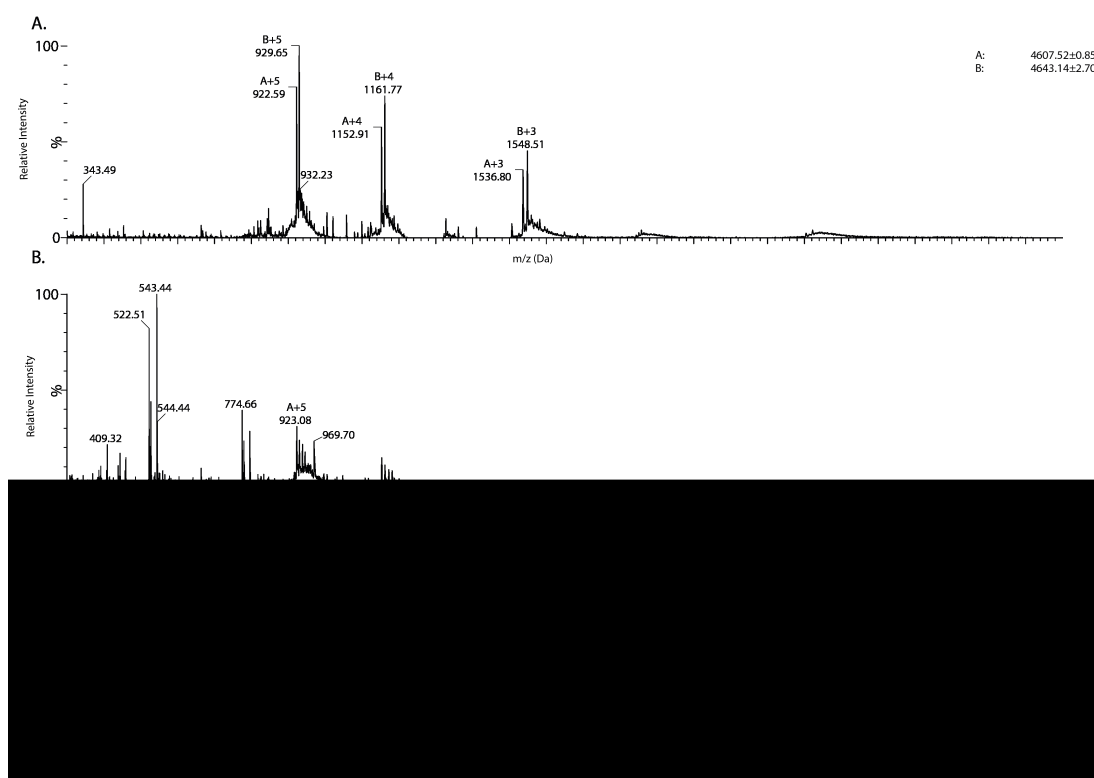


Figure 4.2 | Time-of-flight electron spray positive mass spectrum of CITx conjugating to perillyl 3-carboxypropanoate via the EDC/Sulfo-NHS reaction. **A.** CITx before the reaction. **B.** EDC/Sulfo-NHS reaction between perillyl 3-carboxypropanoate and CITx after 1 hr. **C.** EDC/Sulfo-NHS reaction between perillyl 3-carboxypropanoate and CITx after 2 hr. A: Acetylated CITx B: CITx. The number after the letter corresponds to the charge state of the protein at that particular m/z value. (e.g: B+4 = $4643/1162 = 4$)

The reaction time was increased to 24 hr and then to 48 hr but still no clear peaks were detectable in the spectra to confirm that the conjugation reaction was successful **Figure 4.3**. Even though the Sulfo-NHS ester ligand of P3C was observed by mass spectrometry, due to its semi stable nature, it broke down before it could react with CITx. In order to solve this problem, different buffers was used with varying pH during the P3C/EDC/Sulfo-NHS and CITx/P3C-NHS reactions (**Table 4.1**). Unfortunately, none of these reactions revealed peaks corresponding to P3C conjugating to CITx.

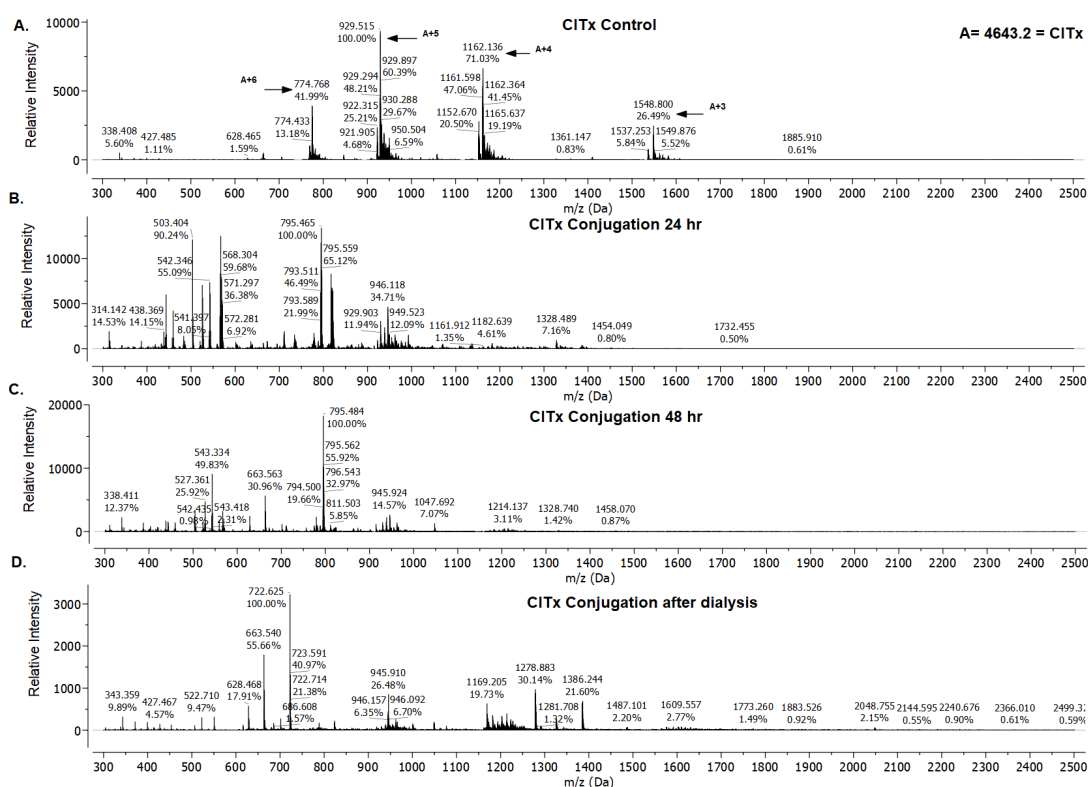


Figure 4.3 | Time-of-flight electron spray positive mass spectrum of CITx conjugating to perillyl 3-carboxypropanoate via the EDC/Sulfo-NHS reaction after 24 and 48 hr. **A.** CITx control before the conjugation reaction. **B.** EDC/Sulfo-NHS reaction between perillyl 3-carboxypropanoate and CITx after 24 hr. **C.** EDC/Sulfo-NHS reaction between perillyl 3-carboxypropanoate and CITx after 48 hr **D.** EDC/Sulfo-NHS reaction between perillyl 3-carboxypropanoate and CITx after removal of all reactants via dialysis. The number after the letter corresponds to the charge state of the protein at that particular m/z value. (e.g: A+4 = 4643/1162 = 4)

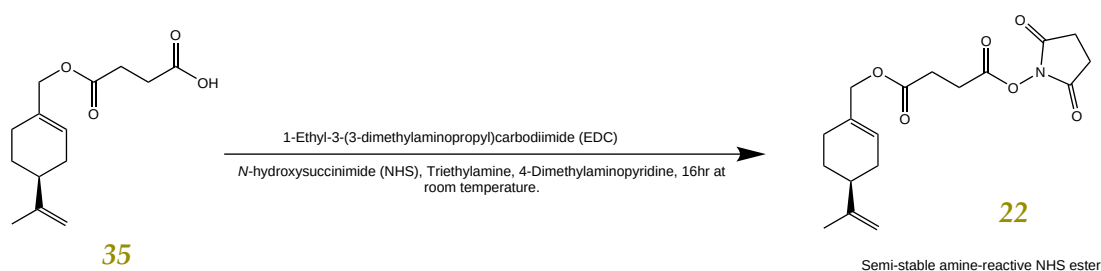
Table 4.1 | Buffer Conditions used during the EDC/Sulfo-NHS coupling of POH to CITx.

Reaction Number	P3C Buffer	CITx Buffer
1	50 mM Phosphate pH 8.0	50 mM Phosphate pH 8.0
2	50 mM Phosphate pH 6.0	50 mM Phosphate pH 8.0
3	80% v/v Acetonitrile	50 mM Phosphate pH 8.0
4	50 mM MES pH 6.0	50 mM Phosphate pH 8.0
5	80% v/v/ Acetonitrile	50 mM Sodium Carbonate pH 9.0

The combined data suggest that further modification of the P3C is necessary for the successful conjugation of the ligand to CITx. Therefore, focus was put on synthesizing a perillyl 3-carboxypropanoate NHS ester ligand (P3C-NHS ester).

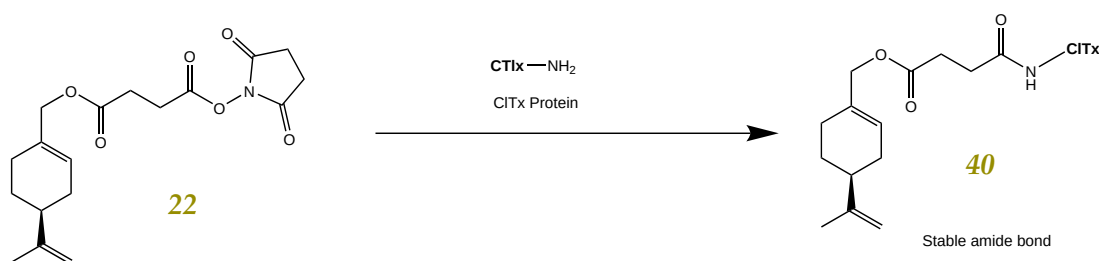
4.0.3 Perillyl 3-carboxypropanoate NHS ester. (P3C-NHS ester)

As shown above, none of the methods using P3C resulted in successful conjugation of P3C to CLTX. Instead, it was decided to modify the P3C to introduce an NHS ester and then directly add this to the CLTX protein. This was shown to be possible during CLTX-FLR conjugation which successfully used the NHS ester group for the conjugation of FLR to CLTX. Therefore, instead of using Sulfo-NHS, N-hydroxysuccinimide (NHS) was used to make perillyl 3-carboxypropanoate NHS ester (P3C-NHS). The NHS ester was synthesized following the same EDC/NHS coupling method as shown in **Scheme 4.5**. TLC was used to confirm completion of the reaction and silica column chromatography was used for the separation of the perillyl 3-carboxypropanoate NHS ester from the remaining reactants. NMR and mass spectrometry confirmed the formation of the compound.



Scheme 4.5 | Formation of perillyl 3-carboxypropanoate NHS ester (P3C NHS ester).

The purified P3C-NHS ester was dissolved in DMSO and then added to CLTX at a molar ratio of 1:10 (CLTX: P3C-NHS) and left to react at room temperature for 24 hr. The reaction mixture was analysed by mass spectrometry **Scheme 4.6**.



Scheme 4.6 | Conjugation of perillyl 3-carboxypropanoate NHS ester (P3C NHS) to CLTX.

As shown in **Figure 4.4**, CITx has conjugated to either one or two (out of the three) lysine residues present on the surface of CITx. Furthermore, the non-conjugated CITx peak can barely be seen after the reaction, suggesting that almost all of CITx has been conjugated to P3C-NHS to form the CITx-P3C conjugate. Therefore, CITx-P3C was carried forward to cell biology experiments in order to test its cytotoxic properties.

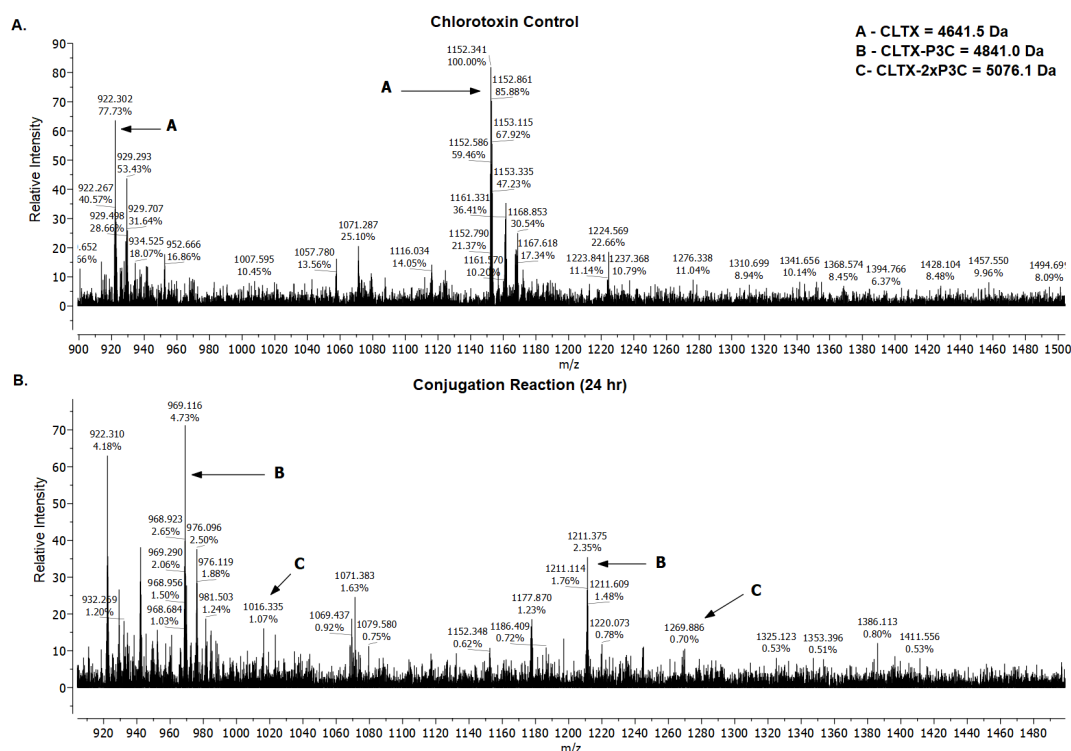


Figure 4.4 | Time-of-flight electron spray positive mass spectrum of CITx conjugating to perillyl 3-carboxypropanoate NHS ester. **A.** CITx control before conjugation. **B.** Reaction between perillyl 3-carboxypropanoate NHS ester and CITx after 24 hr showing the POH conjugated to either one or two of the possible three lysine residues present on the surface of CITx.

4.0.4 Testing the cytotoxicity of CITx-POH

The cytotoxic properties of CITx were assessed using the presto blue assay. U87 cells were cultured for 24 hr at 37 °C in flasks, prior to the addition of CITx-P3C at the indicated concentrations. The cells were cultured for a further 24 hr and the medium from each well was removed, the well washed with HBSS and Presto blue added into each well. The cells were incubated with presto blue for 1 hr at 37 °C, the solution from each well was transferred into a Corning™ 96-Well Solid Black plate and the absorbance at 570 nm was measured using a plate reader **Figure 4.5**.

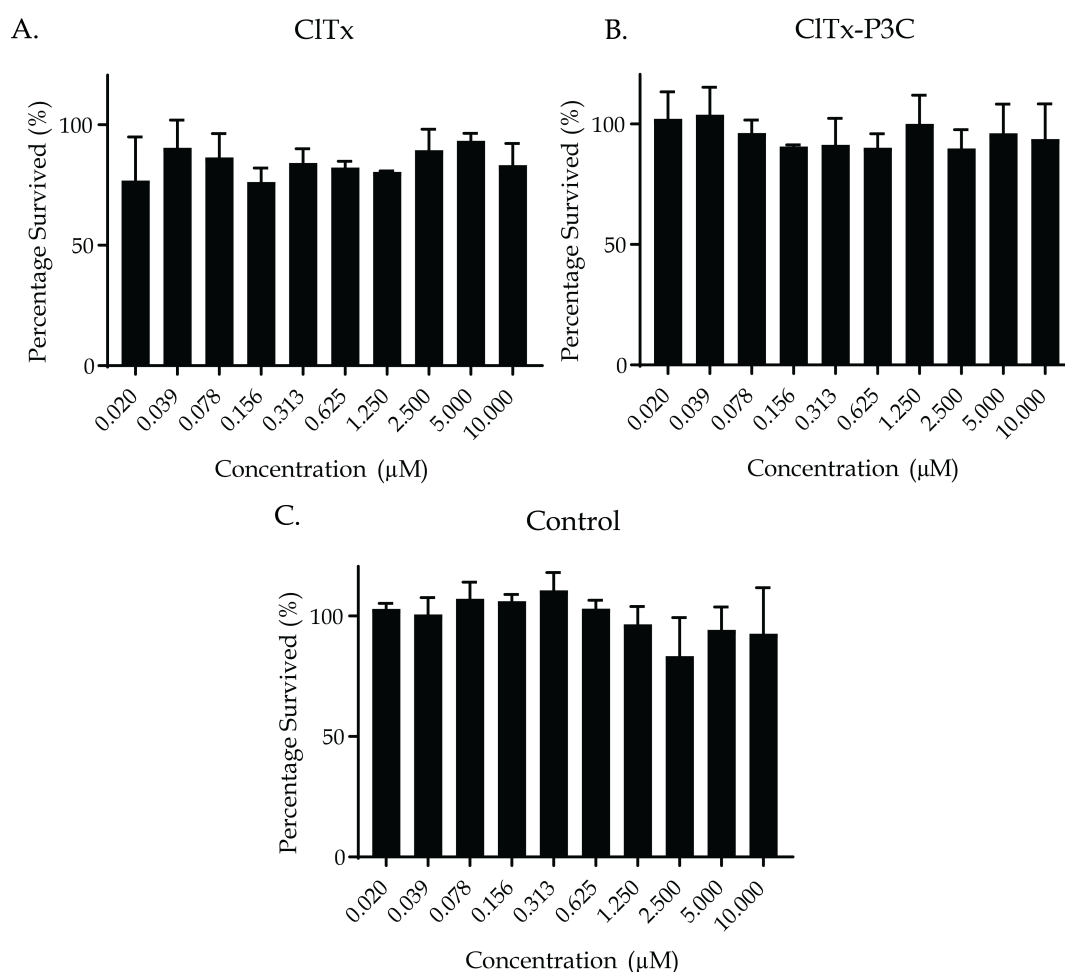


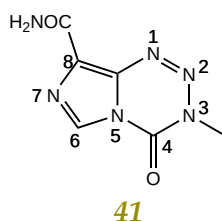
Figure 4.5 | Presto blue cytotoxic assays showing the effect of CITx and CITx-P3C on U87 glioma cells. **A.** Untagged CITx control. **B.** CITx-P3C conjugate. **C.** Media Control. The concentrations (μM) of CITx or CITx-P3C are shown under the corresponding bars. Percentage survival was calculated against a control of media. Error bars show the SD from triplicate repeat experiments.

The data from the CITx control and the CITx-P3C conjugate were comparable suggesting that the P3C has no cytotoxic effect on the U87 glioma cells. The lack of cytotoxicity could be due to failure of CITx-P3C conjugate to bind and enter into the U87 cells or due to the failure of intracellular esterases to cleave off the P3C from CITx. If the conjugated P3C ligand does not detach from CITx inside the cytosol it may not be able to exhibit cytotoxic activity. Alternatively, it may also be that the intracellular concentration of P3C under experimental conditions is not high enough have an effect on the cells, as POH has been reported to have an IC_{50} of 1.8 (mM).⁷⁴ This IC_{50} concentration is nearly 200x the concentration tested in this experiment. Raising CITx-P3C to such high concentrations would not be a financially viable option for pharmaceutical companies because of the cost associated with making large quantities

of CITx. Thus, it was decided to pursue an TMZ analog as a possible CITx conjugate that exhibits potent cytotoxicity at low concentrations.

4.1 N^3 Propargyl temozolomide (N^3P -TMZ)

As discussed previously, TMZ is the main chemotherapeutic drug used for the treatment of GBM. However, at least 50% of patients treated have shown resistance to TMZ.⁹⁶ The emergence of TMZ resistance has necessitated further work to identify TMZ analogues that could be used to treat TMZ resistant GBM. The modification of the functional groups attached on the N^3 position of TMZ has been exploited for the synthesis of novel TMZ variants. Cousin *et al.* synthesized a TMZ analogue by introducing a propargyl group at the N^3 position.⁹⁶

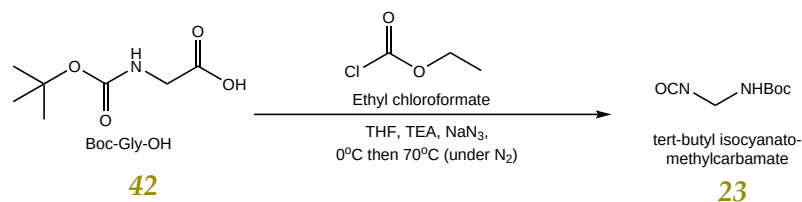


Scheme 4.7 | Structure of TMZ

This propargyl TMZ (N^3P -TMZ) exhibited good cytotoxic activity against TMZ resistant cells such as SNB19M and U373M, with GI_{50} values of 37.8 μ M and 36.1 μ M, respectively, compared to μ M values of 526 μ M and 395 μ M for TMZ.¹⁰⁶ Given its promising potential, synthesis of N^3P -TMZ was attempted with the possibility of modifying its amide bond to allow for conjugation to CITx. These linkers will also contain hydrazone and carbamate bonds to control the speed of release of the N^3P -TMZ from CITx when taken up by the cell to carry out cytotoxicity.

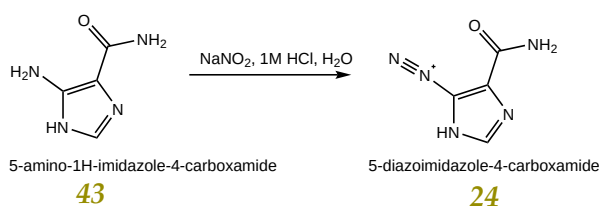
The first stage of the reaction involved the formation of the N -Boc protected temozolomide **25**. This involved synthesizing two precursors, N -Boc protected aminomethyl isocyanate **23** and 5-diazoimidazole-4-carboxamide **24**. The aminomethyl isocyanate was synthesized by modifying the N -Boc protected glycine **42** with the addition of ethyl chloroformate and then sodium azide causing the colourless solution to turn cloudy. After leaving the reaction to proceed for 1 hr, the organic layer was washed and extracted using toluene before heated slowly to 70 °C. The solution was

concentrated, with precaution taken to leave some toluene and prevent dryness, and was immediately used in the next reaction (Scheme 4.8).



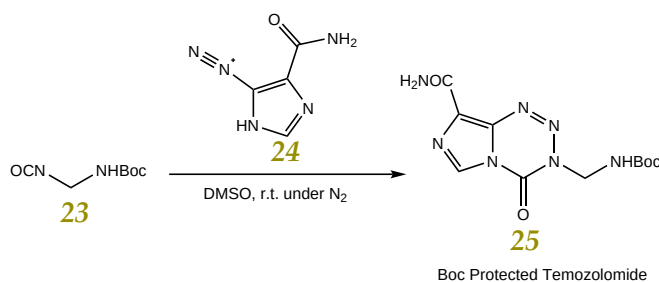
Scheme 4.8 | Synthesis of *tert*-butyl isocyanato-methylcarbamate

The second precursor was formed converting the amino groups in 5-amino-1*H*-imidazole-4-carboxamide **43** to diazonium groups. This was done by diazotation, where the starting material was reacted with sodium nitrite and HCl at 0 °C. After formation of a consistent orange precipitate, the crude product was washed (H₂O, EtOH, Et₂O) and dried to give 5-diazoimidazole-4-carboxamide **24** as an orange solid with a yield of 84%. As with the isocyanate the diazoimidazole carboxamide did not undergo any other purification steps, Scheme 4.9.



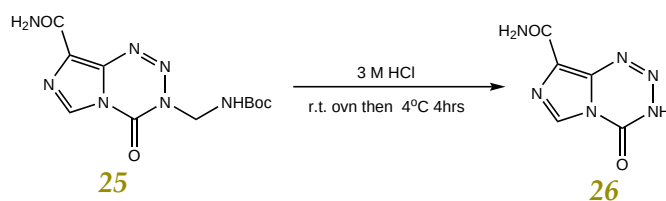
Scheme 4.9 | Synthesis of 5-diazoimidazole-4-carboxamide

The two precursors (*tert*-butyl isocyanato-methylcarbamate and 5-diazoimidazole-4-carboxamide) were then reacted with each other in the presence of DMSO for 16 hr at room temperature to form the *N*-boc protected TMZ **25**. This was then washed (H₂O, EtOAc, Et₂O) and dried to give the product, *N*³-boc temozolomide (*N*³-boc TMZ) as a pale pink solid with good yield of 69%. NMR and mass spectrometry confirmed the synthesis and purification of *N*³-boc TMZ **25** was successful.



Scheme 4.10 | Synthesis of *boc*-protected TMZ

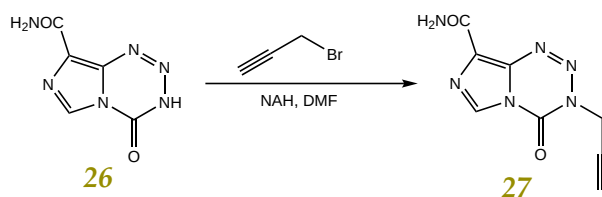
The next stage was the de-protection of the TMZ precursor. This was done by the addition of excess HCl and the reaction was left at room temperature for 16 hr, and then for another 4 hr at 4 °C to remove the N-Boc group. The precipitate was washed (H₂O, EtOAc, Et₂O) and filtered to give the *nor*-TMZ **26** as a pale pink solid with an excellent yield of 81%. Mass spectrometry confirmed the *nor*-TMZ product (**Appendix A4, Figure A.4**). NMR showed the disappearance of the peaks corresponding to the N-Boc group thus further confirming successful deprotection and forming the *nor*-TMZ **26** (**Appendix A5, Figure A.14-A-15**).



Scheme 4.11 | Synthesis of *nor*-TMZ

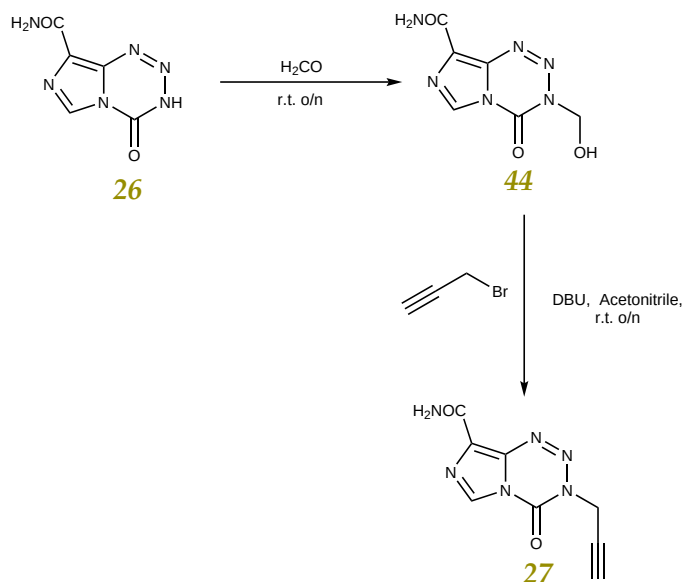
The final stage was the addition of the propargyl group at the *N*³ position of TMZ. This required the initial addition of sodium hydride (1.05 eq.) to deprotonate the *N*³ nitrogen (3 min), followed by the addition of propargyl bromide (2 eq.) to react with the *N*³ nitrogen (30 min at 0 °C and then 2 hr at room temperature). Adding 1.05 eq of sodium hydride and leaving to react for 3 min, as suggested by the original paper⁹⁶ gave no product. Increasing the sodium hydride to 1.5 eq and the reaction time to 1 hr resulted in the formation of the product.

*N*³*P*-TMZ **27** purification via column chromatography using silica, was unsuccessful. This could be due to the acidic nature of silica which could be causing the TMZ to break down. Instead, using alumina, the *N*³*P*-TMZ **27** product, was purified as a brown solid with a 27% yield.



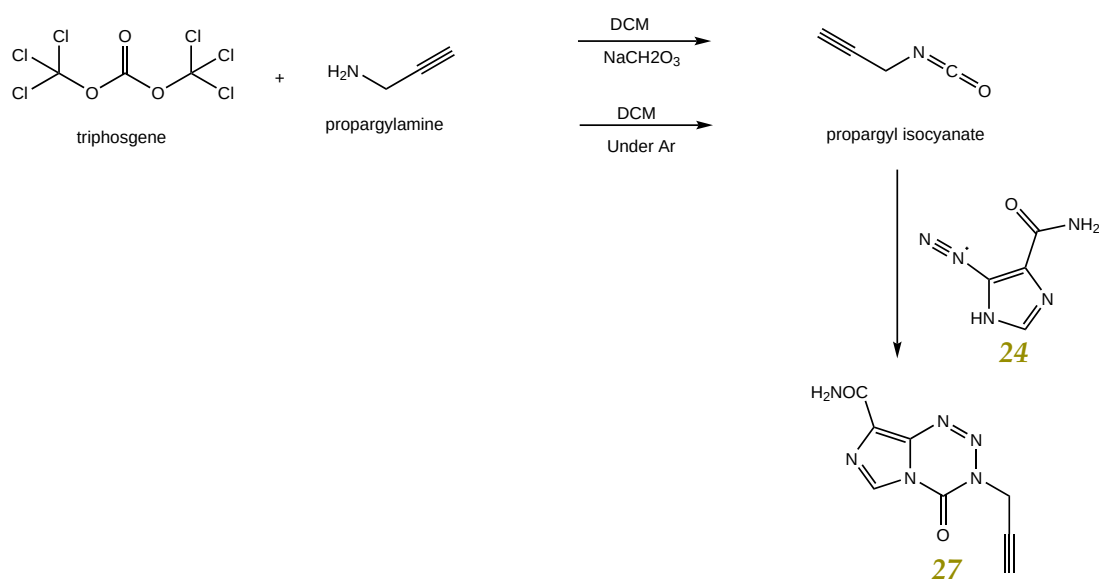
Scheme 4.12 | Synthesis of propargyl TMZ

Because of the relatively low yield of N^3P -TMZ with this method, other synthetic methods were explored to increase the yield of N^3P -TMZ. One of the methods was an alternative method suggested by Cousin *et al.*, which involved forming an alcohol in the N3 position of TMZ and then reacting it with propargyl bromide using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a catalyst.



Scheme 4.13 | Synthesis of propargyl TMZ using a MeOH-TMZ intermediate

This method did produce the methanol substituted TMZ **44** but then the reaction with propargyl bromide gave no product, as no identifiable peaks could be observed with mass spectrometry. Consistent with the mass spectrometry, NMR also showed a crude product. Other attempts of reacting triphosgene with propargylamine to form a propargyl isocyanate was explored which could be directly reacted with 5-diazoimidazole-4-carboxamide to form the N^3P -TMZ, thus eliminating multiple synthetic steps and giving a higher yield. Unfortunately, this again did not work.



Scheme 4.14 | Synthesis of propargyl TMZ using propargyl isocyanate

Since N^3P -TMZ was successfully purified, albeit at low yield, the sodium hydride method was used and repeated until high enough amount of N^3P -TMZ was synthesised to carry out the next steps. At this stage N^3P -TMZ has been purified and possible linkers have been identified that can be synthesized to conjugate N^3P -TMZ to CITx. However, because of the relatively low yield of the N^3P -TMZ synthesis, more reactions will need to be carried out until enough N^3P -TMZ is synthesized to then allow us to carry out its conjugation to TMZ.

5

Further Work

5.1 CITx

Soluble CITx was heterologously over-expressed in *E. coli*, purified, shown to bind to and internalize in U87 glioma cells, and inhibit the metastatic ability of these cells. Further work is required to fully characterize all of the CITx functions. A key experiment to do would be to determine the metastasis inhibitory concentration of CITx. This can be done by repeating the Transwell invasion assay at decreasing concentrations. Similar comparative experiments can also be carried out with synthetic analogues of CITx to identify more potent anti-metastatic CITx based drugs.

More detailed analyses of CITx-FLR binding to U87 glioma cells would also be useful in order to fully characterize and compare the time dependent binding and cellular internalization of CITx and its conjugated analogues. This can be done by measuring the ratio of CITx bound to non-bound cells using flow cytometry in more detailed time courses. Such experiments will allow us to characterize any effects of CITx conjugation with various cytotoxic drugs on cellular binding and uptake to identify potential anticancer drugs.

Flow cytometry is a technique used to analyse the properties of a population of cells. A stream of cells passes through a flow cytometer which arranges the cells into a single file. Then, using a measuring system such as a laser, light signals are passed through each cell and a detector captures these signals and feeds the data into a computer for analysis. Flow cytometry can be used for cell sorting in a method known

as fluorescence-activated cell sorting (FACS). This separates the population of cells into sub-populations based on fluorescence labelling. FACS can be used for quantifying the percentage of cells binding of ClTx-FLR over time. Cells bound to ClTx-FLR can also be sorted (isolated) and further analyse possible cellular effects of the ClTx conjugated analogues.

Conjugating cytotoxic drugs to the three lysine residues (K15, K23 and K27) on the surface of ClTx may potentially influence the ClTx cellular binding and/or internalization activities. Akcan *et al.*⁹⁹ showed that conjugation of cyanine 5.5 NHS-ester to K15, K23 and K27 on the surface of ClTx did not affect the ClTx activity. However, compared to the fluorescein NHS-ester and to the POH, cyanine 5.5 has a longer hydrocarbon chain (5 carbon residues) which places the bulky cyanine 5.5 group further away from the surface of ClTx thus avoiding any steric problems that could interfere with ClTx cellular binding and internalization. *In silico* work carried out by Othman *et al.*,⁵³ suggests that Van der Waals interactions involving the F6 residue and the final C-terminal arginine of ClTx are the biggest contributors in its interaction with the MMP2 on cellular surfaces. Also, the G24R, H10P mutations found in AaClTx resulted in 10X less activity compared to ClTx, suggesting that these residues are also important in the ClTx interaction with MMP2.

Collectively, the experimental data and *in silico* models of ClTx mutations suggest that mutating the K23 and K27 residues to arginine should not influence ClTx binding to MMP2. Therefore, primers were designed using NEBBuilder and carried out Q5-Mutagenesis to construct the K23R and K27R mutations (**Figure 5.1**). Mutations were verified by DNA sequencing (**Figure 5.1**). The K23R, K27R ClTx protein has only one surface lysine (K15) that could potentially be used attach a single conjugate to the ClTx protein.

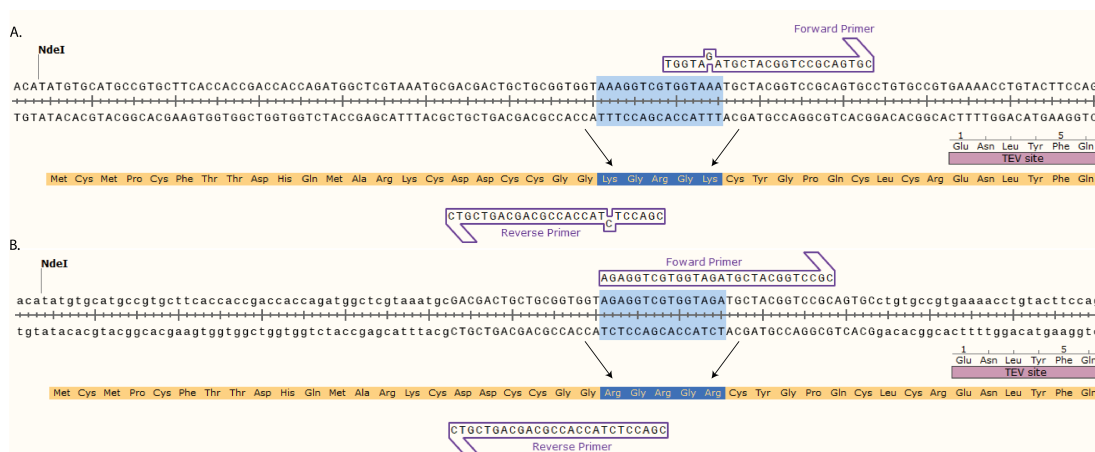


Figure 5.1 | DNA and amino acid sequences showing the K23R and K27R mutations.

A. DNA and amino acid sequences of the wildtype CITx with arrows highlighting the lysines that will be mutated. **B.** DNA and amino acid sequences of the K23R, K27R CITx showing the mutations with arrows.

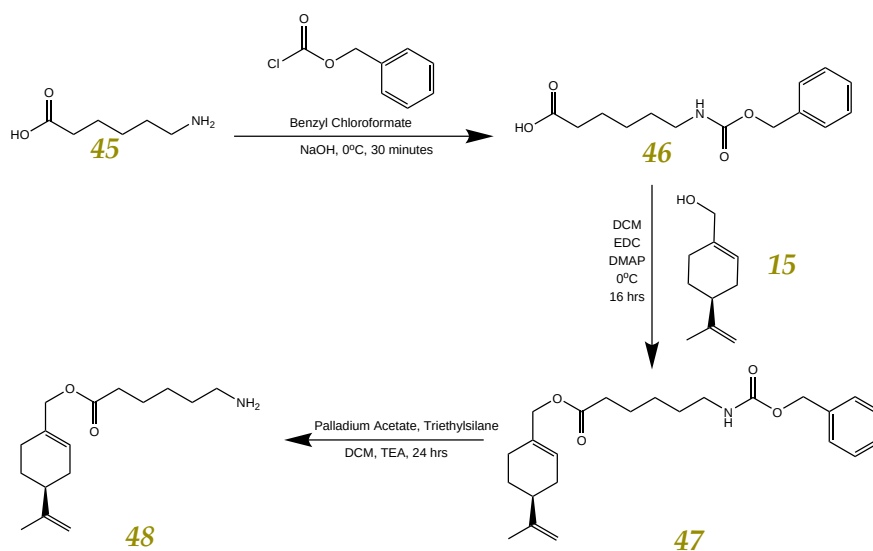
CITx and conjugated analogues can also be tested with other immortalized cell lines such as U251 or D54-MG and more importantly with primary GBM cells obtained directly from patients. Lower grade cell lines can also be tested to assess how treatable these cancers are with CITx and CITx conjugated analogues.

5.2 POH

CITx-P3C conjugate showed no cytotoxic activity against U87 glioma cells. One of the main reasons for the lack of cytotoxic activity could be because of the inability of intracellular esterases to cleave off the POH conjugate from the CITx protein inside the cytosol. Therefore, key experiments to do in the future would be to test the esterase cleavability of P3C. This could be done by identifying and using key esterases in glioma cells to monitor the cleavage CITx-P3C in *in vitro* experiments by mass spectrometry.

Conjugation sites other than lysines on the surface of CITx may also be investigated. For example, instead of the carboxylic groups of the perillyl 3-carboxypropanoate reacting with the amine groups of lysine residues, reactions can take place using the carboxylic groups in the side chains of glutamic and aspartic acids on the surface of CITx with an amine group attached to POH. This approach would require the modification of POH to include an amine group by reacting aminocaproic acid with POH (**Scheme 5.1**). This has been successfully synthesized by Lamont et al. (**Appendix**). First di-tert-butyl-

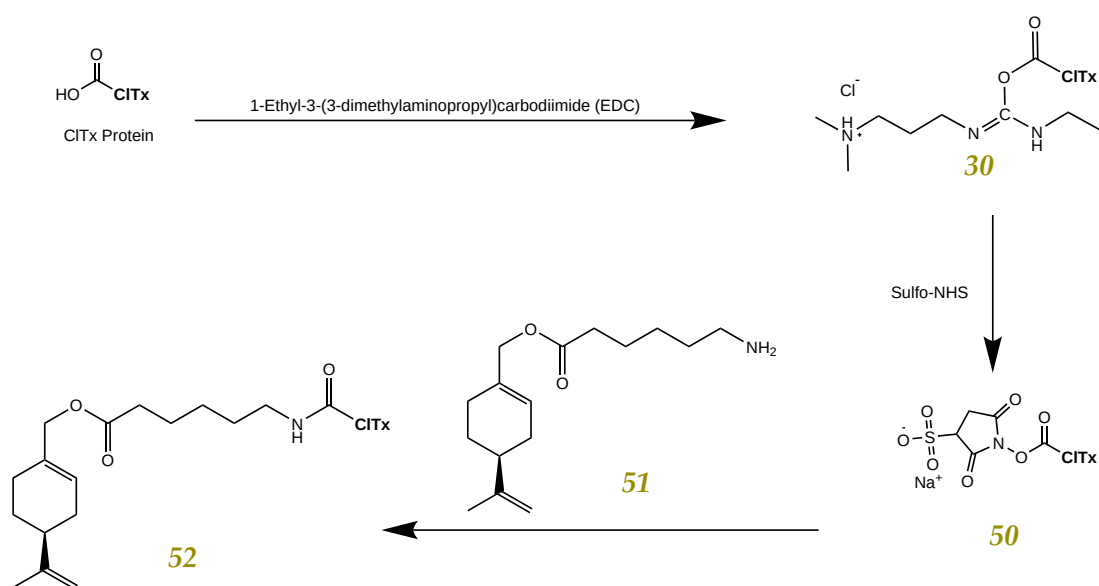
dicarbonate was reacted with 6-aminocaproic acid **45** to give a *N*-boc-aminocaproic acid **46**. This was followed by **46** reacting with POH to give the *N*-boc-perillyl 6-aminohexanoate **47**. Finally using trifluoroacetic acid, **47** was de-protected to give perillyl 6-aminohexanoate (P6A) **48** (53%).



Scheme 5.1 | Formation of perillyl 6-aminohexanoate.

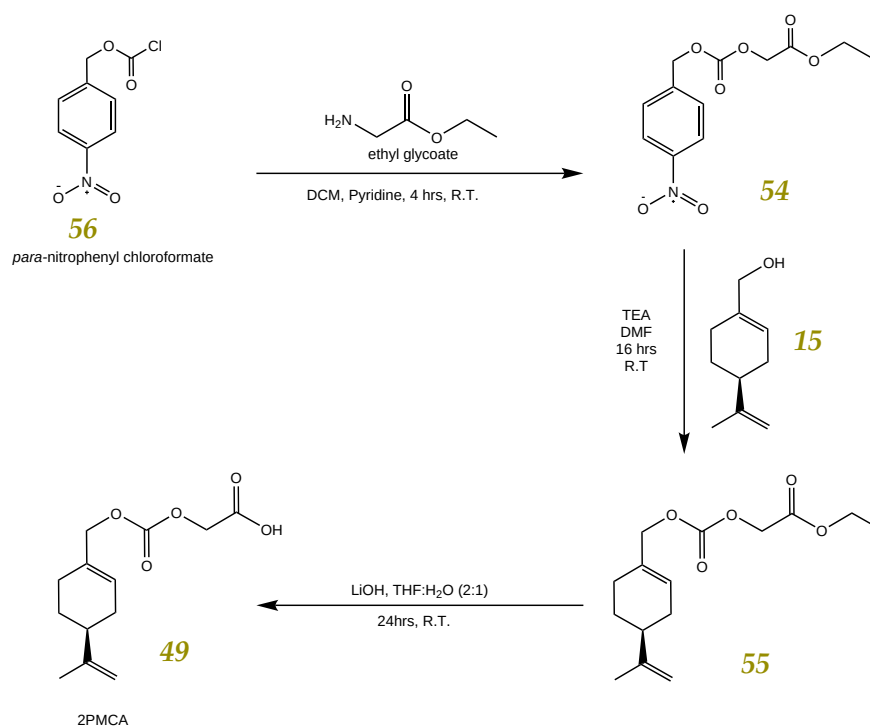
The next stage would be to conjugate P6A to CITx (**Scheme 5.2**). This can be done using the same method as the conjugation of the perillyl 3-carboxypropanoate with the carboxylic groups of aspartic acid and glutamic acids on the surface of CITx reacting with the EDC and Sulfo-NHS to form the semi stable CITx NHS ester **34**. This can be conjugated to P6A to form the CITx-P6A conjugate **Scheme 5.2**. Since P6A has a longer carbon chain, it may facilitate cleaving off of the conjugate from the CITx protein by intracellular esterases.

Both P3C and the P6A have a peptide bond at the conjugation site to CITx and an ester bond at the POH end. If esterase cleavage is the issue with the lack of cytotoxicity with the CITx-P3C conjugate, a similar problem can be expected with the CITx-P6A conjugate. Therefore, another linker can be synthesised so that it has a carbonate group, instead of an ester group, which is pH labile and should readily break down to POH in the cell. This was done by Lamont *et al.* where they synthesised 2-(((perillyl)methoxy)carbonyl)oxy)acetic acid (**49**) (**2PMCA**) (72%).



Scheme 5.2 | Conjugation of perillyl 6-aminohexanoate to CITx

The first step of synthesis was addition of ethyl glycolate in DCM into para-nitrophenyl chloroformate. After a 4-hr reaction, followed by a wash step (H₂O and brine), **54** was purified via column chromatography. It was then reacted with POH and TEA for 16 hr and purified to give **55**. This was then reacted with lithium hydroxide (LiOH) to give 2-(((perillyl)methoxy)carbonyl)oxy)acetic acid (**49**) (**2PMCA**) as a white solid. 2PMCA can then be conjugated to CITx using the EDC/NHS coupling method. As it contains a carbonate bond, it is pH labile and will be more readily released from the CITx to carry out its cytotoxic activity within the cell.



Scheme 5.3 | Formation of 2-(((perillyl)methoxy)carbonyl)oxyacetic acid (2PMCA)

5.3 TMZ

Having synthesized *N*³*P*-TMZ and the next step is modifying the amide functional group so that the TMZ can be linked onto CITx. For the POH ligands, two main types of linkers were of focus. A slow-release carbamate linker, similar to the NEO212 structure, and a pH labile hydrazone linker.

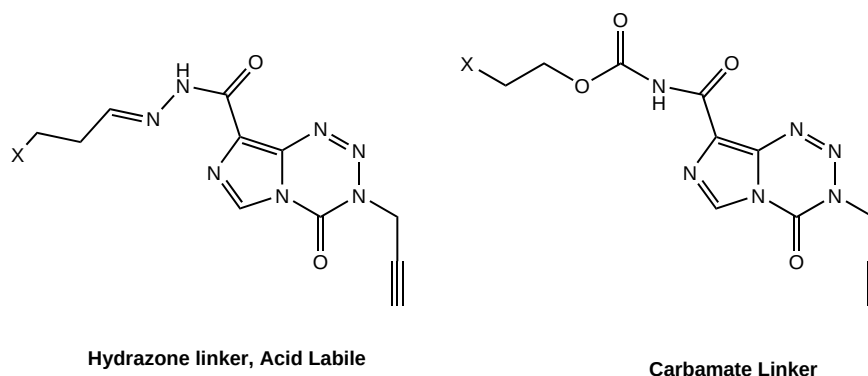
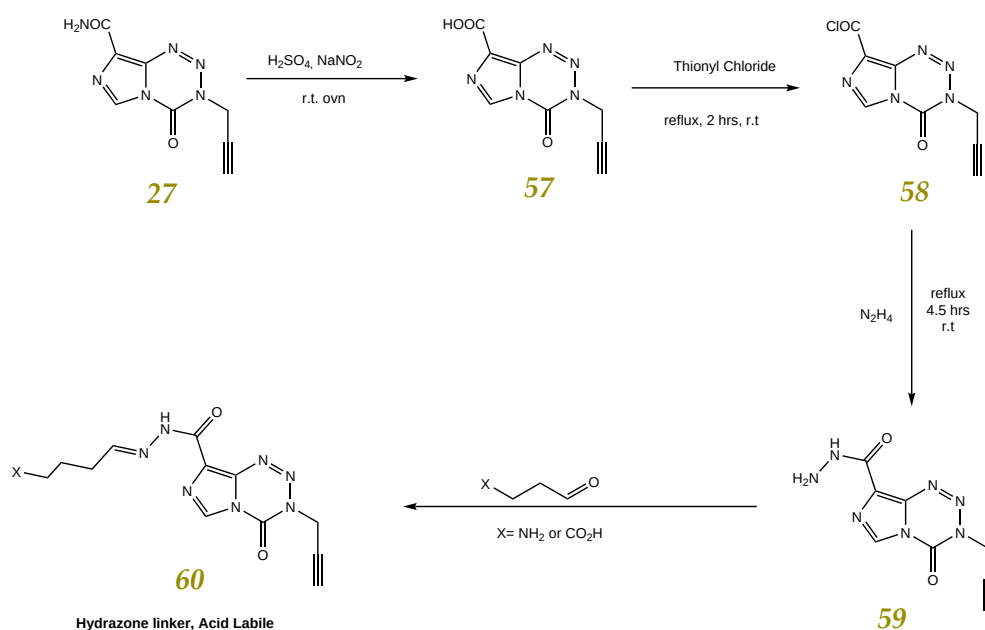


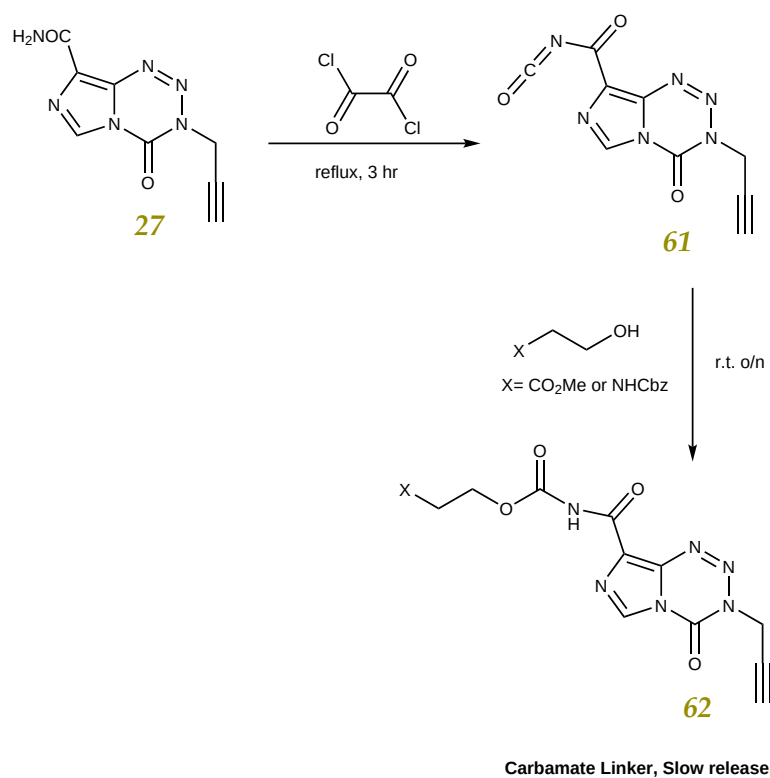
Figure 5.2 | N³P-TMZ ligand analogs

The hydrazone linker for N^3P -TMZ will be prepared by converting the amide group into a carboxylic acid using first H_2SO_4 and then reacting it with sodium nitrite. This is then followed by the addition of thionyl chloride which converts the carboxylic acid to an acyl chloride. Using hydrazine hydrate and methanol, the acyl chloride can be converted to a hydrazine group and then, using ethanol and either 3-aminopropanal or methyl 4-oxobutanoate, to a hydrazone linker with either a protected carboxylic acid group or an amine group. This will allow the ligand to be conjugated to CITx by the EDC-NHS coupling method.



Scheme 5.4 | Formation of hydrazone linker based N^3P -TMZ

The carbamate linker will be synthesized by first reacting the N^3P -TMZ with oxalyl chloride in the presence of 1,2 dichloroethane thus converting the amide group into an isocyanate group. This can then be reacted with methyl 3-hydroxypropanoate or benzyl (2-hydroxyethyl) carbamate to give the carbamate linker with a protected carboxylic acid/amine group at the end. After demethylation/deprotection, the carbamate N^3P -TMZ can finally be conjugated to CITx by the EDC/NHS coupling method.



Scheme 5.5 | Formation of carbamate linker based N^3P -TMZ

Summarizing, up to four different N^3P -TMZ ligands can be synthesised that can be conjugated to CITx and hopefully one (or more) of them will have desirable cytotoxic effects on glioma cells. These ligands could also be tested for cytotoxicity against TMZ resistant cancer cells such as SNB19M and U373M.

5.4 Concluding Statement

CITx, a peptide found in scorpion venom, has been heterologously expressed and purified from *E. coli*. The purification method leads to soluble protein that does not require any refolding steps during the purification protocol. The purified CITx maintained its anti-tumour activity by preventing metastasis of GBM, as shown by Transwell invasion assays using U87 glioma cells. A fluorescein conjugate (CITx-FLR) of CITx showed clear binding to and uptake in the U87, making CITx a viable drug delivery vehicle to specifically target GBM with a range of cytotoxic drugs conjugated to CITx. POH was modified to include a linker region that enabled conjugation to CITx. Two ligands of POH, 4NPC and P3C were successfully synthesized. Conjugation was shown to be successful with the P3C ligand only, but further cellular testing of the conjugate did not reveal a cytotoxic effect. TMZ, the current chemotherapeutic agent of choice against GBM, was then used as a potential conjugate to CITx. A variant of TMZ (N^3P -TMZ), which included a propargyl group instead of a methyl group, was synthesized. N^3P -TMZ was previously shown to be active against TMZ resistant cell lines and therefore would be a potent ligand for CITx.

At this stage, further work needs to be done on N^3P -TMZ to make it ready for conjugation with CITx. Following successful conjugation, the cytotoxic activity of the ligand and the conjugate will need to be tested and compared in both *in vitro* and *in vivo* models. If successful, this can then progress into clinical trials which will create a new type of drug that can combat recurrent TMZ resistant GBM.

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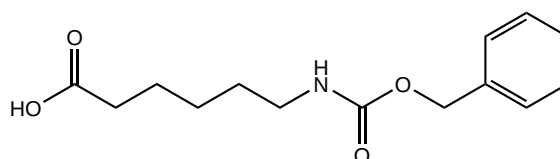
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A

Appendix

A.1 Other POH constructs

All POH constructs shown below were made by Malcolm Lamont from Professor Neil R. Thomas's group, based at the University of Nottingham, Biodiscovery Institute. Below are the methods used for synthesizing all the different constructs mentioned in the previous chapters.

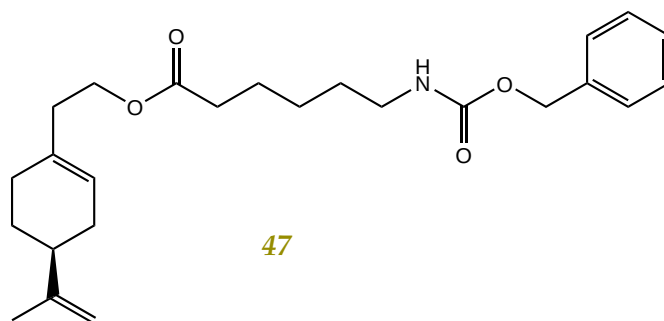


46

Scheme A.1 | 6-(((Benzyloxy)carbonyl)amino)hexanoic acid

To a solution of aminocaproic acid (5.3 g, 40 mmol) in 2.0 M aq. NaOH (0.2 M) at 0 °C was added benzyl chloroformate (6.5 mL, 44 mmol) dropwise. The solution was stirred for 30 min. The solution was extracted with Et₂O × 3. The aqueous layer was acidified with 3 M HCl and further extracted with EtOAc × 3. The organic layers were combined, dried and concentrated and taken forward without further purification (Yield: 10.6 g, 39.8 mmol, 99.4 %).

¹H-NMR (400 MHz, CDCl₃) δ 10.75 (s, 1H), 7.40 – 7.27 (m, 5H), 5.09 (s, 2H), 4.94 (t, *J* = 6.1 Hz, 1H), 3.25 – 3.07 (m, 2H), 2.40 – 2.26 (m, 2H), 1.70 – 1.56 (m, 2H), 1.56 – 1.43 (m, 2H), 1.43 – 1.28 (m, 2H). ¹³C-NMR (101 MHz, CDCl₃) δ 179.28(COOH), 177.04 (NHCOO), 136.62 (Ar), 128.58 (Ar), 128.18 (Ar), 128.01(Ar), 66.75 (CH₂), 40.89 (CH₂), 33.95 (CH₂), 29.64 (CH₂), 26.19 (CH₂), 24.34(CH₂). HRMS (ESI): C₁₄H₁₉NO₄ [M+Na]⁺: calculated 288.1212, found 288.1216.

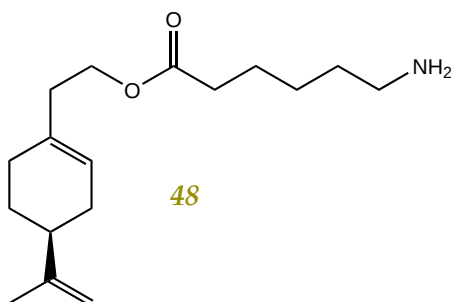


Scheme A.2 | (4-(Prop-1-en-2-yl)-cyclohex-1-en-1-yl)methyl (((benzyloxy)carbonyl)amino)hexanoate

6-

To a solution of **46** (1.0 g, 3.8 mmol) in DCM (0.3 M) at 0°C was added POH (0.6 ml, 3.8 mmol), EDC.HCL (1.1 g, 5.7 mmol) and DMAP (46 mg, 0.4 mmol). The solution was stirred for 16 hr before adding sat. NH₄Cl and the layers separated. The organic phase was washed with 1N HCl × 3, H₂O and brine. The organic layer was dried and concentrated and purified by column chromatography (50% v/v EtOAc/PE) (Yield: 1.3 g, 3.3 mmol, 87.3 %).

¹H-NMR (400 MHz, CDCl₃) δ 7.37 – 7.07 (m, 5H), 5.72 – 5.59 (m, 1H), 5.10 – 4.81 (m, 2H), 4.70 – 4.58 (m, 2H), 4.46 – 4.29 (m, 2H), 3.17 – 2.95 (m, 2H), 2.22 (t, *J* = 7.5 Hz, 2H), 2.13 – 2.01 (m, 2H), 2.01 – 1.93 (m, 2H), 1.93 – 1.81 (m, 1H), 1.80 – 1.70 (m, 1H), 1.64 (t, *J* = 1.1 Hz, 3H), 1.60 – 1.48 (m, 2H), 1.46 – 1.30 (m, 3H), 1.32 – 1.10 (m, 2H). ¹³C-NMR (101 MHz, CDCl₃) δ 173.46 (NHCOO), 156.45 (COOR), 149.50 (C), 136.71 (Ar), 132.65, 128.48 (Ar), 128.07 (Ar), 128.04 (Ar), 125.70 (CH), 108.84 (CH₂), 68.32 (CH₂), 66.51 (CH₂), 40.84 (CH₂), 40.80 (CH), 34.13 (CH₂), 30.45 (CH₂), 29.62 (CH₂), 27.29 (CH₂), 26.39 (CH₂), 26.24 (CH₂), 24.57 (CH₂), 20.77 (CH₃). HRMS (ESI): C₂₄H₃₄NO₄ [M+H]⁺: calculated 400.2488, found 400.2478.

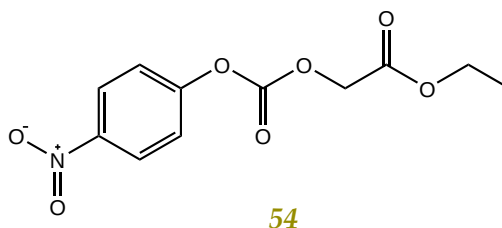


Scheme A.3 | (4-(Prop-1-en-2-yl)cyclohex-1-en-1-yl)methyl 6-aminohexanoate

To a solution of Triethylsilane (1.0 ml, 3.75 mmol) in DCM (0.15 M) was added palladium acetate (55 mg, 0.1 mmol) and triethylamine (0.3 ml, 0.2 mmol). After 5 min stirring, **2** (1.0 g, 1.5 mmol) in DCM (0.1 M) was added dropwise and the reaction stirred for 24 hr. The solution was concentrated and purified by column chromatography (0 – 20 % v/v MeOH in DCM) (Yield: 0.2 g, 0.8 mmol, 53.0 %).

¹H-NMR (400 MHz, CD₃OD) δ 5.83 – 5.66 (m, 1H), 4.77 – 4.64 (m, 2H), 4.46 (s, 2H), 2.97 – 2.88 (m, 2H), 2.38 (t, *J* = 7.3 Hz, 2H), 2.20 – 2.11 (m, 2H), 2.12 – 2.04 (m, 2H), 2.03 – 1.91 (m, 1H), 1.89 – 1.80 (m, 1H), 1.74 (t, *J* = 1.2 Hz, 3H), 1.72 – 1.62 (m, 4H), 1.54 – 1.37 (m, 3H). ¹³C-NMR (101 MHz, CD₃OD) δ 174.94 (COO), 150.64 (C), 134.02 (C), 126.53 (CH), 109.34 (CH₂), 69.32 (CH₂), 42.20 (CH), 40.54 (CH₂), 34.64 (CH₂), 31.53 (CH₂),

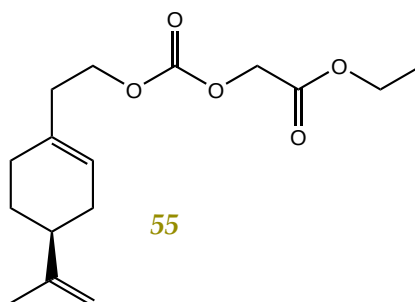
28.55 (CH₂), 28.19 (CH₂), 27.29 (CH₂), 26.87 (CH₂), 25.42 (CH₂), 20.93 (CH₃). HRMS (ESI): C₁₆H₂₈NO₂ [M+H]⁺: calculated 266.2120, found 266.2117.



Scheme A.4 | ethyl 2-(((4-Nitrophenoxy)carbonyl)oxy)acetate

To a solution of ethyl glycolate (0.5 ml, 5.0 mmol) in DCM (0.6 M) was added pyridine (0.7 ml, 7.5 mmol) and para-nitrophenyl chloroformate (1.1 g, 5.5 mmol). After 4 hr stirring, the solution was concentrated and re-dissolved in Et₂O and H₂O. The organic layer was washed with H₂O and brine. The solution was dried, concentrated and purified by column chromatography (DCM) (Yield: 1.2 g, 4.5 mmol, 90.3 %).

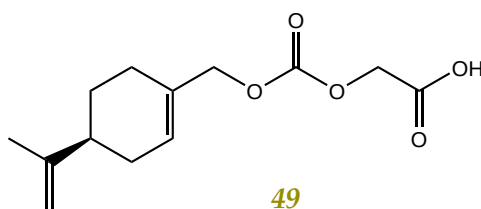
¹H-NMR (400 MHz, Chloroform-d) δ 8.33 – 8.24 (m, 2H), 7.45 – 7.37 (m, 2H), 4.76 (s, 2H), 4.29 (q, *J* = 7.1 Hz, 2H), 1.32 (t, *J* = 7.1 Hz, 3H). ¹³C-NMR (101 MHz, CDCl₃) δ 166.85 (COO), 155.46 (Ar), 152.44 (OCOO), 145.74 (Ar), 125.51 (Ar), 121.88 (Ar), 64.33 (CH₂), 62.13 (CH₂), 14.25 (CH₃).



Scheme A.5 | methyl 2-(((4-(Prop-1-en-2-yl)cyclohex-1-en-1-yl)ethoxy)carbonyl)oxy)acetate

To a solution of **55** (1.2 g, 4.5 mmol) in DMF (0.1 M) was added POH (1.5 ml, 9.5 mmol) and triethylamine (3.8 ml, 27 mmol). After 16 hr stirring the solution was concentrated and re-dissolved in DCM. The organic phase was washed with aq. sat. NaHCO₃ and H₂O, dried and concentrated. The crude product was purified by column chromatography (10 – 20 % v/v EtOAc in PE) (Yield: 0.6 g, 2.2 mmol, 48.8 %).

¹H-NMR (400 MHz, Chloroform-d) δ 5.85 – 5.79 (m, 1H), 4.74 – 4.69 (m, 2H), 4.62 (s, 2H), 4.58 – 4.55 (m, 2H), 4.25 (q, *J* = 7.1 Hz, 2H), 2.23 – 2.08 (m, 4H), 2.04 – 1.91 (m, 1H), 1.90 – 1.81 (m, 1H), 1.76 – 1.70 (m, 3H), 1.57 – 1.41 (m, 1H), 1.29 (t, *J* = 7.1 Hz, 3H). ¹³C-NMR (101 MHz, CDCl₃) δ 167.66 (COO), 154.98 (OCOO), 149.60 (C*), 131.96 (C*), 127.15 (CH), 108.97 (CH₂), 72.76 (CH₂), 63.51 (CH₂), 61.69 (CH₂), 40.82 (CH), 30.57 (CH₂), 27.34 (CH₂), 26.30 (CH₂), 20.88 (CH₃), 14.24 (CH₃). HRMS (ESI): C₁₅H₂₂O₅Na [M+Na]⁺: calculated 305.1365, found 305.1363



Scheme A.6 | 2-((((Perillyl)methoxy)carbonyl)oxy)acetic acid (**49**)

To a solution of **5** (1.2 g, 4.7 mmol) in 2:1 THF:H₂O (0.2 M) was added LiOH (0.1 g, 5.9 mmol). After 24 hr stirring the solution was extracted with DCM. The aq. phase was acidified to pH 4 with 1 M HCl and extracted with ^tBuOMe, dried and concentrated (Yield: 0.84 g, 3.3 mmol, 72.0 %).

¹H-NMR (400 MHz, Chloroform-d) δ 5.91 – 5.73 (m, 1H), 4.74 – 4.69 (m, 2H), 4.68 (s, 2H), 4.57 (s, 2H), 2.26 – 2.05 (m, 4H), 2.05 – 1.91 (m, 1H), 1.91 – 1.79 (m, 1H), 1.73 (s, 3H), 1.57 – 1.39 (m, 1H). ¹³C-NMR (101 MHz, CDCl₃) δ 173.27 (COOH), 154.87 (OCOO), 149.56 (C*), 131.80 (C*), 127.39 (CH), 108.99 (CH₂), 73.00 (CH₂), 62.89 (CH₂), 40.78 (CH), 30.55 (CH₂), 27.31 (CH₂), 26.27 (CH₂), 20.87 (CH₃). HRMS (ESI): C₁₃H₁₈O₅ [M-H]⁻: calculated 253.1081, found 253.1091.

A.2 Mass Spectrum of CITx, POH and TMZ constructs

A.

Protein:

GHHHHHHGMT ALTEGAKLFE **KEIPYITELE** **GDVEGMKFII** **KGESTGDATT**
GTIKAKYICT TGDVPEVWST LVTTLTYGAQ CFARYGPELK DFYKSCMEPG
 YVQERTITFE GDGVFKTRAE VTFENGSVYN RVKLNQGFK KDGHVLGKNL
 EFNFPKCLC IWDQANHL KSAFKIMHEI TQSKEDFIVA DITQMNTFIFG
 GSPVHVVEYH HLTWVTSFGK DPDDDETDLH NIVEVIKAVD LETYRG

Protein Coverage:

Sequence	MH+	% Mass	AA	% AA
EIPYITELEGDVEGMK	1822.88	6.67	22 - 37	6.50
EIPYITELEGDVEGMKFIIK	2324.21	8.50	22 - 41	8.13
EIPYITELEGDVEGMKFIIKGESTGDATTGTIK	3512.77	12.84	22 - 54	13.41
FIKKGESTGDATTGTIK	1708.91	6.25	38 - 54	6.91
GESTGDATTGTIK	1207.58	4.42	42 - 54	5.28
YGPELKDFYK	1259.63	4.61	85 - 94	4.07
SCMPEGVQER	1298.55	4.75	95 - 105	4.47
TITFEGDVQEK	1213.61	4.44	106 - 116	4.47
TITFEGDVFKTR	1470.76	5.38	106 - 118	5.28
TRAEVTFENGSVYNR	1742.85	6.37	117 - 131	6.10
AEVTFENGSVYNR	1485.70	5.43	119 - 131	5.28
NLEFNFPKCLCINWDQANHLK	2657.24	9.72	149 - 171	9.35
DPDDDETDLHNIVEVIK	1966.92	7.19	221 - 237	6.91
Totals:	13537.46	49.50	120	48.78

B.

Protein:

MCNFCPTTDH QMARKDDCC GKGGRGKCYG PQCLCPENLY **FQGHHHHHHG**
MTALTEGAKL FEKEIPYITE LEGDVEGMKF IIKGESTGDA TTGTIKAKYI
 CTTGDVPEVW STLVTTLTYG AQCFARYGPE LKDFYKSCMP EGYVQERTIT
 FEGDGVFKTR AEVTFENGSV YNRVKNLQGG FKDDHVLGK NLEFNFPKCLC
 LCINWDQANH GLKSAFKIMH EITQSKEDFI VADHTQMNTF IGGGPFVHVE
 YHHLTWVTSF GRDPDDDETDLH NIVEVIKAVD VLETYRG

Protein Coverage:

Sequence	MH+	% Mass	AA	% AA
ENLYFQGHHHHHHHGMTALTEGAK	2652.23	8.26	37 - 59	7.99
Totals:	2652.23	8.26	23	7.99

Figure A.1 | Peptide mass fingerprinting of CITx.

A. *In silico* comparison of 70 kDa peptide fragments against the HT-cometGFP sequence. **B.** *In silico* comparison of 70 kDa peptide fragments against the CITx-TEV-HT-cometGFP sequence.

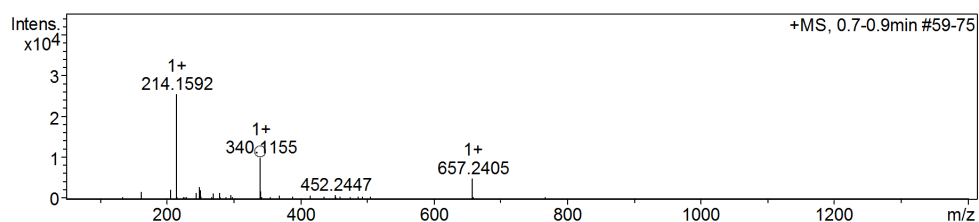


Figure A.2 | Time-of-flight electron spray positive mass spectrum of 4-nitrophenyl perillyl carbonate.

HRMS (ESI): C₁₇H₁₉NNaO₅ [M+Na]⁺ calculated 340.1155; found 340.1155

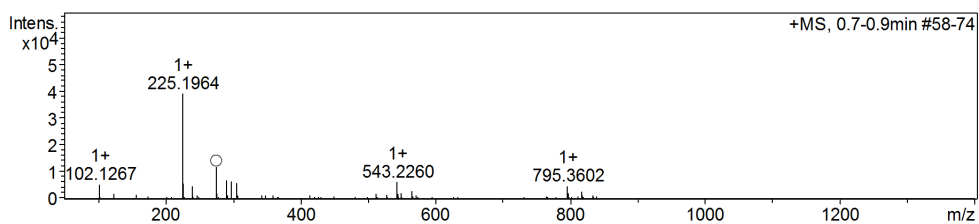


Figure A.3 | Time-of-flight electron spray positive mass spectrum of perillyl 3-carboxypropanoate.

HRMS (ESI): C₁₄H₂₀NaO₄ [M+Na]⁺ calculated 275.1254; found 275.1250

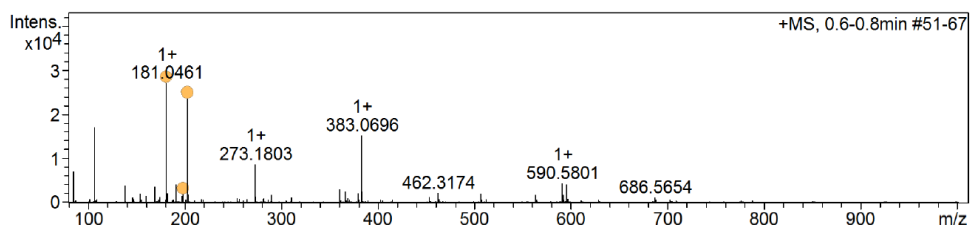


Figure A.4 | Time-of-flight electron spray positive mass spectrum of *nor*-Temozolomide.

HRMS (ESI): C₅H₅N₆O₂ [M+H]⁺ calculated 181.0468; found 181.0461

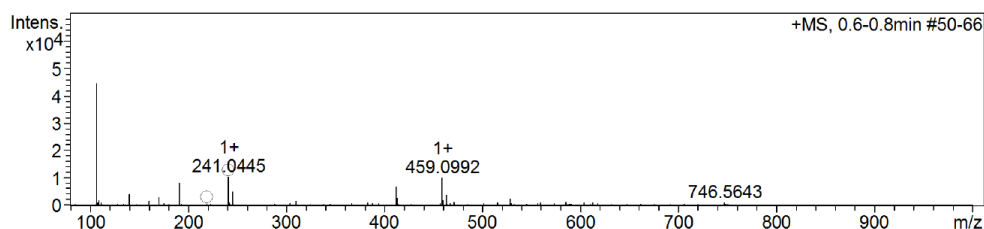


Figure A.5 | Time-of-flight electron spray positive mass spectrum of *N*³*P*-Temozolomide.

HRMS (ESI): C₈H₆N₆NaO₂⁺ [M+Na]⁺ calculated 241.0444; found 241.0445

A.3 NMR of POH and TMZ constructs

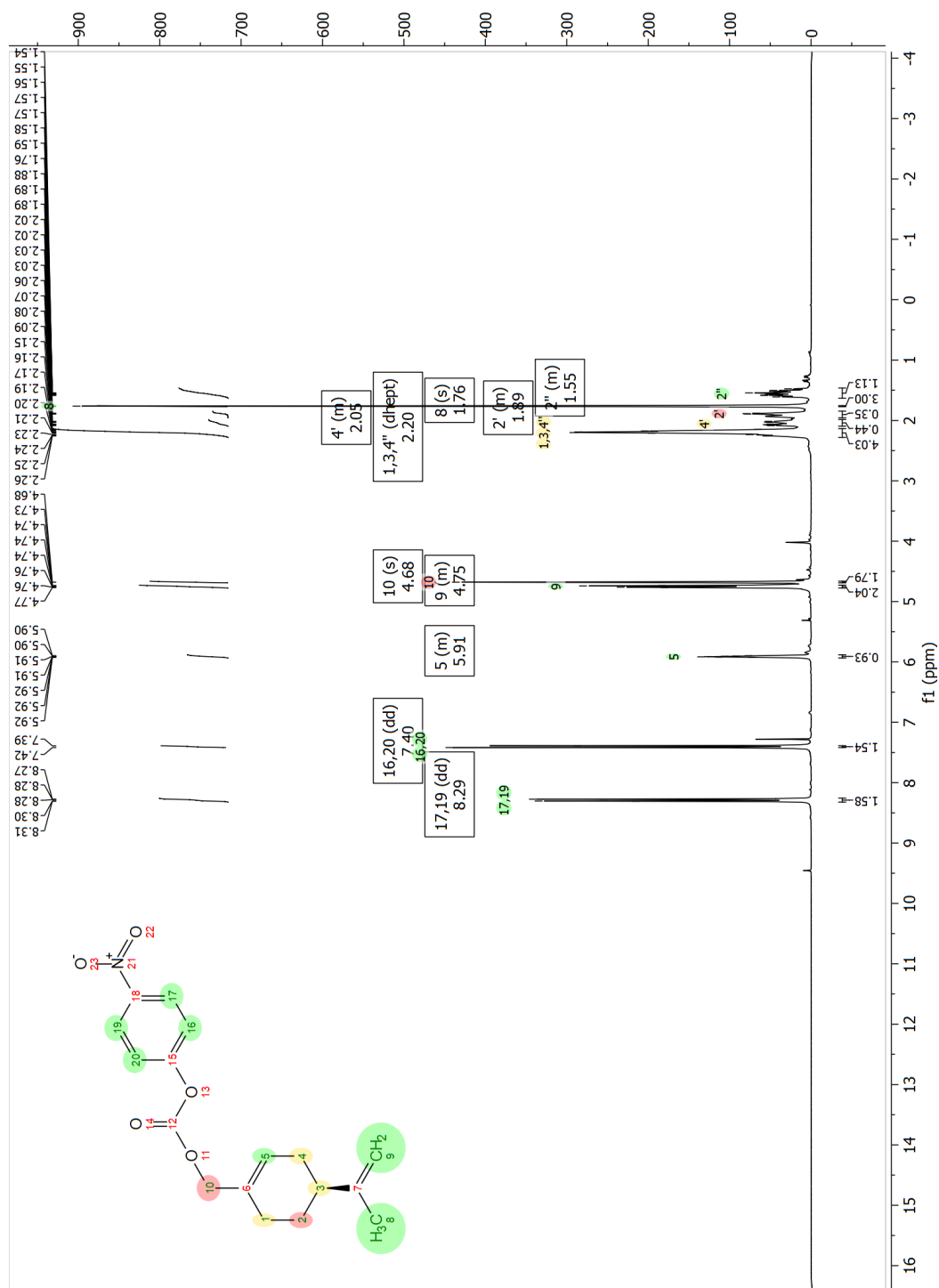


Figure A.6 | ¹H NMR for 4-nitrophenyl perillyl carbonate.

¹H-NMR (400 MHz, CDCl₃): δ 8.33-8.28 (m, 2H) 7.44-7.40 (m, 2H), 5.94-5.91 (m, 1H), 4.79-4.72 (m, 2H), 4.70-4.65 (m, 2H), 2.30-2.14 (m, 4H), 2.10-2.00 (m, 1H), 1.95-1.85 (m, 1H), 1.76 (dd, 3H), 1.62-1.45 (m, 1H)

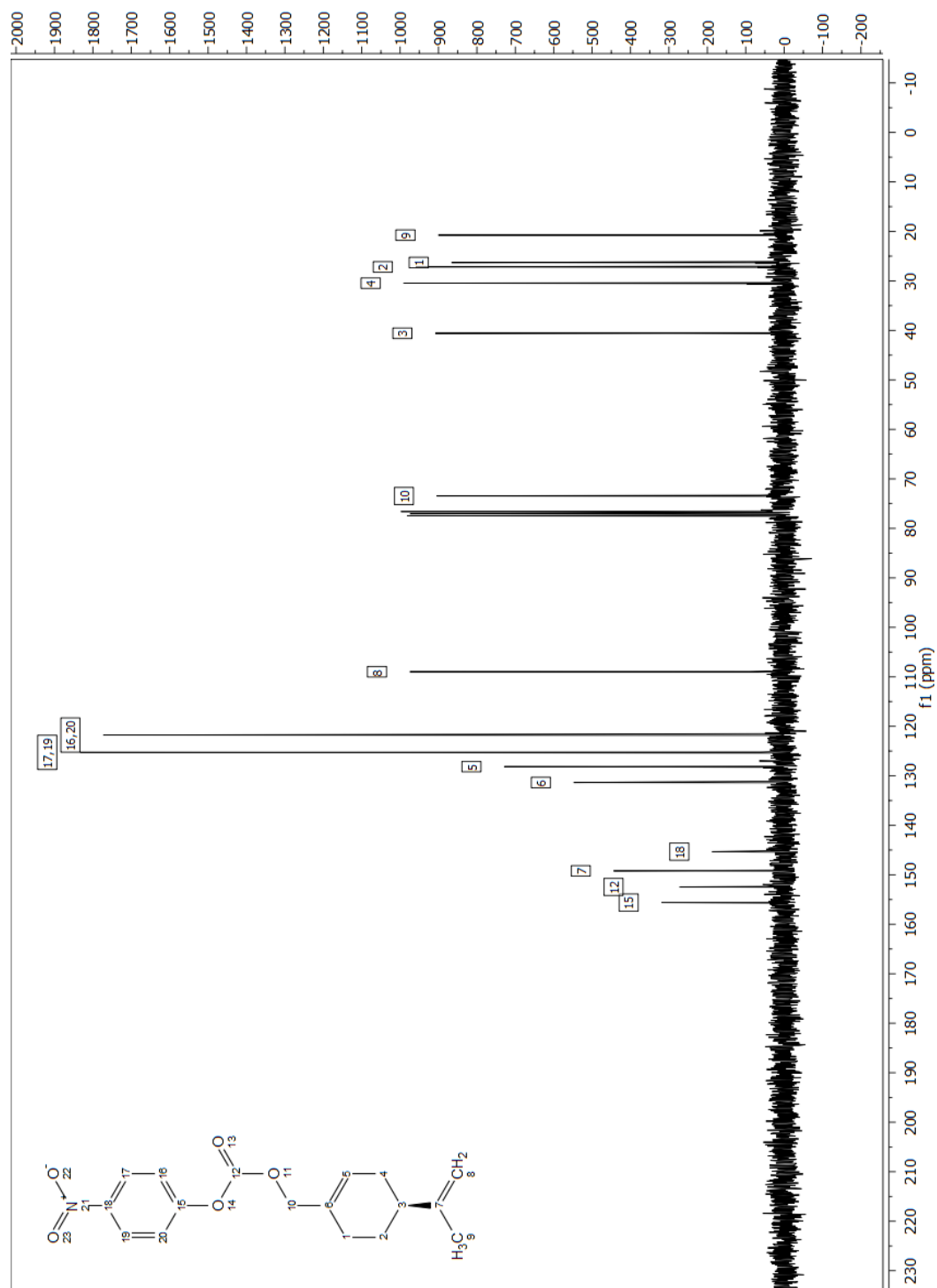


Figure A.7 | ¹³C-NMR for 4-nitrophenyl perillyl carbonate.

¹³C-NMR (100 MHz; CDCl₃): δ 155.5, 152.3, 149.2, 145.3, 131.2, 128.0, 125.2, 121.8, 109.0, 73.4, 40.5, 30.4, 27.1, 26.2, 20.6

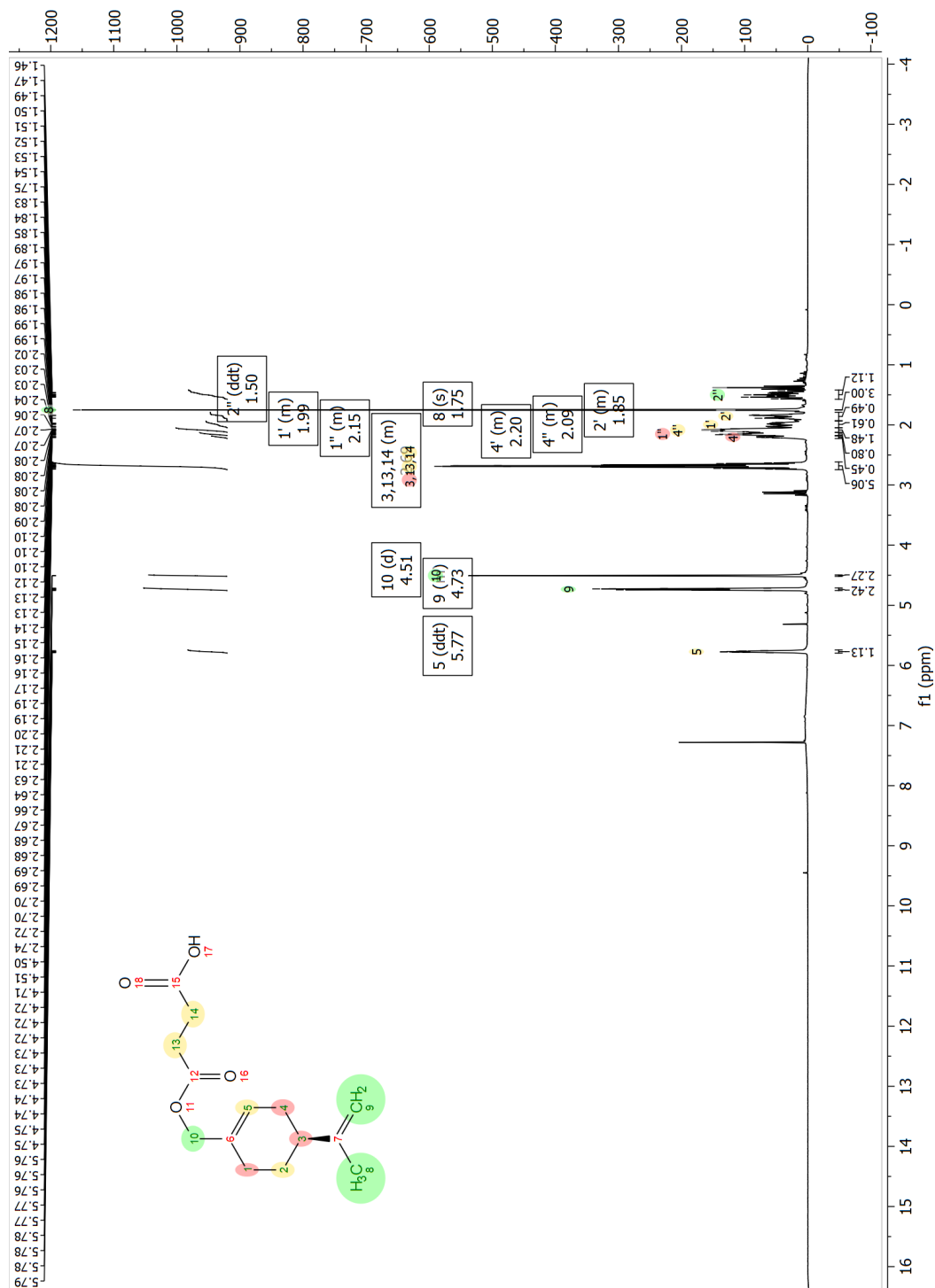
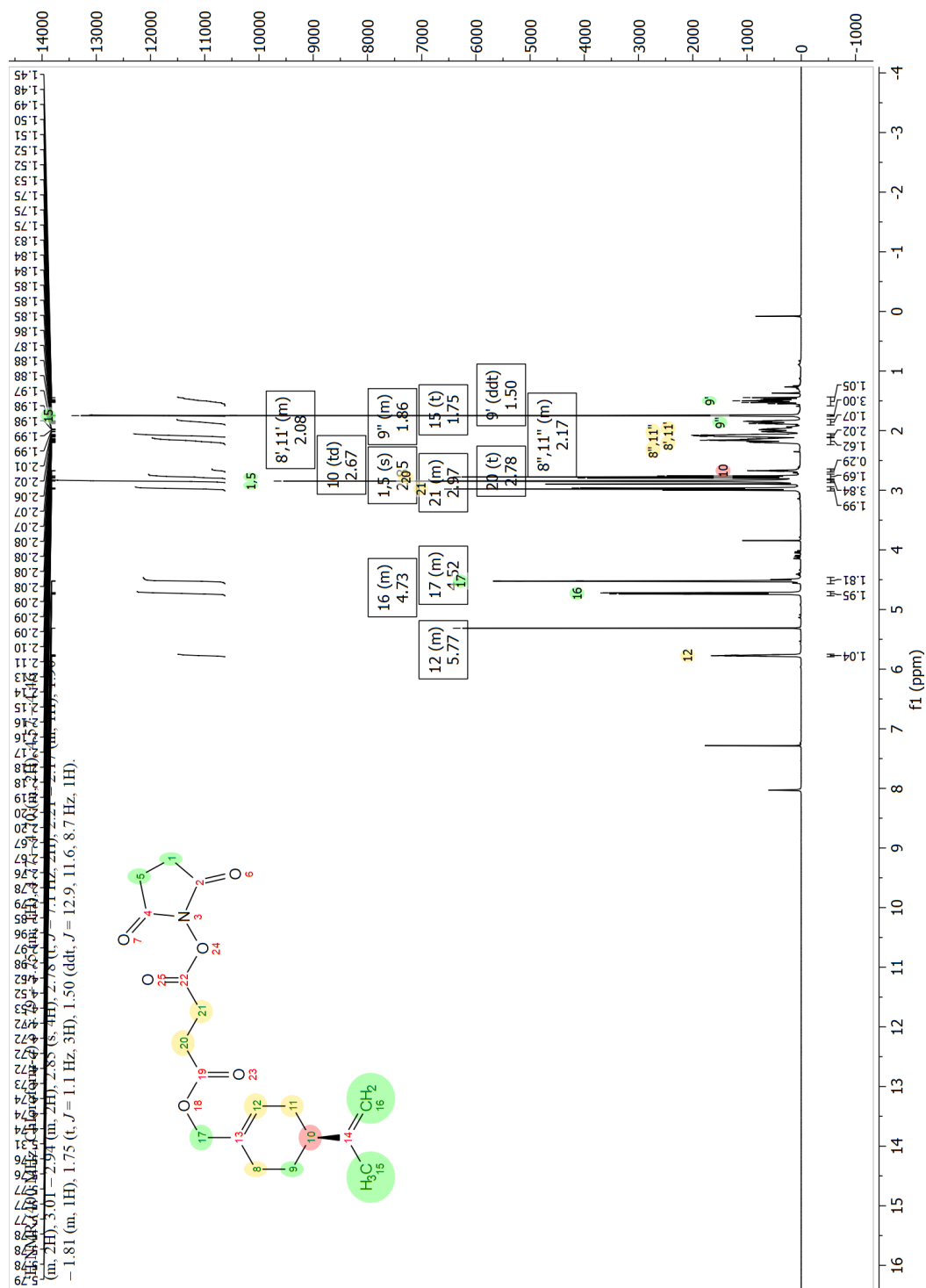


Figure A.8 | ¹H NMR for perillyl 3-carboxypropanoate.

¹H-NMR (400 MHz, CDCl₃): δ 5.75 (m, 1H), 4.73-4.70 (m, 2H), 4.49 (s, 2H), 2.71-2.61 (m, 2H), 2.20-2.16 (m, 1H), 2.15-2.11 (m, 1H), 2.08-2.05 (m, 2H), 1.98-1.93 (m, 1H), 1.87-1.80 (m, 1H), 1.73 (t, 3H), 1.54-1.41 (m, 1H).



^{13}C -NMR (100 MHz; CDCl_3): δ 178.0, 172.2, 149.6, 132.4, 126.0, 108.8, 68.9, 40.8, 30.5, 28.9, 27.3, 26.3, 21.1, 20.7.



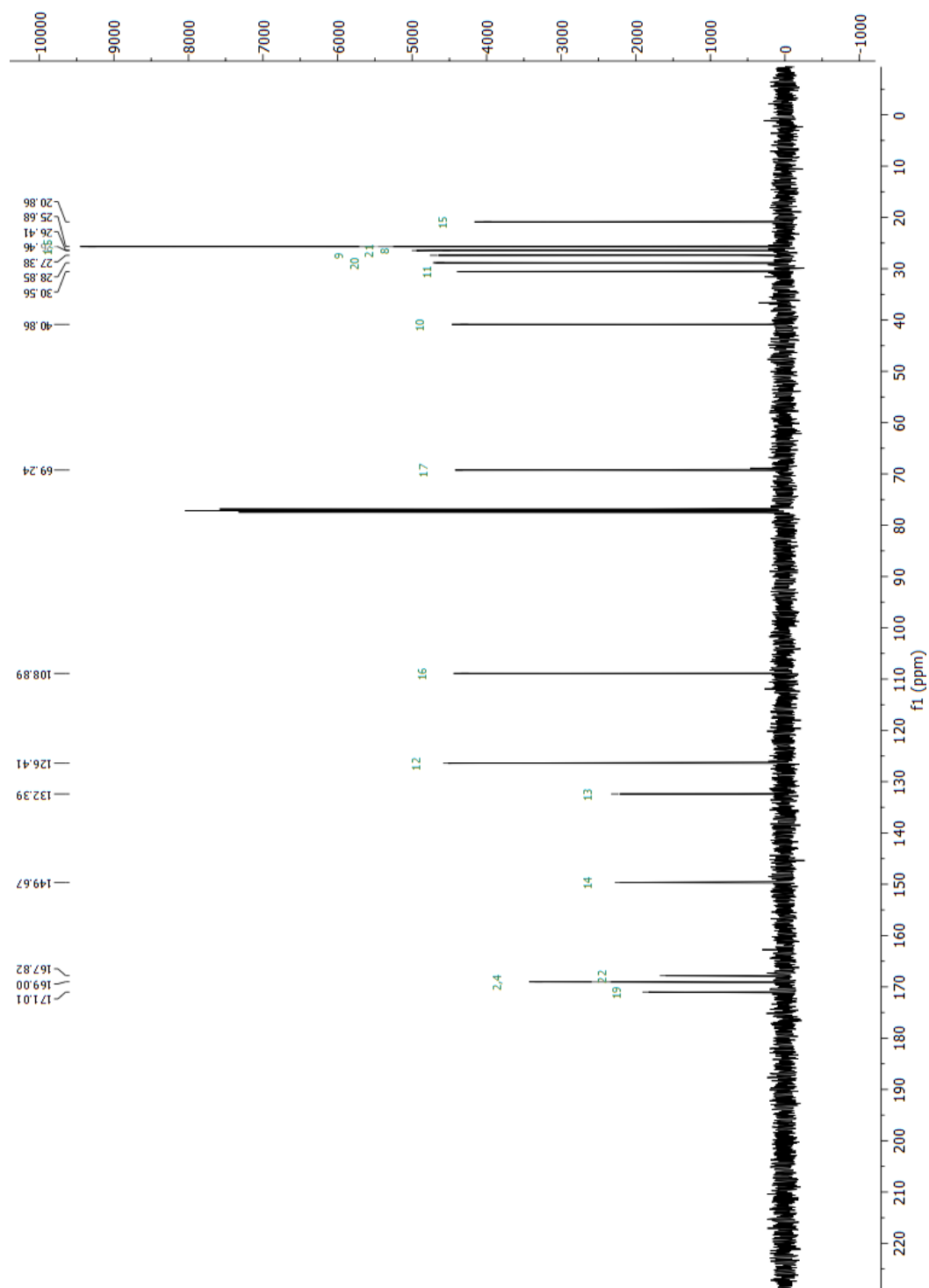
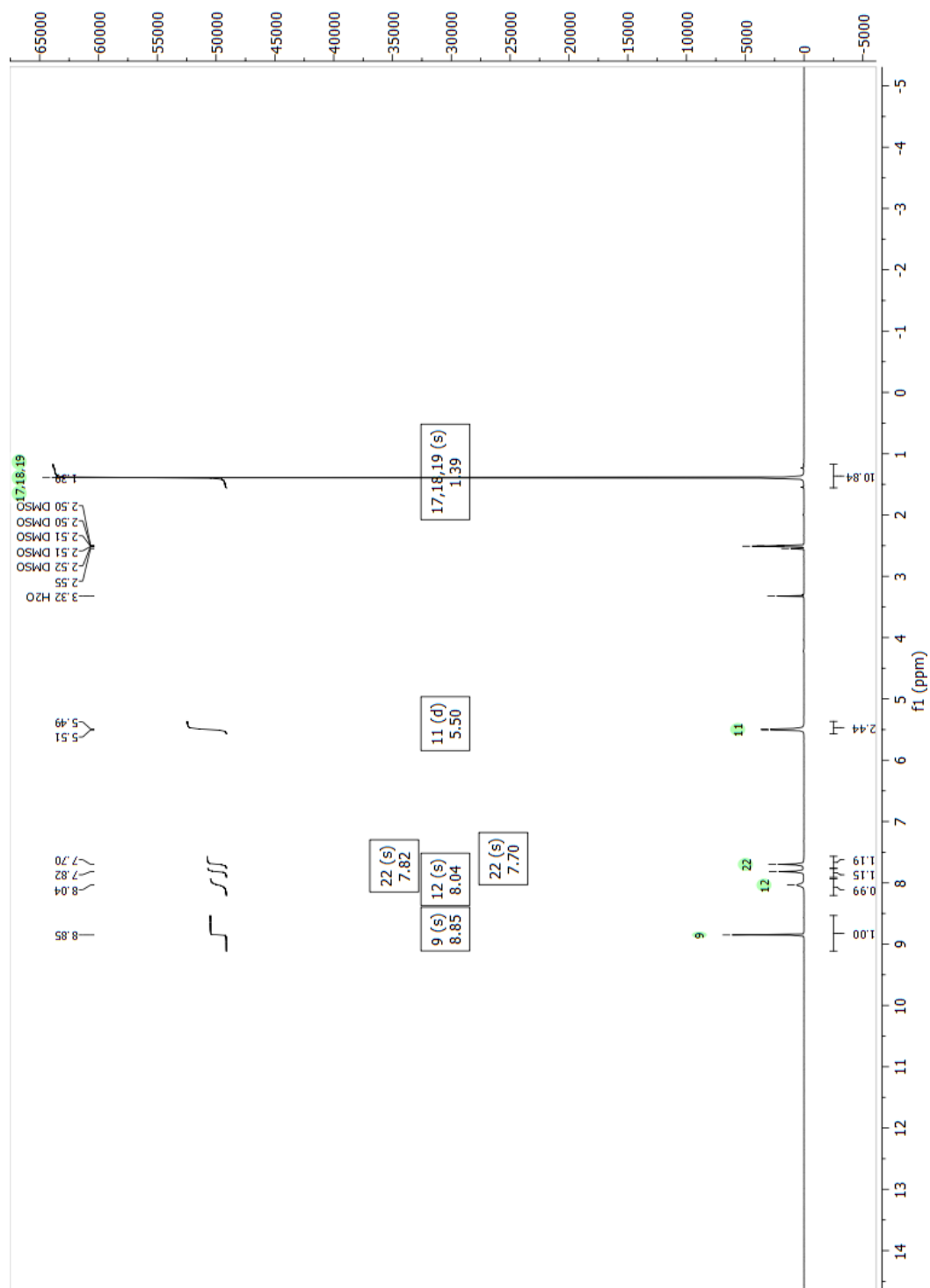


Figure A.11 | ¹³C-NMR for perillyl 3-carboxypropanoate NHS ester.

¹³C-NMR (100 MHz; CDCl₃): δ 171.01, 169.00, 167.82, 149.67, 132.39, 126.41, 108.89, 69.24, 40.86, 30.56, 28.85, 27.38, 26.46, 26.41, 25.68, 20.86.

¹H-NMR (400 MHz, DMSO-d₆): δ 8.85 (s, 1H), 8.04 (s, 1H), 7.82 (s, 1H), 7.70 (s, 1H), 5.50 (d, J = 6.4 Hz, 2H), 1.39 (s, 11H)

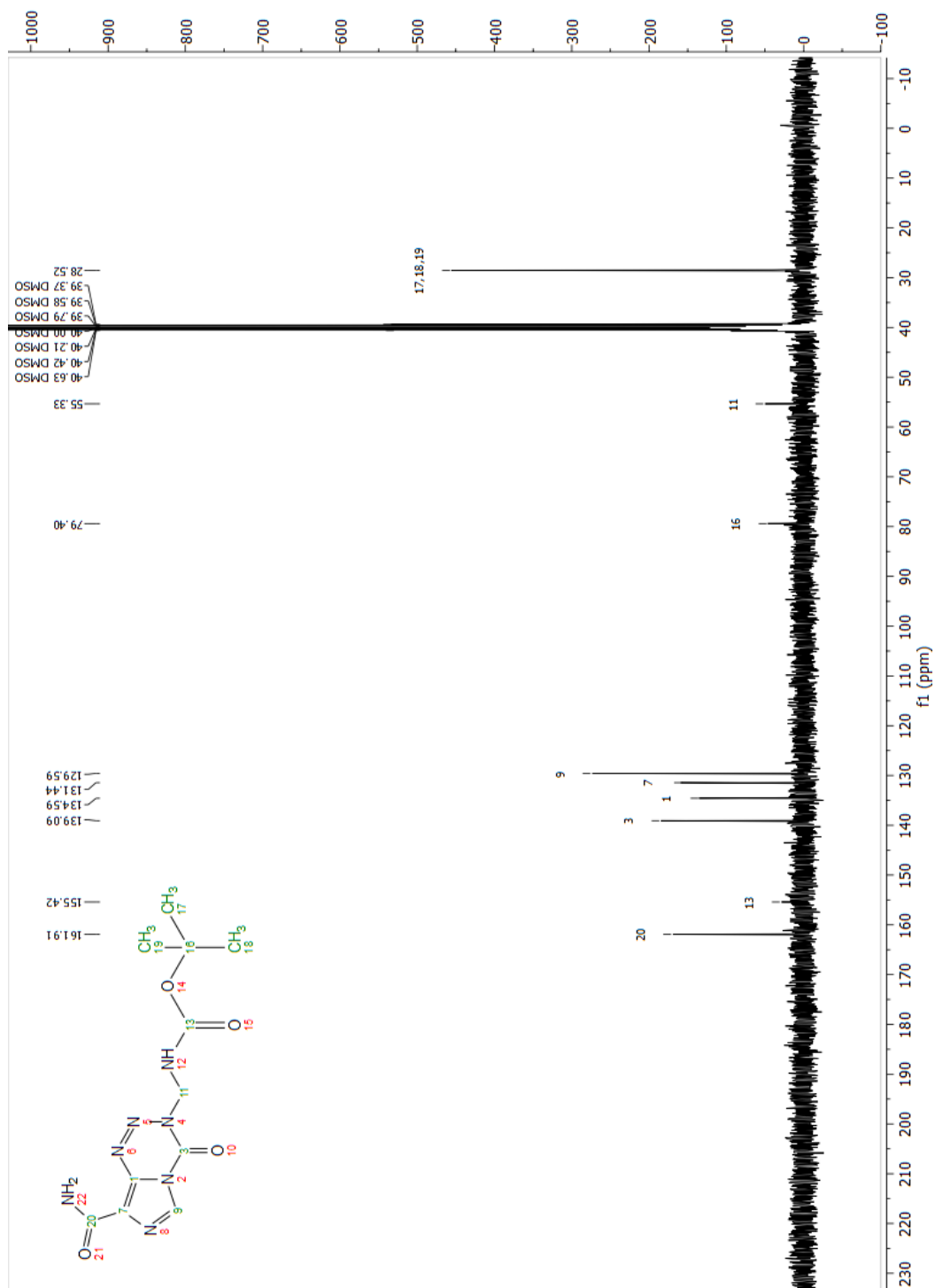


Figure A.13 | ¹³C NMR for *boc*-protected TMZ

¹³C-NMR (100 MHz; DMSO-d₆): δ 161.91, 155.42, 139.09, 134.59, 131.44, 129.59, 79.40, 55.33, 28.52.

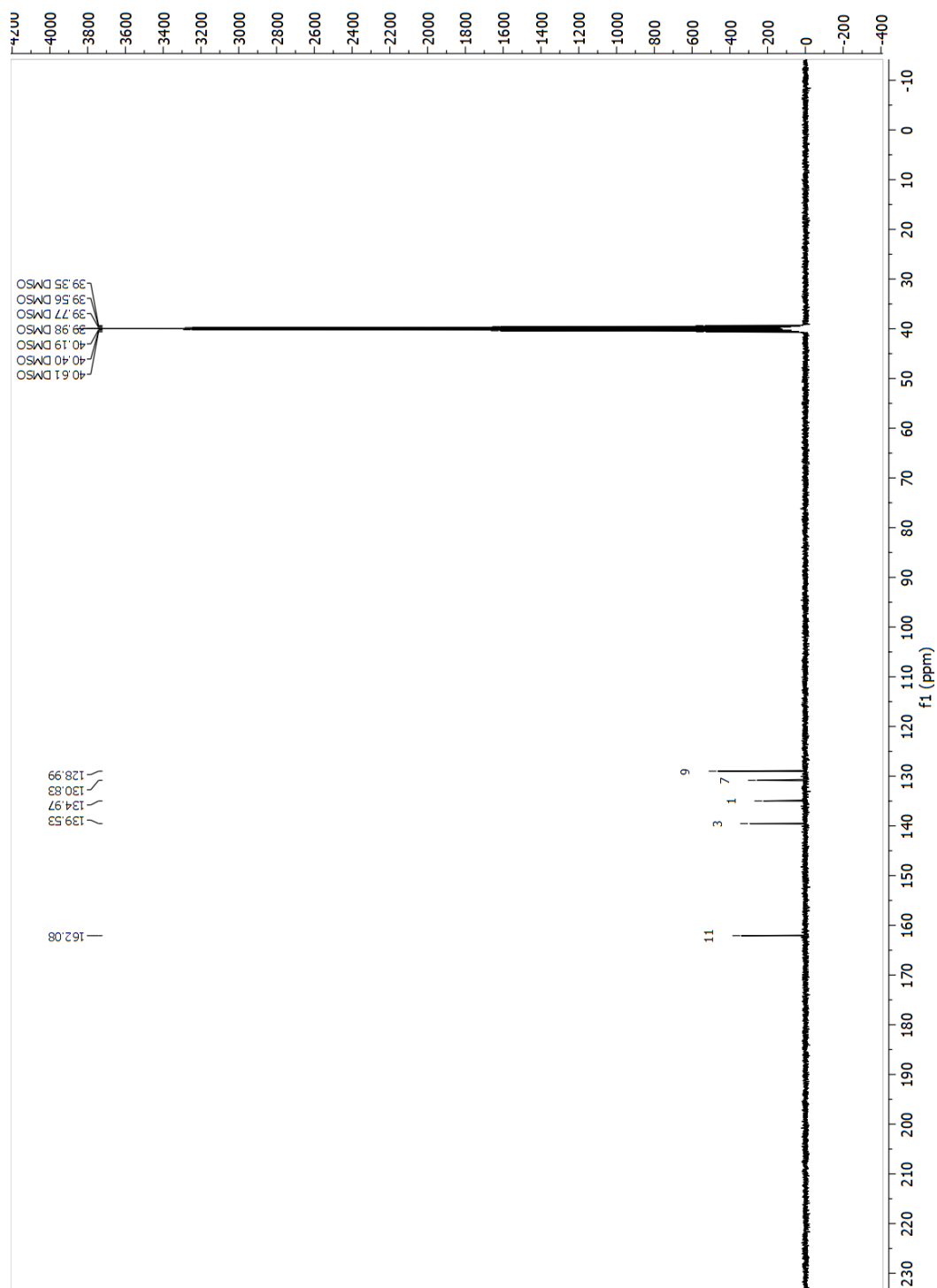


Figure A.15 | ¹³C NMR for *nor*-TMZ

¹³C-NMR (100 MHz; DMSO-d₆): δ 162.08, 139.53, 134.97, 130.83, 128.99.

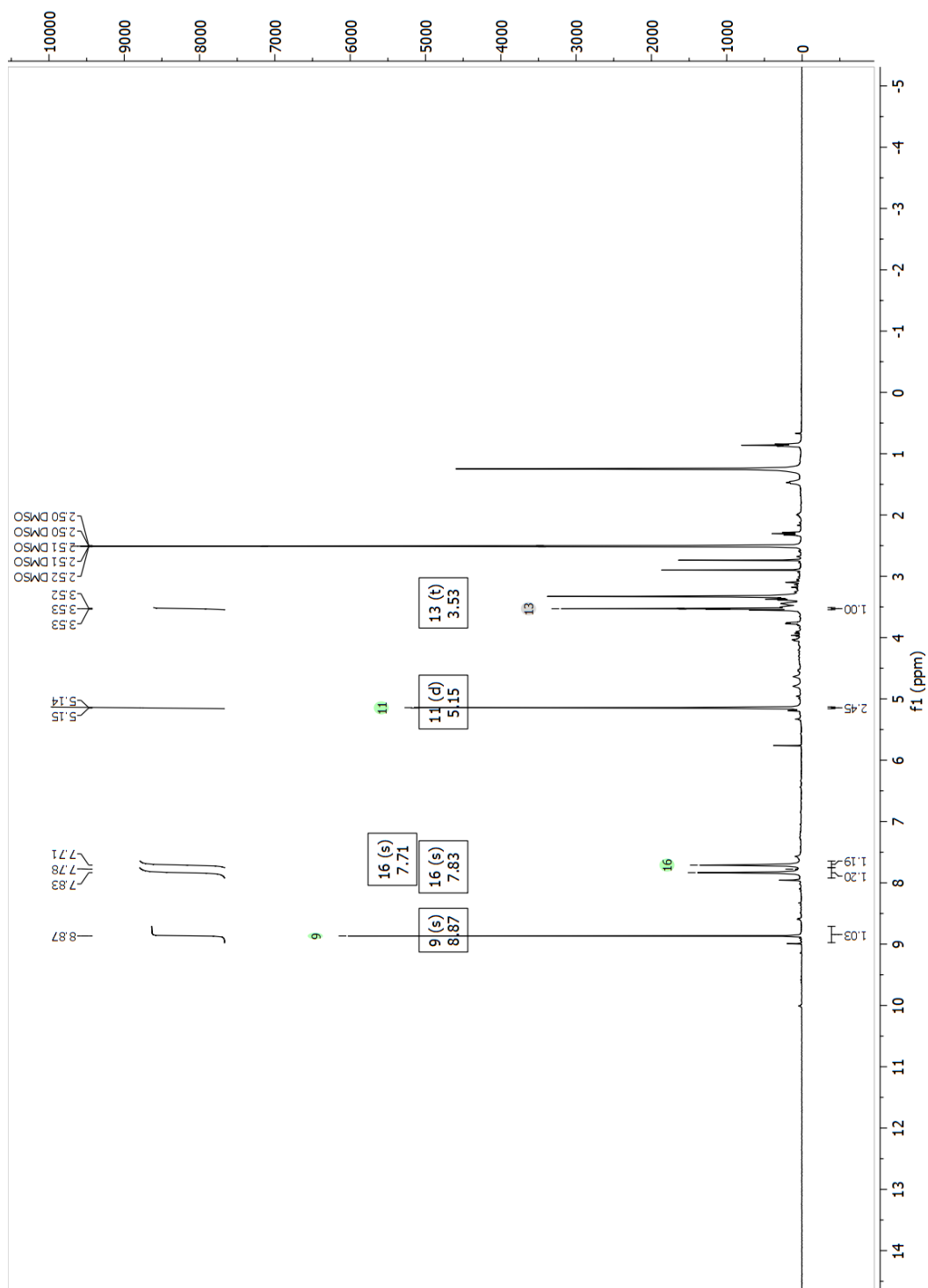


Figure A.16 | ¹H NMR for N³P-TMZ

¹H-NMR (400 MHz, DMSO-d₆): δ 8.87 (s, 1H), 7.83 (s, 1H), 7.78 (s, 1H), 7.71 (s, 1H), 5.15 (d, $J = 2.5$ Hz, 1H), 5.14 (s, 1H), 3.53 (t, $J = 2.5$ Hz, 2H), 3.52 (t, $J = 2.5$ Hz, 2H)

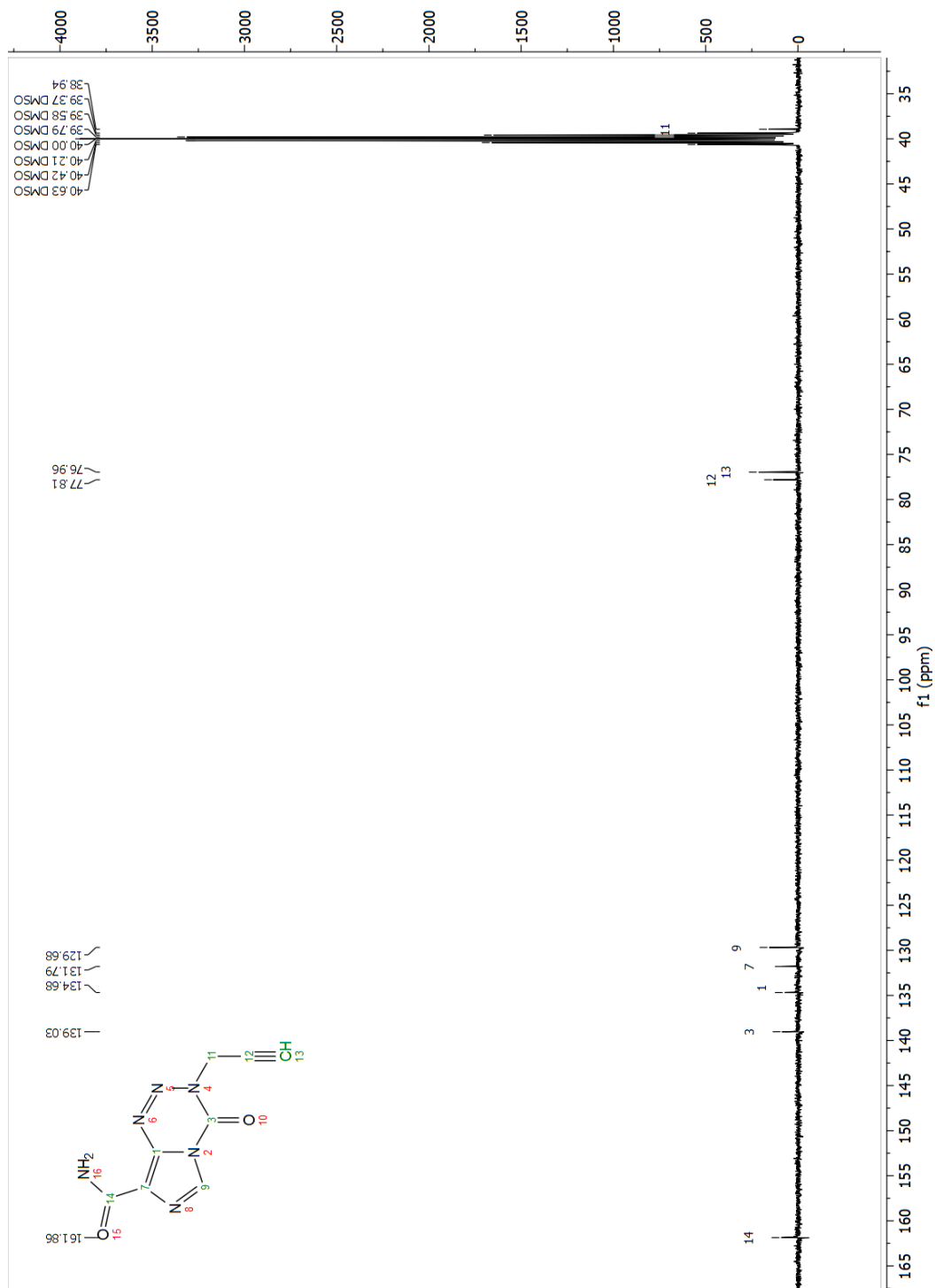


Figure A.17 | ¹³C NMR for *N*³*P*-TMZ

¹³C-NMR (100 MHz; DMSO-*d*₆): δ 161.86, 139.03, 134.68, 131.79, 129.68, 77.81, 76.96, 38.94.