Engineering tumour cells to display unnatural carbohydrates: the development of novel therapeutic strategies for cancer

University of Reading

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

This project introduces novel targeting approaches to increase the selectivity of chemotherapies in cancer treatment. These approaches involve converting the chemotherapies into less cytotoxic prodrugs that can be subsequently activated by bioorthogonal reactions to restore their cytotoxicity at the desired site of action. Two triphenylphosphine-prodrugs (triphenylphosphine-doxorubicin and triphenylphosphine-*N*-mustard, yielded in 39% and 31%, and with 95% and 98% purity, respectively) and two *trans*-cyclooctene-prodrugs (TCO-doxorubicin and TCO-*N*-mustard, yielded in 50% and 45%, and with 97% and 96% purity, respectively) were synthesised and tested for activation by azide and tetrazine activators *via* the Staudinger ligation and tetrazine ligation reactions, respectively.

Two complementary approaches were used for the selective delivery of the activators to tumour cells. In the first, metabolic glycoengineering was used to intercept the biosynthesis of overexpressed tumour associated carbohydrate antigens within cancer cells' surface glycans. Selective installation of azide and tetrazine tags on breast cancer cells using azide (50 μM)- and tetrazine (10 and 20 μM)-modified monosaccharides was successfully achieved (~17-fold and 35-65 folds higher than on non-cancerous fibroblasts). Subsequent activation of the triphenylphosphine- and TCO-prodrugs (> 68 % and up to 100%, respectively) by the engineered azides and tetrazines was demonstrated. In the second approach, PEG activators (PEG-azide and PEG-tetrazine, 10 KDa) were used for targeting cancer *via* passive targeting and the feasibility of their utilisation for prodrug activation was investigated. HPLC-monitored release studies showed 100% release of doxorubicin from the triphenylphosphine-doxorubicin and the TCO-doxorubicin prodrugs after 24 hours and 20 minutes by PEG-azide and PEG-tetrazine, respectively. *In vitro* activation studies on breast cancer cells showed the activation of the triphenylphosphine- and TCO-prodrugs (~68-76% and 100%, respectively) by PEG-azide and PEG-tetrazine. Taken together, these results show the feasibility of utilising metabolic glycoengineering and passive targeting as targeting approaches to increase the selectivity of bioorthogonal prodrugs activation in breast cancer.

Synopsis

Bioorthogonal chemistry refers to reactions that can proceed in biological systems (i.e. in an aqueous environment and at physiological pH) without interfering with biological processes. These reactions have been used to study biomolecules in their native environment and to develop selective diagnostic/imaging and therapeutic strategies. One of the therapeutic applications is *in-situ* prodrug activation in cancer therapy. For this, the bioorthogonal reactions enable the on-demand activation of the prodrugs, however, on-target activation is also a requirement for achieving the selective activation at the desired site of action.

Chapter 1 provides a general introduction to breast cancer, chemotherapy classes and limitations, and the current options for targeted therapy. Moreover, it introduces metabolic glycoengineering and passive targeting as novel targeting approaches for prodrug activation in breast cancer. The rationale of the work presented in this thesis is also provided.

Chapter 2 reviews and critically appraises the types of bioorthogonal reactions reported in the literature with a focus on their *in vivo* applications (cell tracking, imaging, and therapeutic applications) and the targeting mechanisms (metabolic glycoengineering, active, and passive targeting) utilised in these applications. The advances in utilising suitable bioorthogonal ligation and bond cleavage reactions for *in vivo* applications along with the encountered challenges were also identified. Fast reaction kinetics (i.e. k_2: $10^{-1} – 10^4$ M^{-1} S^{-1}) and high selectivity between the bioorthogonal components represent the most critical factors for choosing the suitable reactions for the aimed *in vivo* bioorthogonal application.

Chapter 3 demonstrates the feasibility of utilising metabolic glycoengineering for selective engineering of bioorthogonal azide activators within breast cancer cells' surface glycans using azide-modified monosaccharides *in vitro*. The feasibility of using the Staudinger reaction to activate triphenylphosphine-prodrugs at the azide-engineered breast cancer cells is also demonstrated *in vitro*. The synthesis and characterisation of two novel triphenylphosphine-prodrugs (i.e. triphenylphosphine-doxorubicin and triphenylphosphine-*N*-mustard, yielded in 39% and 31%, respectively) and two 9-azido sialic acid derivatives (i.e. 9-azido sialic acid and Ac_4-9-azido sialic acid, yielded in 46% and 35%, respectively) are reported. Western blotting and confocal imaging assays indicated the selective expression of azide activators within breast cancer cell surface glycans (~ 17-fold higher than non-cancerous L929 fibroblasts). *In vitro* prodrug activation studies indicated the selective activation of the triphenylphosphine-prodrugs on the azide-engineered breast cancer cells (~68 – 100% restoration of parent drugs cytotoxicity). A new Staudinger ligation-based release mechanism is introduced in an **appendix** for urea- and carbamate-linked triphenylphosphine-

prodrugs. Chemical synthesis of model prodrugs bearing a 4-nitroaniline moiety showed the instability of the reactants and the products and only synthesis of the urea model prodrug was successful. Preliminary HPLC-monitored release study showed the incomplete activation of the urea model prodrug (after 120 hours) which suggested the unsuitability of the proposed mechanism for further development into prodrug activation strategy.

In **Chapter 4**, novel tetrazine-modified monosaccharides were synthesised and characterised (i.e. Ac_4ManNTz, Ac_4GalNTz and Ac_4SiaTz, yielded in 67%, 74, and 14%, respectively) and selective labelling of breast cancer cells' surfaces *via* metabolic glycoengineering *in vitro* was demonstrated. Western blotting and confocal imaging indicated that the tetrazine expression level within breast cancer cells' surface glycans was concentration-dependant. The selectivity of tetrazine expression within breast cancer cell surface glycans was demonstrated to be 35-65 folds higher than in non-cancerous L929 fibroblasts. A novel TCO-*N*-mustard prodrug along with a TCO-doxorubicin prodrug were synthesised and characterised (yielded in 45% and 50%, respectively) and tested for prodrug activation on the tetrazine-engineered breast cancer cells by the tetrazine ligation bioorthogonal reaction. *In vitro* prodrug activation studies indicated that restoration of the parent drug's cytotoxicity is dependent on the level of Tz expression where the tetrazine-modified mannosamine and galactosamine derivatives at 20 μM concentration demonstrated the highest Tz expression and hence full recovery of the parent drug's cytotoxicity. A different attempted synthesis approach for the 9-tetrazine sialic acid derivative which does not rely on a protecting group strategy is detailed in an **appendix**. Suggestions to further improve the efficiency of the synthesis is also described.

Chapter 5 introduces the hypothesis of using PEG-derivatised bioorthogonal activators to increase breast cancer selectivity by passive targeting *via* the enhanced permeability and retention effect. The chapter explores the feasibility of derivatising PEG into bioorthogonal activators for prodrug activation in breast cancer cells. PEG-azide conjugates of two MWs (10 and 20 KDa) were tested for activating a triphenylphosphine model ester prodrug by the Staudinger ligation reaction. The 10 KDa conjugate demonstrated faster release rate than the 20 KDa (83% *versus* 44% release, respectively after 24 h) and complete release of doxorubicin from a triphenylphosphine-doxorubicin amide prodrug over the same time interval. A PEG-tetrazine conjugate (10 KDa) was synthesised (yielded in 74%) and demonstrated full activation of a carbonate TCO model prodrug after 24 h and full activation of a TCO-doxorubicin carbamate prodrug after 20 min. *In vitro* cytotoxicity studies on breast cancer cells showed ~68-76% restoration of the parent drug's cytotoxicity for the Staudinger ligation-based prodrug activation, and 100% restoration of the parent drug's cytotoxicity for the tetrazine ligation-based prodrug activation. In an **appendix**, a wound-healing/scratch assay on MDA-MB-231 breast cancer cells to test the anti-migratory activity of the activated TCO-prodrugs is described. The results

showed that TCO-prodrugs have lower anti-migration effects than the parent drugs that was restored upon activation by a PEG-tetrazine activator.

Chapter 6 provides a summary and critical evaluation of the key findings of this project and potential areas for future work. The suitability of using the Staudinger ligation and tetrazine ligation reactions for prodrug activation, and the parameters affecting their prodrug activation efficiency, are discussed with regards of the findings from this project and the literature. Moreover, the data presented in this project demonstrates the feasibility of using metabolic glycoengineering as a novel breast cancer targeting mechanism to deliver azide and tetrazine activators for selective bioorthogonal prodrug activation. Parameters that affect the magnitude of expression of chemical tags by metabolic glycoengineering, and strategies to increase the *in vivo* tumour selectivity of metabolic glycoengineering, are also discussed. Future work will look to further improve the serum stability of the compounds and to further extend the approach to other bioorthogonal reactions and cancer types.

List of Publications

M. M. A. Mitry, F. Greco, and H. M. I. Osborn. *In vivo* Applications of Bioorthogonal Reactions-Chemistry and Targeting mechanisms. *Chem. Eur. J. 2023, 29, e202203942*. (Chapter 2)

M. M. A. Mitry, S. Y. Boateng, F. Greco, and H. M. I. Osborn. Bioorthogonal activation of prodrugs, for the potential treatment of breast cancer, using the Staudinger reaction. *RSC Med Chem. 2023, 14(8):1537-1548*. (Chapter 3)

M. M. A. Mitry, M. Dallas, S. Y. Boateng, F. Greco, and H. M. I. Osborn. Selective activation of prodrugs in breast cancer using metabolic glycoengineering and the tetrazine ligation bioorthogonal reaction. *Accepted for publication in Bioorg. Chem, March 2024. DOI: 10.1016/j.bioorg.2024.107304.* (Chapter 4)

M. M. A. Mitry, F. Greco, and H. M. I. Osborn. Polymeric PEG-based bioorthogonal triggers for prodrug activation in breast cancer. (2024). *In preparation for submission to Polym. Chem.* (Chapter 5)

List of Conferences Attended

M. M. A. Mitry, F. Greco, and H. M. I. Osborn. Engineering tumour cells to display unnatural carbohydrates: the development of novel therapeutic strategies for cancer. **Pharmacy PhD Showcase, April 2021, University of Reading, UK. Oral flash presentation.**

Medicinal Chemistry Ireland Online Meeting, July 2021, online event.

M. M. A. Mitry, F. Greco, and H. M. I. Osborn. Using metabolic glycoengineering for targeted treatment of cancer. **The 29th Annual GP2A medicinal chemistry conference, August 2021, online. Poster presentation**.

M. M. A. Mitry, S. Y. Boateng, F. Greco, and H. M. I. Osborn. Bioorthogonal Chemistry for Prodrug activation in breast cancer therapy. **Pharmacy PhD Showcase, March 2022, University of Reading, UK. Oral presentation.**

M. M. A. Mitry, F. Greco, and H. M. I. Osborn. Activation of prodrugs for cancer chemotherapy using azide-conjugated PEG polymers. **The 13th International Symposium on Polymer Therapeutics: From Laboratory to Clinical Practice, May 2022, Valencia, Spain. Poster.**

M. M. A. Mitry, S. Y. Boateng, F. Greco, and H. M. I. Osborn. Engineering tumour cells to display unnatural carbohydrates: the development of novel therapeutic strategies for cancer. **RSC Chemical Biology meets Drug Discovery 2022, September 2022, Francis Crick Institute, London, UK. Poster.**

M. M. A. Mitry, S. Y. Boateng, F. Greco, and H. M. I. Osborn. Engineering tumour cells to display unnatural carbohydrates: the development of novel therapeutic strategies for cancer. **Charles River PhD Poster Competition, September 2022, Charles River, Chesterford Research Park, UK. Poster.**

M. M. A. Mitry, S. Y. Boateng, F. Greco, and H. M. I. Osborn. Bioorthogonal Chemistry for Prodrug activation in breast cancer therapy. **Pharmacy PhD Showcase, March 2023, University of Reading, UK. Oral presentation.**

M. M. A. Mitry, S. Y. Boateng, F. Greco, and H. M. I. Osborn. Bioorthogonal activation of prodrugs, for the potential treatment of breast cancer, using the Staudinger reaction. **The 31st Annual GP2A medicinal chemistry conference, August 2023, Marseille, France. Talk**.

M. M. A. Mitry, F. Greco, and H. M. I. Osborn. PEG-based bioorthogonal activators for prodrug activation in breast cancer. **The 14th APS PharmSci international conference, September 2023, Reading, UK. Poster** (poster prize winner).

Table of Contents

Chapter 1: General introduction **1**

Chapter 2: *In vivo* bioorthogonal chemistry applications: types of reactions and targeting mechanisms **38**

Chapter 3: Metabolic glycoengineering using azide-modified monosaccharides for triphenylphosphine-prodrugs' selective activation in breast cancer cells **62**

Appendix: Proposed alternative Staudinger release mechanism using urea and carbamate-linked triphenylphosphine-prodrugs **93**

Chapter 4: Metabolic glycoengineering using tetrazine-modified monosaccharides for TCO-prodrugs' selective activation in breast cancer cells **107**

Appendix: Alternative synthetic approaches for accessing the Ac_4SiaTz compound **156**

Chapter 5: Activation of triphenylphosphine-prodrugs and *trans*-cyclooctene-prodrugs in breast cancer cells using PEG-azide and PEG-tetrazine activators, respectively **163**

Appendix: *In vitro* wound-healing scratch assay to test the effect of the activated prodrug on the migration of MDA-MB-231 cells **193**

Chapter 6: General discussion **197**

Abbreviations

5-FU: 5-fluorouracil

ADIBO: azadibenzocyclooctyne

ANOVA: analysis of variance

ASCO: American society of clinical oncology

AT: active targeting

BC: breast cancer

BCN: bicyclononyne

CPT: camptothecin

CuAAC: copper-catalyzed azide−alkyne cycloaddition

Cy5: cyanine-5

DAPI: 4',6-diamidino-2-phenylindole

DBCO: dibenzocyclooctyne

DCC: *N,N*'-dicyclohexylcarbodiimide

DCL-AAM: histone deacetylase/cathepsin L-responsive acetylated azidomannose

DIC: *N,N'*-Diisopropylcarbodiimide

DMAP: 4-dimethylaminopyridine

DMSO: dimethyl sulfoxide

Dox: doxorubicin

DPP: diphenylphosphine

DPPBA: 2-diphenylphosphinobenzoic acid

EDG: electron donating group

EPR: enhanced permeability and retention

ER: oestrogen receptor

ESMO: European society for medical oncology

EWG: electron withdrawing group

FBS: fetal bovine serum

FDA: food and drug administration

Gal: galactosamine

GalNAc: *N*-acetyl galactosamine

GlcNAc: *N*-acetyl glucosamine

hEPCs: human embryonic stem cells-derived endothelial progenitor cells

HER2: human epidermal growth factor receptor 2

HPLC: high performance liquid chromatography

HPMA: *N*-(2-hydroxypropyl)methacrylamide

HRs: hormone receptors

IC_{50}: half maximal inhibitory concentration

IEDDA: inverse electron demand Diels–Alder

IR: infra-red

IT: intratumoral

IV: intravenous

J: coupling constant

m/z: mass/charge ratio

m: multiplet

mAb: monoclonal antibody

Man: mannosamine

ManNAc: *N*-acetyl mannosamine

ManNAz: *N*-azidoacetylmannosamine

MFI: mean fluorescence intensity

MGE: metabolic glycoengineering

MMAE: monomethyl auristatin E

MMT: matrix metalloproteases

MOA: mechanism of action

mp: melting point

MsCl: methanesulfonyl chloride

MTD: maximum tolerated dose

mTOR: mammalian target of rapamycin

Mw: molecular weight

Neu5Ac: *N*-acetyl neuraminic acid

NICE: national institute for health and care excellence

NMR: nuclear magnetic resonance

NO: nitrogen oxide

NP: nanoparticle

PBS: phosphate buffer saline

PDC: polymer-drug conjugate

PEG: poly(ethylene glycol)

PET: positron emission tomography

PFAAs: perfluoroaryl azides

pH: power of hydrogen

PI3K: phosphatidylinositol 3-kinase

PLGA: poly lactic-co-glycolic acid

ppm: parts per million

PR: progesterone receptor

PSA: poly sialic acid

PSA: prostate-specific antigen

PT: passive targeting

RaNi: Raney-nickel

ROS: reactive oxygen species

RT: room temperature

s: singlet

SEM: standard error of mean

SERD: selective estrogen receptor degrader

Sia: sialic acid

sLe^a: sialyl Lewis^a

sLe^x: sialyl Lewis^x

SPAAC: strain-promoted azide−alkyne cycloaddition

SPECT: single-photon emission computerised tomography

sTn: sialyl Tn

SuFEx: sulfur(VI) fluoride exchange

t: triplet

TACAs: tumour-associated carbohydrate antigens

TBDMS: *tert*-butyldimethylsilyl

TBDMSCl: *tert*-butyldimethylsilyl chloride

TBS: tris-buffered saline

TCO: *trans*-cyclooctene

TEA: triethylamine

TFA: trifluoroacetic acid

Tz: tetrazine

WHO: world health organisation

Chapter 1

General introduction

1.1. Background

Cancer is one of the leading causes of death worldwide resulting in nearly 10 million deaths in 2020 (almost one in six deaths is attributed to cancer) with over 16 million deaths expected by 2040.[1,2] Of the identified 200 types of cancer, breast, lung, colorectal, and prostate cancer are the most prevalent according to the latest data (2020) by the World Health Organization (WHO).[1,3]

Chemotherapy is one of the main treatment options for cancer. However, despite its efficiency and preference for use, it can cause side effects throughout the body due to its systemic administration.[4] The research presented in this thesis aims to develop novel targeting methods through a medicinal chemistry approach to increase the selectivity of established chemotherapy drugs (doxorubicin and nitrogen mustard) for cancer (breast cancer has been chosen as a model to test the approach). The proposed approach includes the use of bioorthogonal chemistry (the Staudinger ligation and tetrazine ligation reactions) to develop novel chemotherapeutic prodrug activation systems.[5] Cancer targeting mechanisms (Metabolic glycoengineering (MGE) and passive targeting *via* the enhanced permeability and retention (EPR) effect) are also explored to increase the cancer-selectivity of the prodrug activation systems.

This chapter provides a brief background to key areas of relevance to the presented project, specifically, breast cancer, chemotherapy and its limitations, current targeted breast cancer therapy approaches, the concepts of MGE and the EPR effect as breast cancer targeting mechanisms, and the concept of bioorthogonal prodrugs **(Fig. 1)**. Thereafter, the project aims and structure of the thesis are presented.

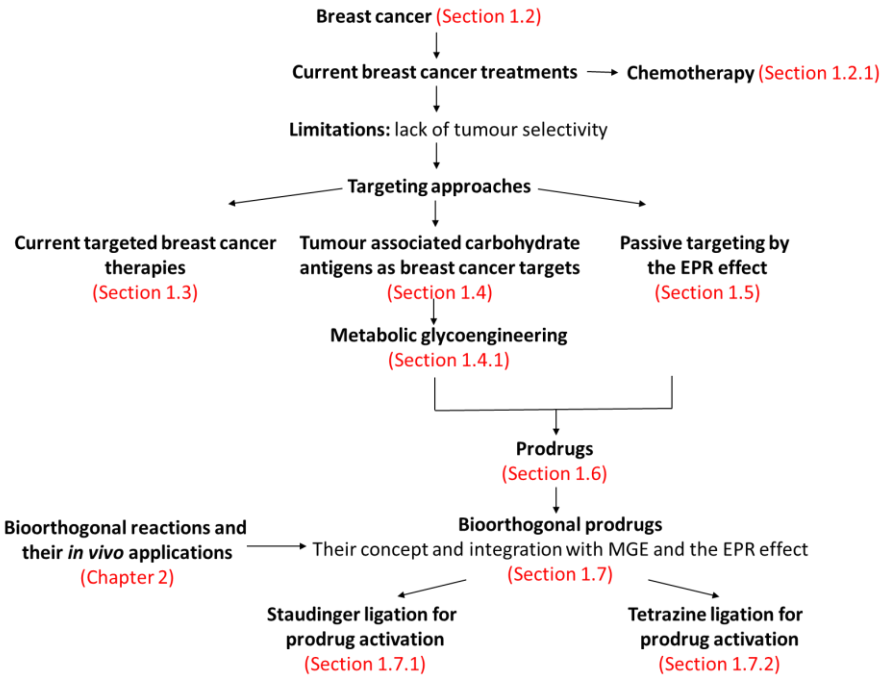


Figure 1. Schematic representation of the organisation of the general background to this project.

1.2. Breast cancer: current status and general overview of treatment options

Since 2020, breast cancer has become the most prevalent cancer type in the UK and worldwide (around 2.26 million diagnosed cases and 685,000 deaths in 2020 worldwide) and the leading cause of death from cancer in women.[6] Over the last three decades, the incidence of breast cancer has doubled in over 60 countries, and breast cancer-related deaths have doubled in over 40 countries. Indeed, it is predicted that breast cancer cases will reach 2.7 million diagnosed cases and 0.87 million deaths by 2030.[7,8]

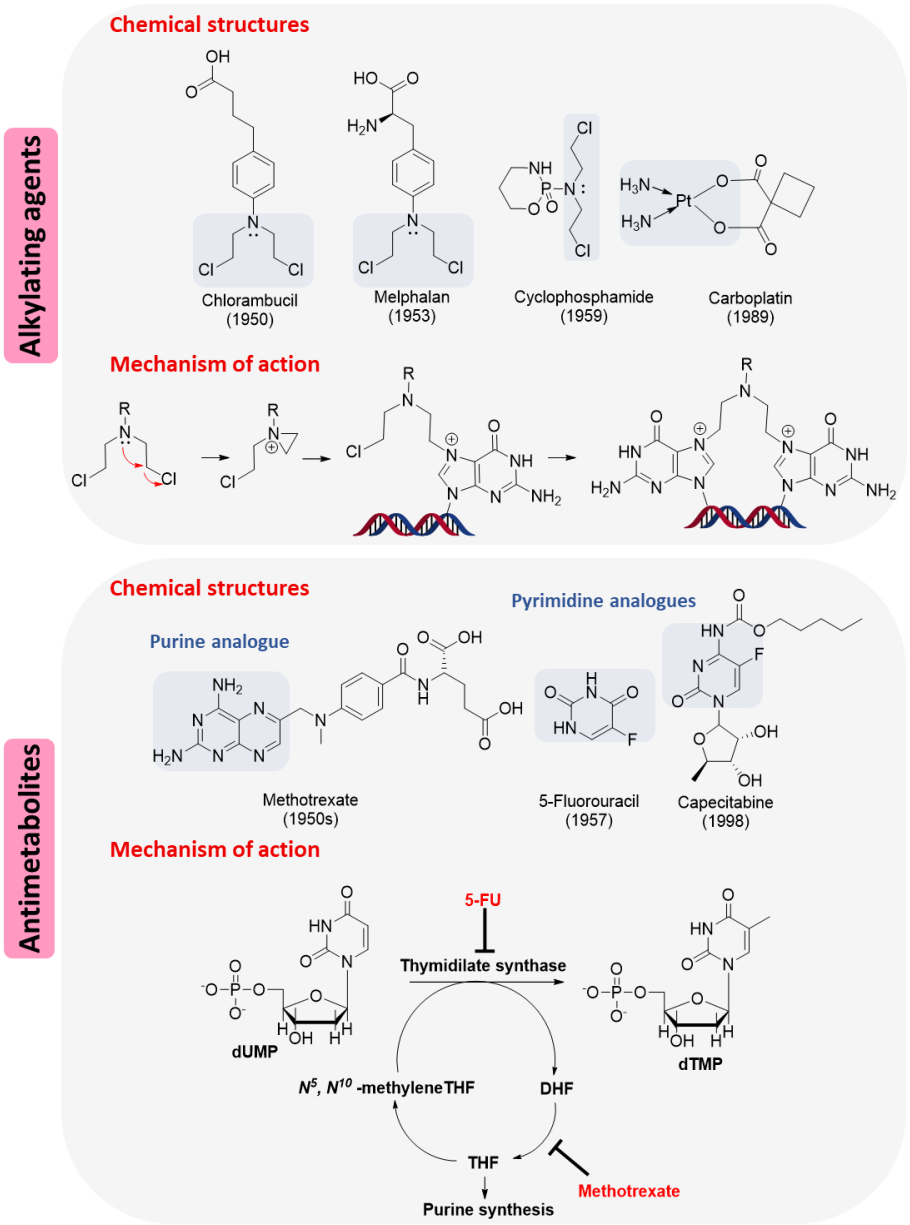
Treatment approaches for breast cancer generally include surgery, radiotherapy, chemotherapy, hormonal therapy, and targeted therapy.[9] Surgery is usually considered the primary treatment option for breast cancer, especially in early-diagnosed cases when the tumour is localised, and lumpectomy (breast-conserving surgery) or mastectomy can be performed.[10,11] According to the European Society for Medical Oncology (ESMO) guidelines for early breast cancer, breast-conserving surgery is preferred and the choice between this and mastectomy depends on tumour size/stage, patient choice, and the tumour clinical phenotype.[12] Chemotherapy is a crucial aspect of breast cancer treatment due to its cytotoxic effectiveness and ability to limit metastases which contributes to positive clinical outcomes.

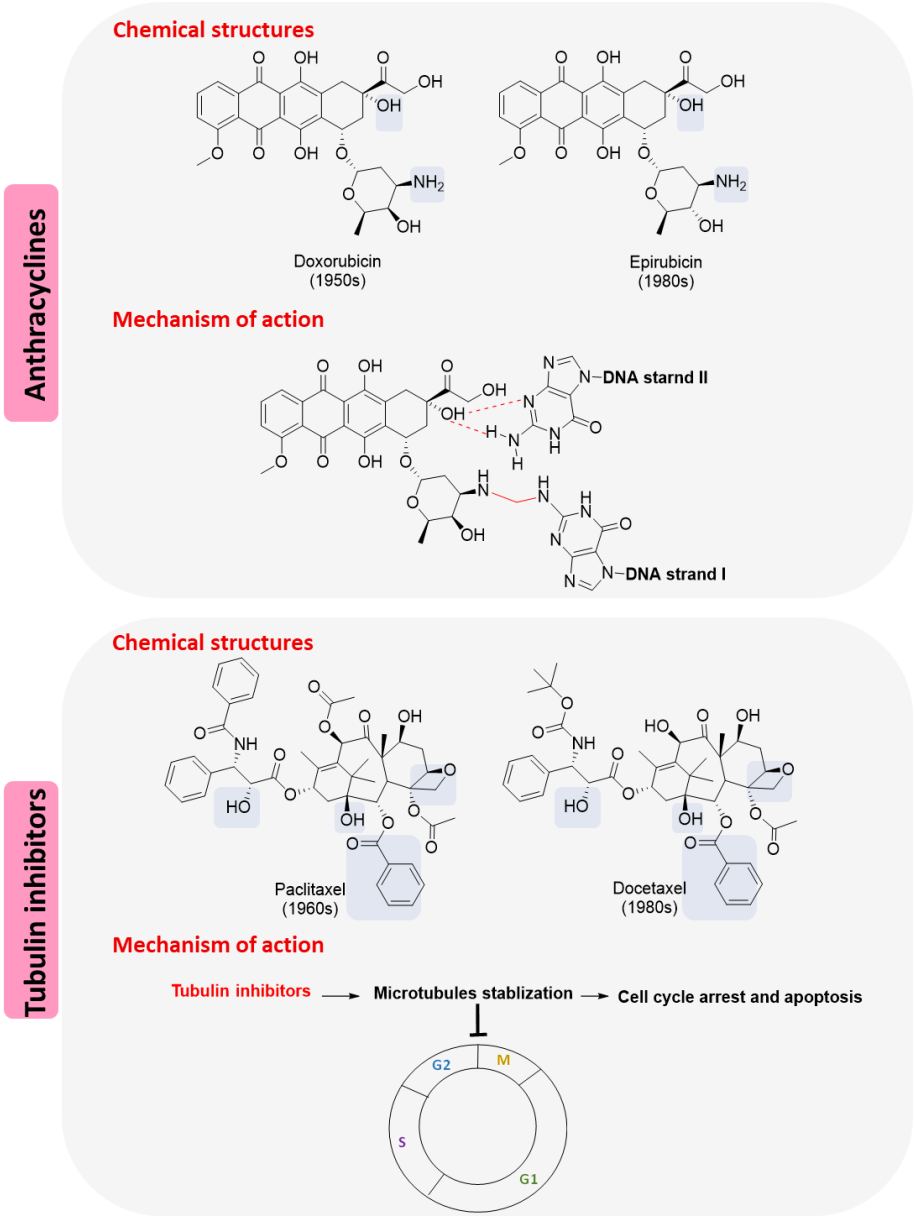
1.2.1. Chemotherapy in breast cancer

The use of chemotherapeutic drugs in breast cancer treatment is an approach in which drugs are administered systemically to induce cancer cell death (cytotoxic) or inhibit their proliferation (cytostatic). These drugs comprise alkylating agents, antimetabolites, anti-tumour antibiotics (anthracyclines), and tubulin inhibitors (**Table 1, Fig. 2**). Conventional chemotherapies in breast cancer treatment are either neoadjuvant (administered before surgery to minimise the tumour size and facilitate its removal) or adjuvant (administered after surgery to eliminate the cancer cells left after surgery to reduce the reoccurrence rates and metastasis). Chemotherapy can also be chemoradiotherapy (given with radiotherapy) or palliative chemotherapy (in advanced cancer stages for relieving symptoms and improving quality of life).[13–15]

Table 1: Summary of the chemotherapeutic drugs currently used in breast cancer treatment

Class	Examples	Mechanism of action (MOA)	Ref
Alkylating agents	-*N*-Mustards such as Melphalan, Chlorambucil, and Cyclophosphamide -Platinum compounds such as Carboplatin	DNA binding, cross-linking the DNA strands, and preventing cell replication.	[16,17]
Antimetabolite drugs	Methotrexate, 5-Fluorouracil, and Capecitabine	Structural analogues that 1- replace natural DNA nucleotides, 2- inhibit enzymes involved in DNA synthesis which leads to DNA damage.	[18,19]
Anti-tumour antibiotics (anthracyclines)	Doxorubicin and Epirubicin	-DNA intercalation forming a drug-DNA adduct to prevent DNA replication. -Topoisomerase II inhibitors that disrupt topoisomerase II-mediated DNA repair.	[20,21]
Tubulin inhibitors	Paclitaxel and Docetaxel	Interfere with cell mitosis through stabilisation of cellular microtubules and thus prevent cell division.	[22,23]


Alkylating agents
Chemical structures
Chlorambucil (1950)
Melphalan (1953)
Cyclophosphamide (1959)
Carboplatin (1989)
Mechanism of action
Antimetabolites
Chemical structures
Purine analogue
Pyrimidine analogues
Methotrexate (1950s)
5-Fluorouracil (1957)
Capecitabine (1998)
Mechanism of action
5-FU
Thymidilate synthase
dUMP
dTMP
N^5, N^{10} -methyleneTHF
DHF
THF
Methotrexate
Purine synthesis



DHF: dihydrofolate; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; THF: tetrahydrofolate.

Figure 2. Chemical structures of the currently used chemotherapeutic drugs in breast cancer treatment, their historical development, essential functional groups for activity, and their mechanisms of action.

The use of chemotherapy has improved the survival rate of patients diagnosed with breast cancer (early stage and metastatic),[24] however, the lack of selectivity of these drugs towards cancer cells leads to undesirable toxicity towards normal cells. This normal cell toxicity is the reason for the serious side effects of the chemotherapies which comprise anaemia, alopecia, bone marrow suppression, and low platelet counts.[25] More serious toxicities are also associated with some chemotherapies. For example, platinum compounds, such as carboplatin, are associated with nephrotoxicity, ototoxicity, and neurotoxicity, and doxorubicin is associated with cardiotoxicity which limit their cumulative doses and long-term use.[26–28] The lack of selectivity in chemotherapy also affects the concentration of the drug that reaches the tumour. A high intratumoral concentration of the drug is required to

maximise its effect, but this is not normally achieved.[29] This is one of the main reasons that maximum tolerated doses of the chemotherapies are usually administered, resulting in cumulative dose toxicities and long-term side effects.[25,30]

As a result of these challenges that accompany conventional chemotherapy, interest has turned towards the development of targeted cancer therapy and tumour selective drug-delivery systems. These approaches aim to decrease the drawbacks associated with systemic toxicity, low selectivity, and low bioavailability of conventional chemotherapies.

1.3. Current targeted breast cancer therapies

Targeted cancer therapy differs from conventional chemotherapy by targeting specific characteristics that are unique to, or overexpressed by, cancer cells such as overexpressed proteins and mutated targets.[31] In breast cancer, targeted therapy can be classified according to the molecular subtype of breast cancer (i.e. according to the absence or presence of breast cancer biomarkers). **Table 2** summarises the subtypes of breast cancer and their relevant biomarkers.[32–35]

Table 2: Classification of breast cancer subtypes according to biomarkers.[32–35]

	Luminal A	**Luminal B**	**HER2-enriched**	**Triple negative**
Hormone receptors (HRs) (Oestrogen ER and Progesterone PR)	+ ER +/- PR	+ ER +/- PR	-	-
Human epidermal growth factor receptor 2 (HER2) protein	-	- /+	+	-
Ki-67 protein (a cellular marker for proliferation)	Low level	High level	High level	High level

In 1998, Trastuzumab, also called Herceptin (an anti-HER2 antibody), became the first FDA-approved targeted breast cancer therapy in clinical use.[36,37] Subsequent to this, targeted breast cancer therapy has seen many developments. **Table 3** shows the FDA-approved and currently clinically used breast cancer targeted therapies, their classes, and their limitations.

Table 3: FDA-approved breast cancer targeted therapies, their classes, and limitations.

BC-targeted therapy according to the BC subtype		Class	Limitations/adverse effects	FDA approval	Ref
HER2+ targeted therapy	Trastuzumab Pertuzumab Margetuximab	Anti-HER2 antibody	Frequent cardiotoxicity that can lead to heart failure and pulmonary toxicity.	Trastuzumab: approved in 1998 Pertuzumab: approved in 2017 Margetuximab: approved in 2020	[38,39]
	Lapatinib Neratinib Tucatinib	Tyrosine kinase inhibitors	Modest antitumour efficacy, should be used in combination with trastuzumab and other chemotherapies as second- and third-line treatments.	Lapatinib: approved in 2007 Neratinib: approved in 2017 Tucatinib: approved in 2020	[40–42]
	Trastuzumab-Emtansine (T-DM1) Trastuzumab-deruxtecan (T-Dxd)	HER2 ADC	-Only used as adjuvant therapy. -Risk of thrombocytopenia, hepatotoxicity (T-DM1), pulmonary, and haematological toxicities (T-Dxd).	Trastuzumab-Emtansine: approved in 2013 Trastuzumab-deruxtecan: approved in 2022	[43–45]
HER2-, HR+ targeted therapy	Palbociclib Ribociclib Abemaciclib	CDK4/6 inhibitors	-Given with oestrogen receptor blockers or aromatase inhibitors to increase cell sensitivity. -Diarrhoea in >80% of patients due to off-target effects. -Thromboembolism and haematological toxicity due to CDK6 inhibition.	Palbociclib: approved in 2015 Ribociclib: approved in 2017 Abemaciclib: approved in 2017	[46–50]
	Everolimus	mTOR inhibitors	Immunosuppressant effect, pneumonitis, neutropenia, stomatitis, diarrhoea, and alopecia.	Approved in 2012	[51,52]
	Alpelisib	PI3K inhibitors	Hyperglycemia, rash, diarrhoea, and pneumonitis.	Approved in 2019	[53,54]

Triple negative targeted therapy	Sacituzumab govitecan	TROP2 ADC	Neutropenia, anaemia, nausea, vomiting, and diarrhoea.	Approved in 2021	[55–57]
	Pembrolizumab	Immune checkpoint inhibitor	Immune-related side effects e.g. severe skin reactions, thyroiditis, pneumonitis, hepatitis, nephritis, and colitis.	Approved in 2021	[58,59]

BC hormonal therapy		**Class**	**Limitations/adverse effects**	**FDA approval**	**Ref**
ER+ targeted therapy	Tamoxifen	Oestrogen-receptor blocker	Blood clots, high stroke risk, and increased endometrial cancer risk.	Approved in 1978	[60]
	Anastrozole Letrozole Exemestane	Aromatase inhibitors	Reduction in bone density, joint and muscle pain, elevated cholesterol and blood pressure, and CNS side effects.	Anastrozole: approved in 1995 Letrozole: approved in 1997 Exemestane: approved in 1999	[61,62]
	Fulvestrant	Selective oestrogen receptor degrader (SERD)	Hepatotoxicity, haematological disorders, and muscle pain.	Approved in 2007	[63,64]

ADC: antibody drug conjugate; CDK: cyclin-dependant kinase; HER2: human epidermal growth factor receptor 2; HR: hormone receptor; mTOR: mammalian target of rapamycin; PI3K: phosphatidylinositol 3-kinase; T-DM1: Trastuzumab-Emtansine; T-Dxd: Trastuzumab-deruxtecan; TROP-2: trophoblast cell surface antigen 2.

Despite the advances of targeted breast cancer therapy, they face limitations in their clinical use.

-In HER2+ breast cancer, conventional chemotherapy is recommended for use in combination with the HER2-targeted therapy. For example, according to the guidelines from the National Institute for Health and Care Excellence (NICE)[65] and the American Society of Clinical Oncology (ASCO),[66] Trastuzumab is recommended for use in combination with a taxane (paclitaxel or docetaxel) to maximise the efficiency of both and for safety considerations (decrease in the administered dose). However, HER2+ breast cancer is known to have high drug resistance and HER2 expression heterogeneity which significantly reduces the effectiveness of anti-HER2 treatment. For example, it has been reported that only one third of HER2+ breast cancer patients benefit from anti-HER2 antibody therapy (in terms of increasing time interval of disease progression and increasing survival rate) due to the structural modification of HER2, which contributes to resistance and low antibody binding.[67] Also, HER2 tyrosine kinase domain mutation causes resistance to tyrosine kinase inhibitors.[68]

-In HR+ breast cancer, CDK4/6, mTOR, and PI3K inhibitors are shown to have good clinical response rates, for example, CDK4/6 inhibitors (examples in **table 3**) are reported to increase the progression-free survival rate by more than one year when given with hormonal therapy.[69–71] However, they have many side effects (as shown in **table 3**). This is mainly because they intercept pathways that regulate many cellular processes like protein synthesis, cell cycle progression, cell survival, and metabolism and the crosstalk between these signalling pathways contributes to drug resistance.[72]

-Triple negative breast cancer is the most proliferative and invasive subtype, and has fewer treatment options as it lacks HR expression (making it ineligible for hormonal therapy) and does not overexpress HER2 (making it ineligible for Trastuzumab).[73] The treatment options mainly comprise conventional chemotherapies,[74] or TROP-2 antibody loaded to deliver a cytotoxic chemotherapy drug (SN-38) which is highly cytotoxic against triple negative breast cancer cells but is unsuitable for systemic administration,[75] or immunotherapy antibodies that are administered with conventional chemotherapies.[76]

Despite the advances introduced in targeted breast cancer therapy over the past 25 years, they still face toxicity risks, serious adverse effects **(table 3)**, and development of drug resistance which emphasises the need for the identification and development of novel breast cancer targeting approaches.

1.4. Tumour-associated carbohydrate antigens (TACAs) as breast cancer targets

Carbohydrates (also known as glycans) are abundant within the human cell membrane surface biomolecules, where they are either bound to membrane phospholipids (i.e. to form glycolipids) or proteins (i.e. to form glycoproteins). Biosynthesis of glycans (on their lipid or protein scaffolds) involves the action of two main bio enzymes, specifically glycosyltransferases and glycosidases, which result in

the production of a vast array of glycan structures.[77] Glycosylation (i.e. addition of sugar moieties to the cell surface proteins) by the glycosyltransferases can either be *O*-glycosylation, where the sugar is added to the hydroxyl group of serine or threonine residues (forming *O*-linked glycoproteins), or *N*-glycosylation, where the sugar is added to the amino group of asparagine residues (forming *N*-linked glycoproteins). The terminal positions in the *O*-linked and *N*-linked glycoproteins often display sialic acid residues.[78] The cellular functions of these surface glycans mainly comprise maintaining membrane integrity, cell-cell interactions, and host-pathogen interactions.[79,80]

In cancer, various alterations occur to the cell surface glycosylation pattern as a result of variations in the expression level of the glycosyltransferase and glycosidase enzymes. The resulting alterations usually influence the size and/or the branching of glycoproteins resulting, for example, in overexpression of complex-*N*-branched glycoproteins, expression of shortened *O*-linked glycans, and altered expression of sialoglycoproteins.[81,82] This means that the altered expression in the glycosylation enzymes in cancer causes the production of glycoproteins with specific tumour-associated variations in their glycan structures, also known as Tumour-Associated Carbohydrates Antigens (TACAs).[82,83] Within these glycosylation enzymes, sialyltransferases, which are responsible for the addition of sialic acid residues to the growing chains of glycoproteins, are reported to be highly upregulated in many cancer types including breast cancer.[84,85] This upregulation leads to a condition called hypersialylation where specific terminal glycan epitopes (i.e. TACAs) are overexpressed.[86] These TACAs are characterised by increased size and branching which creates additional sites for more terminal sialic acid residues to be added by the upregulated sialyltransferases.[87–89] TACAs usually mediate various tumour hallmarks such as cell-cell interactions, oncogenic signalling, and most importantly, metastasis.[84,90] TACAs are considered as hallmarks of tumour phenotypes, which suggests their clinical importance as biomarkers and therapeutic targets. During the last two decades, TACAs have been investigated to serve as selective markers for cancer diagnosis[91] and for developing carbohydrate-based anti-cancer vaccines.[92] This research project aims to use TACAs as novel targets for breast cancer therapy.

In breast cancer, the most commonly expressed TACAs are sialyl Lewis^{x} (sLe^{x}), sialyl Lewis^{a} (sLe^{a}), sialyl Tn (sTn), and polysialic acid (PSA) **(Fig. 3)**.[93,94] The common feature between these TACAs is the abundance of sialic acid. Therefore, this project proposes the use of sialic acid-rich TACAs as novel selective targets for delivering breast cancer chemotherapies through metabolic glycoengineering (MGE).

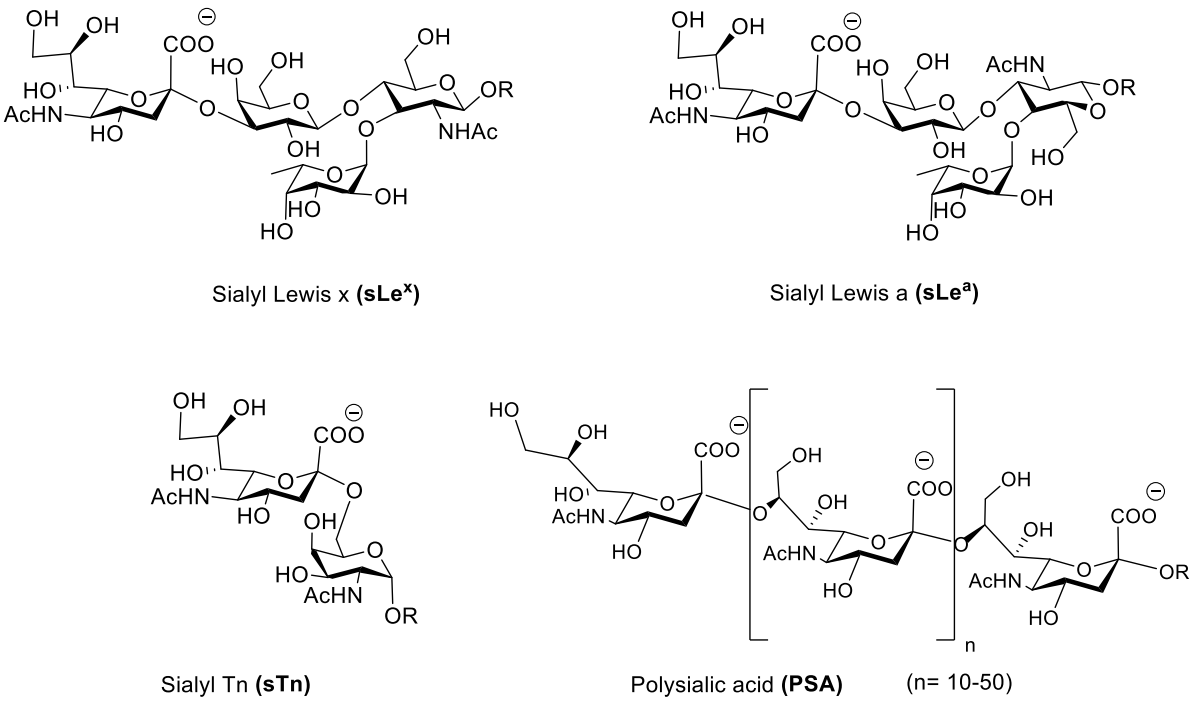
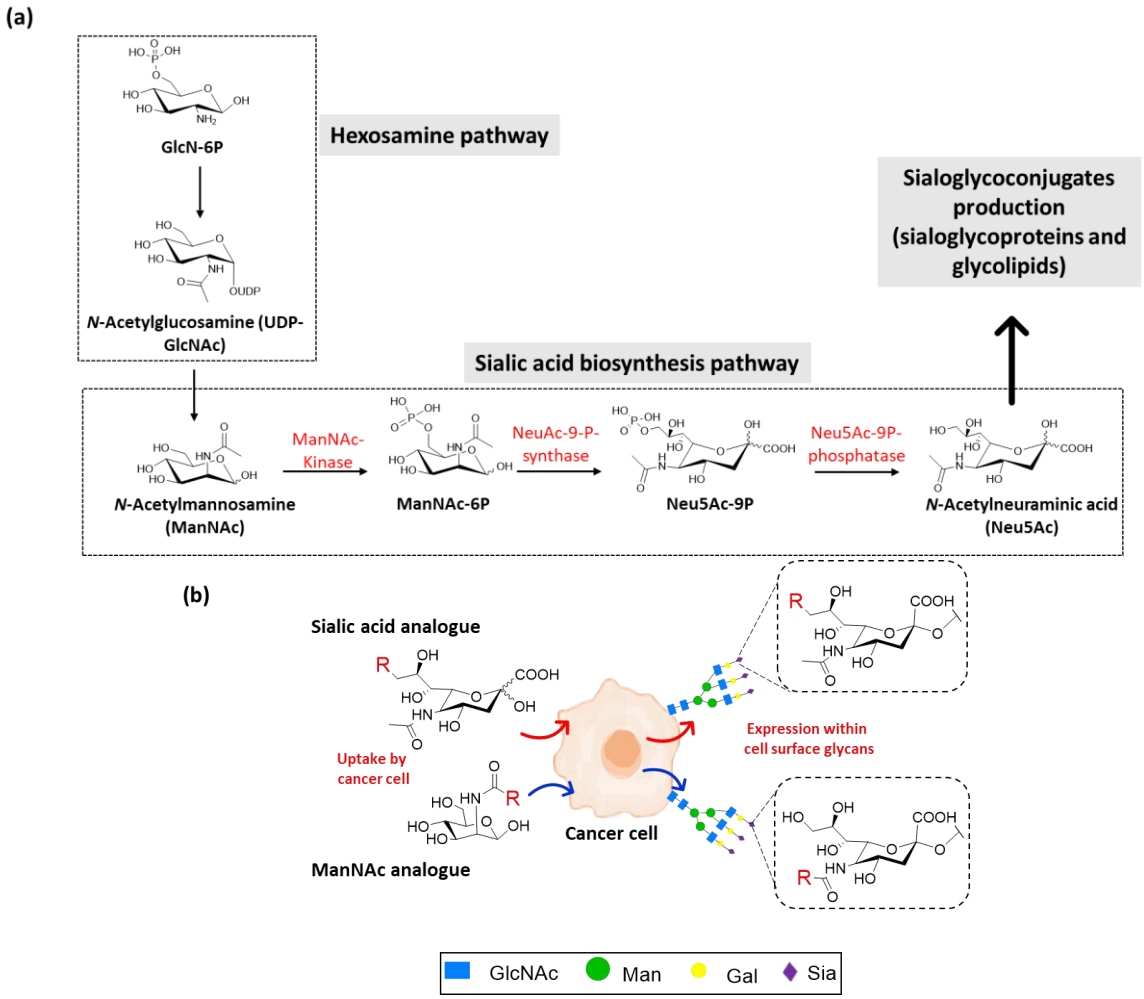


Figure 3. Chemical structures of TACAs overexpressed in breast cancer: sialyl Lewis^x (sLe^x), sialyl Lewis^a (sLe^a), sialyl Tn (sTn), and polysialic acid (PSA).[82]

1.4.1. Metabolic glycoengineering (MGE)

MGE (initially called chemoenzymatic labelling) is a technique introduced in the late 1980s in which diverse chemical functional groups are incorporated within cell surface glycans through the use of chemically-modified monosaccharide precursors bearing non-natural functional groups.[95,96] These chemically-modified monosaccharide precursors are processed by the cellular biosynthesis enzymes in a similar manner to the natural monosaccharide precursors. Hence, they can intercept the glycosylation pathways for the biosynthesis of the surface glycans allowing expression of the non-natural functional groups within the cell surface glycans.[97,98] The sialic acid biosynthesis pathway has proved the most suitable for MGE due to its tolerance for chemically-modified neuraminic acid and mannosamine analogues **(Fig. 4)**.[99–101]

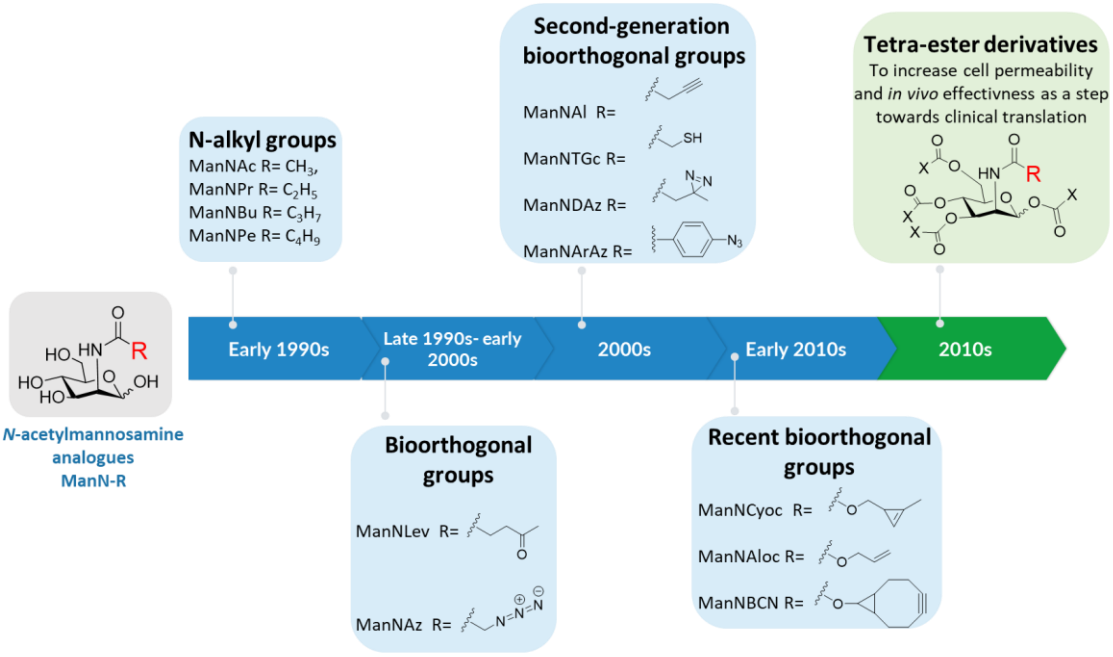


Gal: galactosamine; GlcNAc: *N*-acetyl glucosamine; Man: mannosamine; ManNAc: *N*-acetyl mannosamine; ManNAc-6P: *N*-acetyl mannosamine-6-phosphate; Neu5Ac: *N*-acetyl neuraminic acid; Neu5Ac-9P: *N*-acetyl neuraminic acid-9-phosphate; Sia: sialic acid; UDP-GlcNAc: *N*-acetyl glucosamine-uridine diphosphate.

Figure 4. Sialic acid MGE overview. (a) Sialic acid (*N*-acetylneuraminic acid) biosynthetic pathway. (b) Schematic view of interception of the sialic acid biosynthetic pathway by sialic acid and mannosamine analogues leading to the interception of sialic acid biosynthesis and eventually, the cell surface expression of non-natural chemical functional groups (R) within the sialic acid residues in cell surface glycans. (Figure adapted from references [97,102])

MGE is an established strategy and over the past three decades, it has enabled the generation of glycans with broad structural diversity *in vitro* and *in vivo*. MGE has been exploited mainly using *N*-acetylmannosamine analogues **(Fig. 5)** that bear different chemical functional groups. These functional groups are mainly characterised by being small in size to enable the analogue to be processed by the enzymes in the sialic acid biosynthesis in living cells. The structural diversity in these analogues has allowed glycans to be generated for various biological applications. For example, *N*-alkyl analogues **(Fig. 5)** facilitated the study and modulation of receptor signalling to decrease the infectivity of viruses that utilise sialic acids as cell-surface receptors (e.g. influenza virus). Installation of elongated alkyl chains into cell surface sialic acids decreased the virus attachments to mammalian cells and thereby decreased the viral infectivity.[103–106] Moreover, the introduction of bioorthogonal groups

(physiologically inert small reactive functional groups) on cell surface glycans acted as anchors for larger moieties to install biotin or fluorophores on the cell surface, mainly for cell imaging applications at cellular level and *in vivo*.[107–113] However, rapid serum clearance and poor cellular uptake of these monosaccharides (> 5mM is required for cellular experiments) represented a challenge for their clinical translation (> 10 gm per day is required for human dosage).[102] Therefore, recently, the derivatisation of ManNAc analogues focused on acetylation of the hydroxyl groups at positions 1, 3, 4, and 6 to increase the lipophilicity and thereby the membrane permeability of these compounds. This acetylation strategy decreased their serum clearance, increased their cellular uptake, and decreased the concentrations needed for cellular and *in vivo* experiments (from millimolar range to micromolar range).[114–116]



ManNAc: *N*-acetylmannosamine; ManNPr: *N*-propylmannosamine; ManNBu: *N*-butylmannosamine; ManNPe: *N*-pentylmannosamine; ManNLev: *N*-levulinoylmannosamine; ManNAz: *N*-azidoacetylmannosamine; ManNAl: *N*-alkynylmannosamine; ManNTGc: *N*-thioglycolylmannosamine; ManNDAz: *N*-diazirinmannosamine; ManNArAz: *N*-arylazidomannosamine; ManNCyoc: *N*-cyclopropeneacetylmannosamine; ManNAloc: *N*-allyloxycarbonylmannosamine; ManNBCN: *N*-bicyclononynylmannosamine.

Figure 5. Timeline showing the development of *N*-acetylmannosamine analogues reported to be utilised in MGE (Data in figure taken from reference [102]).

Based on the reported foundation of MGE, this research hypothesises targeting the overexpression of TACAs in breast cancer using MGE as part of the strategy to activate prodrugs for breast cancer. The altered glycosylation pattern and the overexpressed sialic acid residues within the TACAs along with the high metabolic rates of cancer cells (including the increased uptake of sugars)[117,118] are proposed to result in expression of the desired chemical tags preferably on the cancer cell surface with higher density. These chemical tags can act as selective activators for prodrugs (prodrugs are further discussed in sections 1.6 and 1.7) at cancer cells.

Therefore, in this research, the use of MGE to intercept the sialic acid biosynthesis pathway to selectively engineer specific chemical moieties for subsequent activation of prodrugs for the targeted treatment of breast cancer is proposed.

The following section introduces the enhanced permeability and retention (EPR) effect, which is a complementary targeting mechanism for solid tumours, including breast cancer, that is also exploited in the research reported herein.

1.5. Passive targeting by the enhanced permeability and retention (EPR) effect

The enhanced permeability and retention (EPR) effect is a phenomenon indicating the preferential accumulation of macromolecules in solid tumours. This phenomenon was first reported and named the EPR effect by Maeda and co-workers in 1986, after observing preferential accumulation of a polymer-protein conjugate (styrene-maleic anhydride and neocarzinostatin) in tumours.[119] The tumour environment has the pathological characteristics of hyperpermeability of tumour vasculature together with the impaired (i.e. decreased) lymphatic drainage. These features are developed in tumours to facilitate the supply of oxygen and nutrients, to meet the requirements for the rapid tumour growth.[120–122]

This high permeability allows for passive delivery of macromolecules such as polymers, polymer-drug conjugates (PDCs), liposomes, and nanocarriers to the solid tumours and the decreased lymphatic drainage allows their accumulation with concentrations reaching 10-50-fold higher compared to normal tissues.[123] This phenomenon contributes to increased cancer targeting and selectivity of the aforementioned therapeutic systems, which makes the EPR effect a valuable cancer targeting mechanism.[124,125] The magnitude of the EPR effect has been reported to be affected by many factors, mainly the tumour type, tumour size, and tumour perfusion.[126,127] **Table 4** shows the effect of tumour type and size on the magnitude of the EPR effect in terms of tumor/normal ratios of nanomedicine accumulation.[126,127]

Table 4: The effect of tumour type and size on nanomedicine accumulation according to tumour/normal ratios. (Data taken from references [126,127])

Tumour type	**Normalised average T/N ratio (~)**
Pancreatic adenocarcinoma	14
Colon, colorectal, and rectal cancers	12
Breast cancer	11
Stomach cancer	10
Brain cancer and brain metastasis	8
Ovarian cancer	8
Tumour size	**Normalised average T/N ratio (~)**
Medium (tumour stage ≤ IIB, area ≤ 25 cm^2)	4
Very large (tumour stage: IIB/IV, recurrent, unresectable, metastatic, area ≥ 140 cm^2)	5
Large (between upper two values)	28

As shown in **table 4**, breast cancer is the third highest solid tumour for accumulation of nanomedicines by the EPR effect, which suggests its suitability for targeted delivery of nanomedicines. Over the past three decades, many researchers have successfully investigated the loading of breast cancer chemotherapeutic agents into nanocarriers, or their conjugation to polymers, for selective delivery of conventional chemotherapies. **Table 5** summarises examples of using passive targeting for selective delivery of conventional breast cancer chemotherapies.

Table 5: Examples of passive targeting systems for breast cancer chemotherapies

Drug delivery technique	Brand /formulation name	API	Status	Ref
PEGylated liposomes	Doxil®	Doxorubicin	FDA-approved in 1995	[128]
Non-PEGylated liposomes	Myocet®	Doxorubicin	Approved for clinical use in the European Union and Canada in 2000	[129]
Albumin-bound nanoparticles	Abraxane®	Paclitaxel	FDA-approved in 2005	[130]
PLGA and PLGA-PEG nanoparticles	N.A.	Docetaxel	*In vivo* models	[131–133]
Liposomes	N.A.	Daunorubicin + Tamoxifen	*In vivo* models	[134,135]
PLGA-PEG nanoparticles	N.A.	Vincristine	*In vivo* models	[136]
Drug-drug conjugate loaded into nanocapsules	Camptothecin–Floxuridine conjugate	Camptothecin + Floxuridine	*In vivo* models	[137]
Polymer-drug conjugate	HPMA-doxorubicin copolymer	Doxorubicin	Phase II clinical trials	[138]

HPMA: *N*-(2-hydroxypropyl)methacrylamide; PEG: poly(ethylene glycol); PLGA: poly lactic-co-glycolic acid.

The above examples (**Table 5**) suggest the suitability of using passive targeting to target breast cancer. Therefore, in this project, passive targeting by the EPR effect was hypothesised as a targeting mechanism to selectively deliver prodrug activators to breast cancer for subsequent selective activation of chemotherapeutic prodrugs.

The next sections explore the concepts of prodrugs and bioorthogonal prodrugs and how MGE and the EPR effect could potentially be used to achieve selective activation of prodrugs in breast cancer.

1.6. Prodrugs

Generally, prodrugs are defined as molecules with little or no pharmacological activity that undergo biotransformation to a pharmaceutically active form. Since the introduction of prodrugs in 1958, they

have been widely implemented to improve the pharmaceutical (i.e. safety, clinical efficacy, and stability), pharmacodynamic (i.e. enhanced therapeutic index by lowering toxicity) and pharmacokinetic (i.e. enhanced absorption and decrease pre-systemic metabolism) properties of drugs.[139] Conventionally, prodrug activation is achieved by either intrinsic stimuli or exogenous stimuli.[140] Intrinsic stimuli involve overexpressed enzymes[141], or microenvironmental factors (low pH and hypoxia)[142,143], or elevated reactive oxygen species (ROS)[144,145] **(Table 6).** Exogenous stimuli involve on-demand activation strategies using light[146] and chemical reactions.[147] However, the former, despite comprising the most approved prodrug strategies, usually face selectivity challenges due to limited differences between normal and diseased tissues. For example, breast cancer prodrugs, cyclophosphamide,[148] tegafur (5-fluorouracil prodrug),[149] and tamoxifen [150] are activated by cytochrome (P450) enzymes which are overexpressed in tumours (colorectal, breast, ovarian, and lung), as well as the liver.[140,151]

Table 6: Examples of doxorubicin prodrugs that are activated by intrinsic stimuli.

Prodrug activation strategy	**Example**	**Status**	**Ref**
Overexpressed enzymes			
-Matrix metalloproteases (MMPs)	-Leucyl-doxorubicin is a doxorubicin prodrug modified with a prostate-specific antigen (PSA)-specific peptide.	Phase I completed	[152]
	- Ac–γE–ProCitGly~hofTyrLeu–Dox is a doxorubicin prodrug cleaved by MMP-2, MMP-9, and MMP-14 and further activated by extracellular proteases.	*In vivo* models	[153]
Microenvironmental factors			
-Hydrolysis in the acidic tumour environment	-Poly HPMA-Dox conjugate is a prodrug conjugating doxorubicin with an *N*-(2-hydroxypropyl)methylacrylamide polymer by a pH-sensitive hydrazone bond.	Phase I/II	[154]
	-Aldoxorubicin is a prodrug conjugating doxorubicin with a thiol-reactive maleimide moiety by a pH-sensitive hydrazone bond.	Phase III	[155]
-Elevated ROS levels	Doxorubicin conjugated with ROS-biocleavable photosensitisers that are cleaved by endogenous ROS and exogenous laser irradiation.	*In vivo* models	[156,157]

Many targeting approaches have been reported for use in conjugation with enzyme-activated prodrug therapy to improve selectivity. However, they still face tumour heterogeneity and off-target activation limitations. These approaches are under clinical development and include antibody-directed enzyme/prodrug therapy (ADEPT), polymer-directed enzyme/prodrug therapy (PDEPT), and gene-directed enzyme/prodrug therapy (GDEPT) which is also known as suicide gene therapy (SGT). **Table 7** summarises the concepts behind these approaches and their limitations.

Table 7: Concepts of targeted enzyme prodrug therapy approaches and their limitations

Targeted approach	Concept	Limitations	Ref
ADEPT (active targeting)	A tumor-specific monoclonal antibody (mAb) is attached to the activating enzyme to direct it to tumour cells and subsequently activate the prodrug at the tumour site.	-Tumour heterogeneity of the overexpressed tumour antigens -Off-target activation by unbound mAb-enzyme in circulation	[158,159]
PDEPT (passive targeting)	A polymeric prodrug that contains an enzyme cleavable link is localised in the tumour by passive targeting for subsequent activation by a polymer-enzyme conjugate.	-Off-target and premature activation by the circulating polymer-enzyme conjugate in the blood	[160]
GDEPT	Physical delivery of a gene encoding for a foreign enzyme (i.e. from a viral origin) to the tumour cell (usually by liposomal gene delivery) to subsequently activate the prodrug at the tumour site after the enzyme expression.	-Immunogenicity concerns -Mutagenicity concerns	[159,161]

1.7. Bioorthogonal prodrugs

On-demand prodrug activation strategies using chemical reactions provide a promising alternative to enzyme activation. Bioorthogonal chemistry refers to a group of specific chemical reactions that can proceed under physiological conditions (aqueous environment and physiological pH) with high selectivity and without interfering with biological processes or interacting with other biomolecules. Bioorthogonal chemistry was first introduced by Carolyn R. Bertozzi in 2003 [162] and due to the physiological advantages of these reactions, their use in diagnostic and therapeutic applications, including prodrug activation strategies, has received considerable attention. **Chapter 2** details the up-to-date classes of bioorthogonal reactions, their mechanisms for chemoselective ligations and bond

cleavage applications, and the targeting strategies utilised to increase the selectivity of their *in vivo* applications, and hence this is not covered further here.

Bioorthogonal prodrug systems usually comprise two components, a prodrug and an activator to achieve on-demand activation. A targeting strategy is crucial to selectively deliver either entity to the desired site of action in order to promote the selective activation of the prodrug (i.e. on-target activation). As discussed earlier, MGE and passive targeting by the EPR effect are suitable strategies for breast cancer targeting and hence have been investigated in this project for selective delivery of the activator **(Fig. 6)** (on-target activation). Two bioorthogonal bond-cleavage reactions have been investigated in this project for the bioorthogonal prodrug activation system, specifically the Staudinger ligation and tetrazine ligation reactions (on-demand activation). The following sections, **1.7.1** and **1.7.2**, discuss the reaction mechanisms that are proposed for prodrug activation.

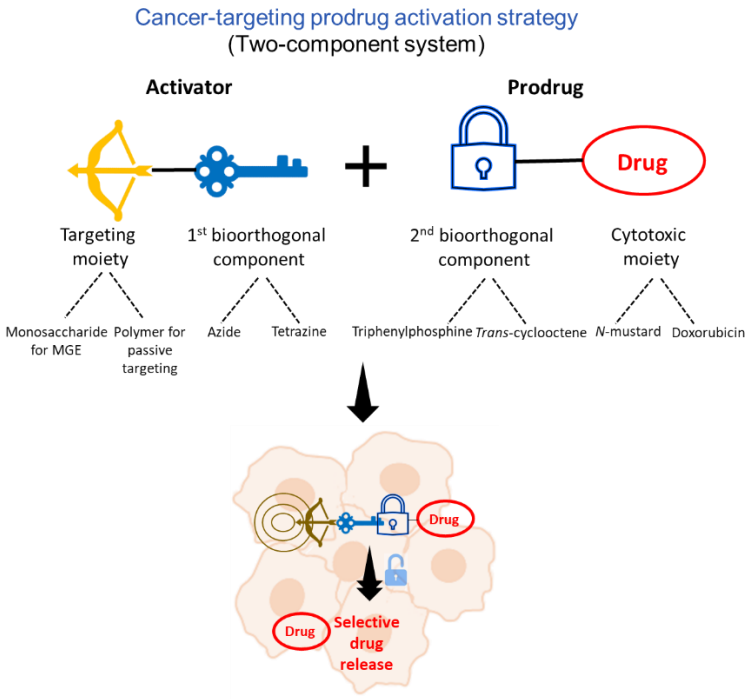


Figure 6. Schematic illustration of the selective prodrug activation strategy presented in this thesis.

1.7.1. Staudinger ligation reaction for prodrug activation

After its first report by Hermann Staudinger and Jules Meyer in 1919 for synthesis of aza-ylides and natural products,[163] the Staudinger reaction was adapted by Carolyn Bertozzi in 2000 to achieve selective ligation between two molecules for cell imaging and for studying cell surface glycans and was called the Staudinger ligation reaction.[107,164–168] The history of the Staudinger reactions, their mechanisms, and their biological applications are reviewed in depth in **Chapter 2 section 2.1**. The modified-Staudinger ligation reaction is a reaction between an azide and a triphenylphosphine moiety that gives an aza-ylide intermediate. In the presence of aqueous medium, it undergoes spontaneous

intramolecular rearrangement to give the corresponding phosphine oxide with a stable amide linkage as illustrated in **figure 7**.[107]

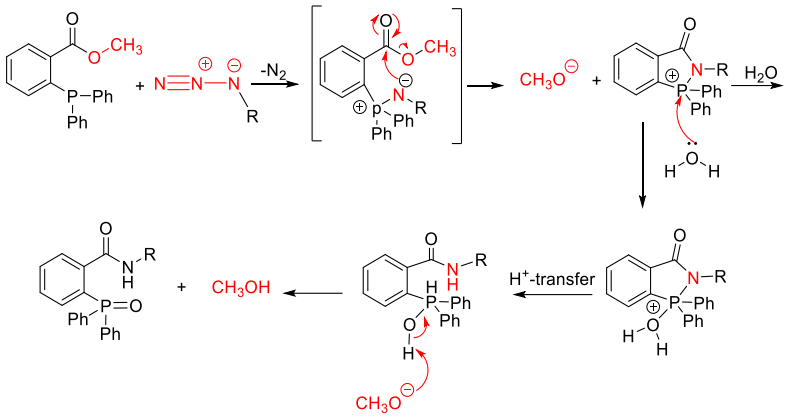


Figure 7. Mechanism of the Staudinger ligation reaction.

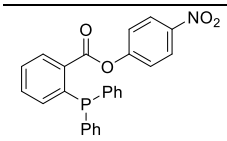
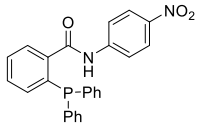
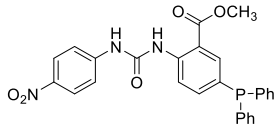
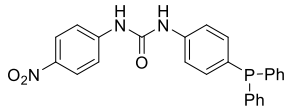
The reaction has found widespread use in the field of chemical biology due to the advantages of using the azide moiety in biological systems. For example, the azide moiety, despite being a chemically reactive moiety, is physiologically stable, has no *in vivo* toxicity, can be easily derivatised, is resistant to oxidation, and is relatively small in size.[169] The Staudinger reaction and the Staudinger ligation have been used in prodrug activation strategies with various release mechanisms (1,6-elimination after azide reduction to amine and 1,6-quinone methide rearrangement of the aza-ylide intermediate, respectively) [170,171] (also reviewed in **Chapter 2 section 2.1**). In this project, drug release by the original Staudinger ligation mechanism is explored whereby the methoxy group in the triphenylphosphine moiety (see **figure 7**) is replaced by a chemotherapeutic drug such as doxorubicin or a *N*-mustard, to develop triphenylphosphine-modified bioorthogonal activatable prodrugs.[172].

As discussed earlier in sections 1.4 and 1.5, MGE and the EPR effect are proposed to be suitable breast cancer targeting mechanisms. Azide activators (i.e. azide-modified monosaccharides and azide-modified polymer) are proposed to be selectively delivered to breast cancer by these two targeting mechanisms for subsequent activation of triphenylphosphine-prodrugs by the Staudinger ligation bioorthogonal reaction (see **figure 6**).

A complementary approach of utilising the Staudinger reactions for prodrug activation was previously investigated in our laboratory. This approach utilised various model prodrugs that comprise 4-nitrophenol and 4-nitroaniline moieties instead of cytotoxic moieties and were tested for 4-nitrophenol and 4-nitroaniline release *via* HPLC-monitoring using a set of azide activators.[172] The model prodrugs included ester and amide models (entry 1 and 2 in **table 8**) that were hypothesised to release 4-nitrophenol and 4-nitroaniline, respectively by Staudinger ligation mechanism, and urea models (entry 3 and 4 in **table 8**) that were hypothesised to release 4-nitroaniline by the Staudinger

reaction mechanism.[171] **Table 8** summarises the model prodrugs and their respective azide activators, and also the release results.

Table 8: Summary of results from previous work reported by our laboratory [172]

Prodrug	Azide activator	Release observed* (estimated not reported)	Proposed release mechanism	Method of detection
 Ester model prodrug	Na azide Benzyl azide PEG-azide (1 KDa) PEG-azide (14 KDa)	No 4-nitrophenol release ~ 4% release over 180 hours ~ 6% release over 24 hours ~ 8% release over 180 hours	Staudinger ligation	HPLC
 Amide model prodrug	Na azide 9-azido sialic acid	No 4-nitroaniline release ~ 6% release over 36 hours	Staudinger ligation	HPLC
 Urea model prodrug	Na azide	No 4-nitroaniline release	Staudinger reaction	HPLC
 Urea model prodrug	Na azide	No 4-nitroaniline release	Staudinger reaction	UV

*not reported in that thesis but were estimated from the presented graphs.

The above studies were preliminary in nature and did not progress to *in vitro* testing. However, they did provide initial proof of the feasibility of using the Staudinger ligation reaction for drug release using organic azide activators and PEG-azide activators.

Based on this, the approach reported here required the design, synthesis, and characterisation of new amide-linked triphenylphosphine-prodrugs that comprise cytotoxic moieties. Different urea and carbamate-linked model prodrugs (comprising the electrophilic ester trap in an *ortho*-position to the diphenylphosphine moiety and for activation by the Staudinger ligation) were also designed and synthesised using different synthetic approaches. Most importantly, the selectivity of the proposed targeting mechanism and efficiency of prodrug activation were biologically evaluated on breast cancer cells.

1.7.2. Tetrazine ligation reaction for prodrug activation

Tetrazine ligation is an Inverse Electron Demand Diels-Alder (IEDDA) reaction which involves a [4+2] cycloaddition between a tetrazine (Tz) (i.e. diene) and a *trans*-cyclooctene (TCO) (i.e. dienophile). The mechanism of the Tz ligation reaction and their biological applications are reviewed in **Chapter 2 section 2.6.2**. The Tz ligation reaction was first modified for bond cleavage and drug release in 2013 by Robillard *et al* where a carbamate-linked doxorubicin was attached at the allyl position of the TCO moiety, which was activated upon its reaction with 1,2,4,5-Tz activators (reaction mechanism illustrated in **figure 8**).[173]

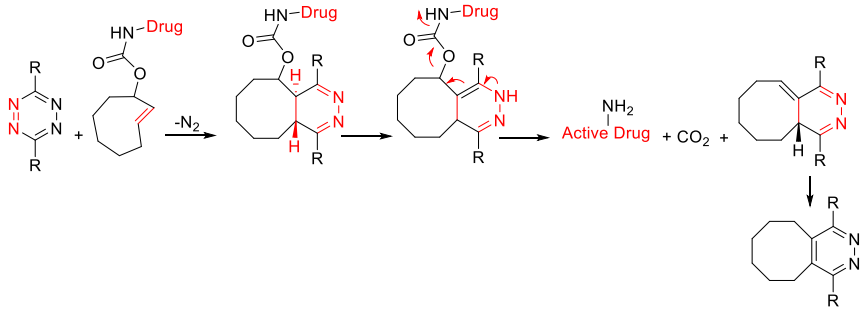


Figure 8. Mechanism of the Tz ligation reaction for prodrug activation.

The Tz ligation reaction is the most utilised bioorthogonal reaction in bioorthogonal prodrug activation strategies due to its very fast *in vivo* kinetics (k_2 up to 10^4 $M^{-1}s^{-1}$) which enables click-and-release of the active drug from the prodrug upon its reaction with the Tz activator.[174] As hypothesised earlier with the Staudinger ligation reaction, developing breast cancer-targeted Tz activators (i.e. Tz-modified monosaccharides and Tz-modified polymer) could promote the selective localisation of the Tz moiety in breast cancer tissues by MGE and the EPR effect, respectively. Subsequently, this will allow the preferable activation of TCO prodrugs in breast cancer tissues (see **figure 6**).

1.8. Project aims

The overall aim of this PhD project was to investigate novel bioorthogonal approaches for the selective delivery and activation of chemotherapeutic prodrugs in breast cancer. To achieve selective drug release at the tumour site, efficient targeting mechanisms to selectively deliver the prodrug activators are required. In this context, two targeting strategies, specifically MGE and passive targeting through the EPR effect, were employed within this thesis.

The overall organisation of this thesis is summarised in **figure 9**. The background review chapters comprise **Chapter 1** (general introduction) and **Chapter 2** where the current bioorthogonal reactions (including the bond cleavage prodrug activation reactions) are comprehensively reviewed, and the targeting mechanisms utilised for *in vivo* applications are critically appraised. Then, the experimental chapters look at using monosaccharide-based bioorthogonal activators and PEG-based bioorthogonal

activators. Specifically, in **Chapter 3**, the use of the MGE to selectively incorporate azide activators within breast cancer cell surface glycans, using azide-modified monosaccharide precursors, was evaluated. This was determined using different *in vitro* approaches (i.e. western blotting and confocal microscopy imaging). The selective activation of novel synthesised triphenylphosphine-prodrugs in the azide-engineered breast cancer cells by the Staudinger ligation reaction was also evaluated. **Chapter 4** further evaluated the selectivity of the MGE approach by evaluating the selective incorporation of Tz activators within breast cancer cell surface glycans using a synthesised library of Tz-modified monosaccharide precursors. The selective activation of novel synthesised TCO prodrugs in the Tz-engineered breast cancer cells by the tetrazine ligation reaction was also evaluated. As for the PEG-based bioorthogonal activators, the synthesis of PEG-based activators, the effect of PEG size on the reaction rate, and the feasibility of their usage for bioorthogonal activation of prodrugs in breast cancer *in vitro* were evaluated in **Chapter 5**. Finally, **Chapter 6** presents a general discussion of the key findings, along with critical evaluation of the work presented, and areas of future research. The overview of the overall body of work presented herein is summarised in **figure 9**.

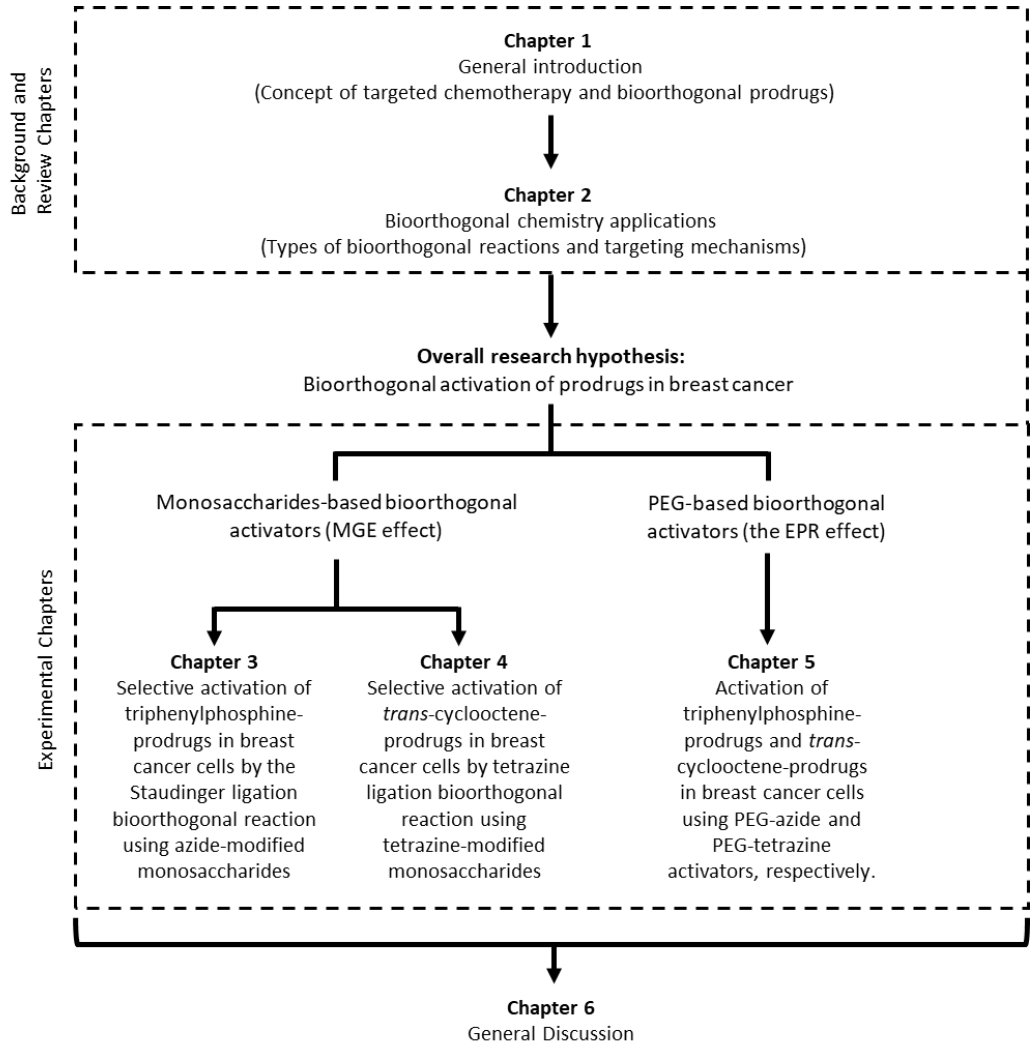


Figure 9. Schematic representation of the content of this PhD thesis.

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Chapter 2

In vivo bioorthogonal chemistry applications: types of reactions and targeting mechanisms

Chapter summary: *In this chapter, bioorthogonal reactions, their in vivo applications, and targeting mechanisms are discussed and critically evaluated. Focus is also placed on identifying the challenges that need to be taken into consideration for maximising the impact of bioorthogonal reactions for in vivo applications.*

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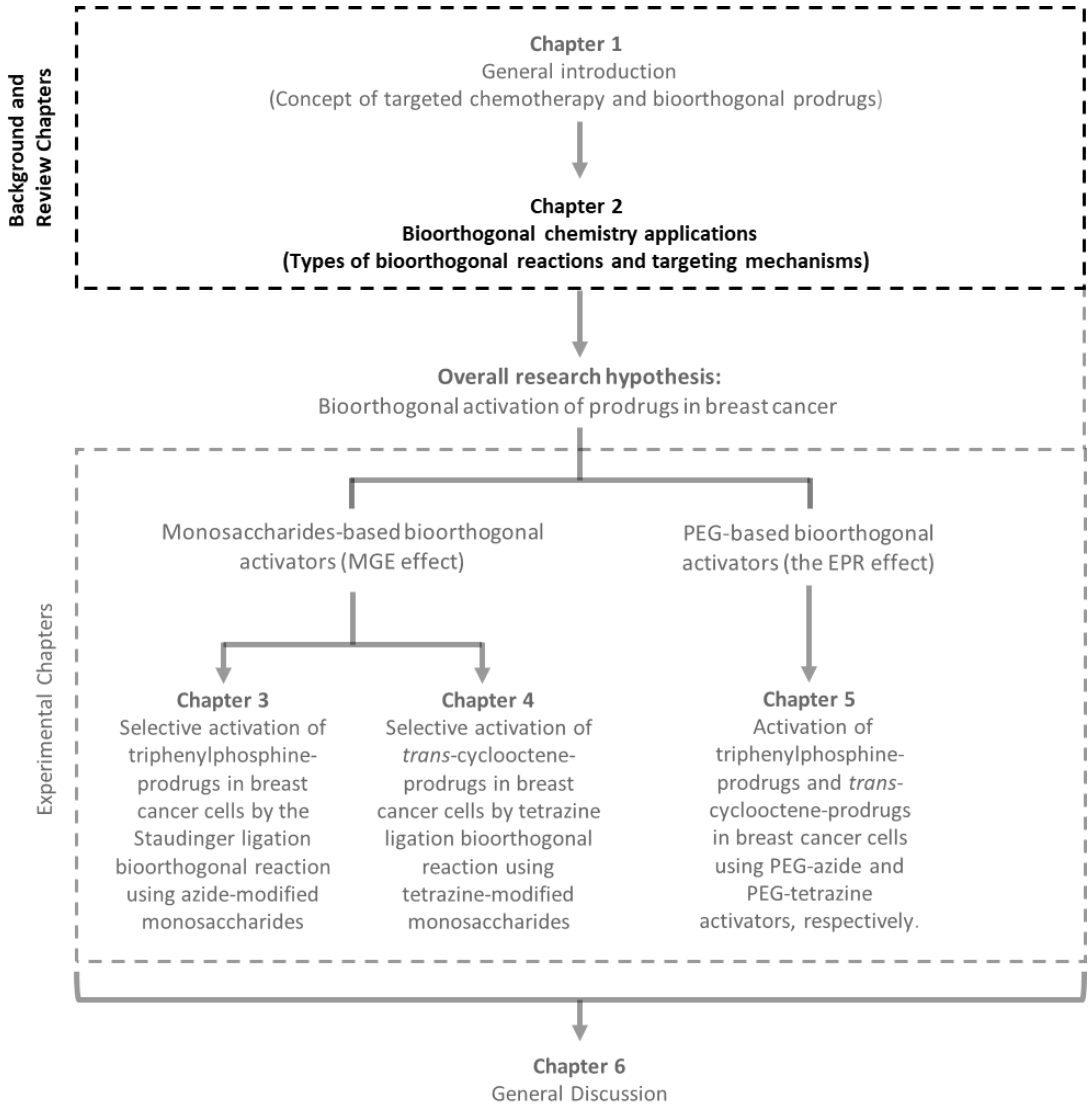

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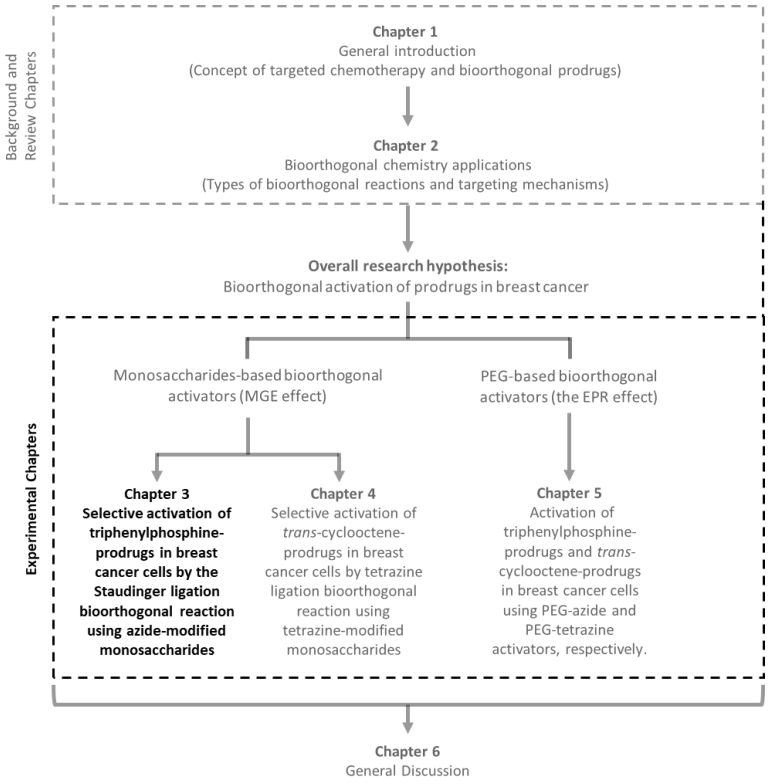
Chapter 3

Metabolic glycoengineering using azide-modified monosaccharides for triphenylphosphine-prodrugs' selective activation in breast cancer cells

Chapter summary: *This chapter is structured into 3 parts: the main paper, its associated supplementary information, and an appendix. In the* ***main paper****, the selective activation of triphenylphosphine-prodrugs in MCF-7 breast cancer cells versus the non-cancerous L929 cells by the Staudinger ligation bioorthogonal reaction was evaluated. The synthesis of two novel azide-modified sialic acid monosaccharides and two novel triphenylphosphine-doxorubicin and N-mustard prodrugs is described. The results showed the selective expression of azide reporters on MCF-7 breast cancer cells via metabolic glycoengineering which was confirmed by western blotting and confocal microscopy imaging. These azide reporters serve as activators for the triphenylphosphine-prodrugs and in vitro studies showed good levels of restoration (~ 68 – 100%) of the parent cytotoxic drugs' activities on the azide-engineered MCF-7 breast cancer cells.* ***The supplementary information*** *provides the* IC_{50} *graphs and the NMR spectra of the compounds.* ***The appendix*** *details another proposed Staudinger release mechanism using urea and carbamate-linked triphenylphosphine-prodrugs.*

Bibliographic details: *M.M.A. Mitry, S. Y. Boateng, F. Greco, H.M.I. Osborn, Bioorthogonal activation of prodrugs, for the potential treatment of breast cancer, using the Staudinger reaction, RSC Med. Chem. 2023, 14(8), 1537-1548.*

Author Contributions: *F. Greco and H.M.I. Osborn designed, reviewed and supervised the study. M.M.A. Mitry designed the triphenylphosphine-prodrugs and the in vitro assays. M.M.A. Mitry carried out all the experimental work (synthesis and characterisation of compounds, western blotting and the confocal microscopy imaging assays under the supervision of S. Y. Boateng, the in vitro activation studies, and data analysis). M.M.A. Mitry constructed figures and prepared the original draft of the manuscript. All authors edited the manuscript.*



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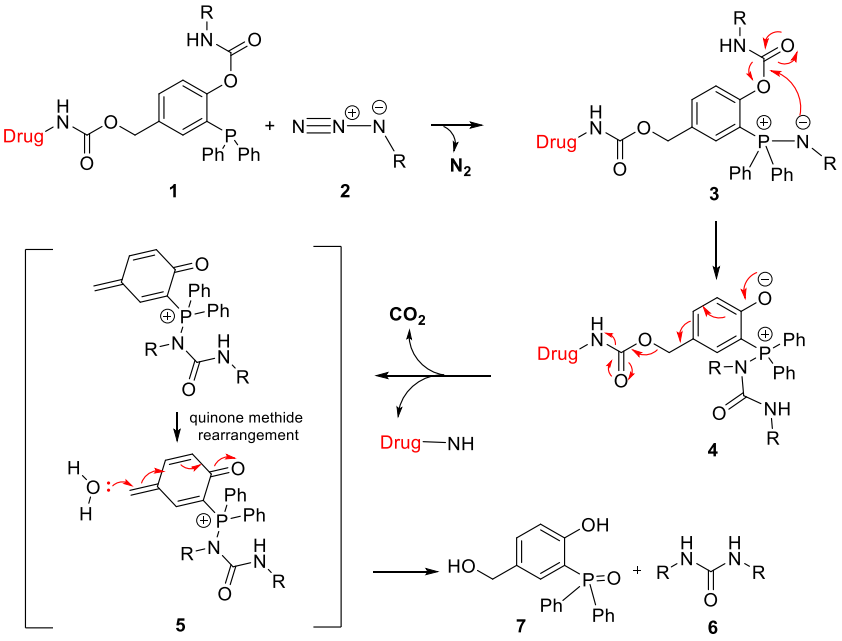
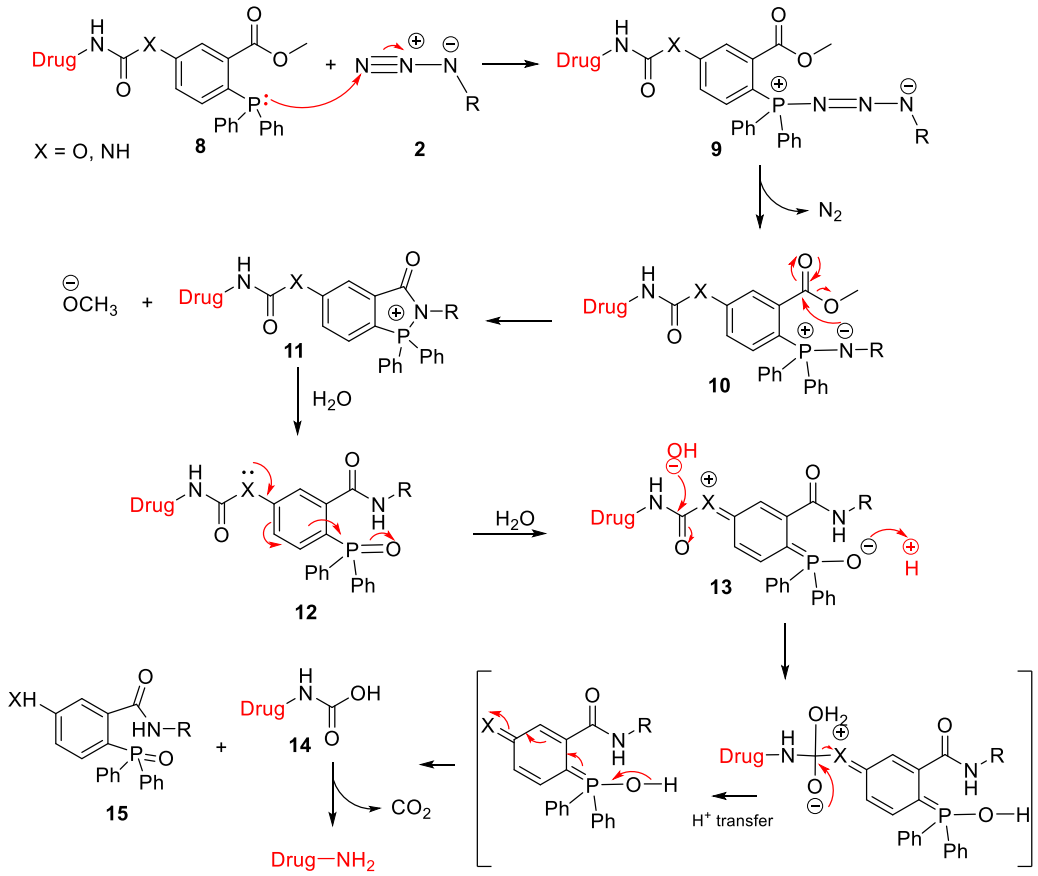
Appendix: Proposed alternative Staudinger release mechanism using urea and carbamate-linked triphenylphosphine-prodrugs.

Introduction

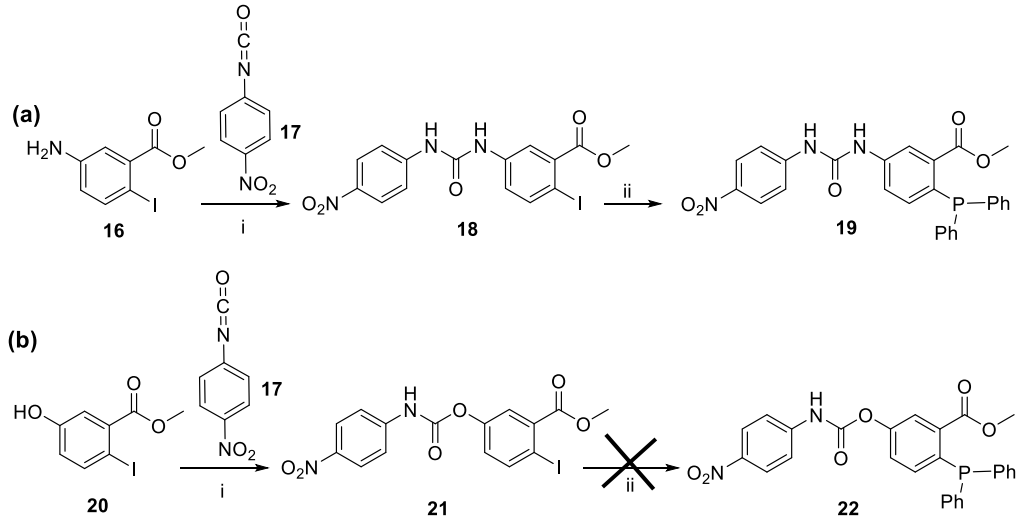
In addition to the strategy detailed in the paper,[1] a new complementary drug release mechanism relying on the Staudinger ligation reaction was investigated. A previously reported Staudinger ligation release reaction detailed by Azoulay *et al*[2] demonstrated rearrangement of the aza-ylide intermediate **3** formed during the Staudinger ligation. Intramolecular cyclisation of **3** results in the formation of a phenol anion **4** which subsequently effects a 1,6-elimination to release the drug moiety **(Scheme 1)**. The HPLC-monitored release results showed that incubation of a doxorubicin prodrug with a short-chain (n=3) polymer azide activator resulted in the release of 90% of doxorubicin after 3 h at 37 °C in aqueous solvent. In that approach, the drug was linked *via* a carbamate bond to the *meta*-position of the diphenylphosphine moiety and to the *para*-position of the electrophilic ester trap. In this project, the synthesis of prodrugs where drugs are linked *via* a urea or carbamate linkage to the *meta*-position of the ester electrophilic trap and the *para*-position of the diphenylphosphine moiety was proposed. This was in order to explore the effect of changing the position of the phosphine group *versus* the Azoulay system on the release kinetics of the drug. The proposed release mechanism from this system is demonstrated in **scheme 2**. Two model prodrugs having the 4-nitroaniline moiety as a replacement for a cytotoxic drug were proposed to be synthesised as outlined in **scheme 3** which was based on reported methods.[3–6] The model prodrugs were then set to be tested for activation by azide activators.



Scheme 1: Drug release mechanism by Staudinger ligation reported by Azoulay *et al*.[2]



Scheme 2: Proposed drug release mechanism from urea and carbamate-linked triphenylphosphine-prodrugs by azide activators.



(a) (i) Anhydrous DCM, TEA, N_2 atmosphere, RT, overnight, 30%; (ii) DPP, anhydrous CH_3CN, anhydrous TEA, $Pd(OAc)_2$, N_2 atmosphere (Schlenk line), reflux, overnight, 25%.

(b) (i) Anhydrous THF, TEA, N_2 atmosphere, ice bath for 1 hr then RT overnight, 14%; (ii) DPP, anhydrous CH_3CN, anhydrous TEA, $Pd(OAc)_2$, N_2 atmosphere (Schlenk line), reflux, overnight.

Scheme 3: General synthesis for urea and carbamate-linked triphenylphosphine model prodrugs

Results

Synthesis of urea and carbamate model prodrugs (19 and 22)

The synthesis of the urea and carbamate model prodrugs **19** and **22,** respectively, commenced by linking 4-nitroaniline (which acts as a model for the cytotoxic moiety) *via* a urea or carbamate linkage to a 5-amino, or 5-hydroxy-2-iodobenzoate derivative, respectively, through their reaction with 4-nitrophenyl isocyanate. The reaction mixture was kept strictly under a N_2 atmosphere and anhydrous solvents were used due to the high instability of the 4-nitrophenyl isocyanate. The yields for the urea-iodo intermediate **18** and the carbamate-iodo intermediate **21** after purification were 30% and 14%, respectively.

The next step was the substitution of the iodine with the diphenylphosphine group *via* a palladium-catalysed coupling reaction. Palladium diacetate is reported to be one of the most utilised transition metal-catalysts in C-P, C-halogen, and C-C bond formation reactions.[7–10] Due to the high air sensitivity of the reagent, the reaction was carried out under strictly inert conditions using a Schlenk line and anhydrous degassed solvents. However, due to the oxidation-prone nature of the diphenylphosphine reagent and the triphenylphosphine products, the urea analogue **19** showed inconsistency in the yield and was finally afforded in a relatively low yield of 25%. The synthesis of the carbamate analogue **22** was not successful.

The urea model prodrug **19** was then analysed for release of 4-nitroaniline by Staudinger ligation upon its incubation with azide activators in HPLC-monitored release study.

4-Nitroaniline HPLC-monitored release study

The release of 4-nitroaniline from the synthesised model urea prodrug **19** was probed using HPLC, to determine whether this prodrug analogue was suitable for drug release by the Staudinger ligation mechanism. A RP-HPLC analytical method for 4-nitroaniline detection in the presence of the urea model prodrug **19** was optimised and a calibration curve for 4-nitroaniline was generated to quantify the amount of the 4-nitroaniline released.[11] Benzyl azide and 9-azido sialic acid were used as activators and were incubated with the urea model prodrug **19** under aqueous conditions (H_2O:CH_3CN, 1:1) at 37 °C and the release was monitored by HPLC over 120 h (**Fig. 1**). For the benzyl azide activator, 27% of the 4-nitroaniline was released after 24 h. Then, a slow gradual increase in the release of 4-nitroaniline was observed reaching a maximum of 44% after 96 h that plateaued at 120 h. When 9-azido sialic acid was used as the activator, no release of 4-nitroaniline was detected in the first 48 h of incubation. Then a 25% release was observed at 72 h that was followed by a small increase in the percentage released (27% at 96 h) which then plateaued at 29% after 120 h of monitoring. The faster and higher release of 4-nitroaniline when using benzyl azide *versus* 9-azido sialic acid may be due to

the lower steric hindrance of the other parts of the benzyl azide molecule which may have made the azide group more accessible to activate the prodrug. This observation is in line with the release pattern obtained when a model ester triphenylphosphine-prodrug comprising a 4-nitrophenol was incubated with various azide activators (4-nitrophenol release caused by benzyl azide > 10 KDa PEG-azide > 20 KDa PEG-azide) (discussed more in **Chapter 5**), which clearly indicate that the size of the azide activator influences the rate of release by the Staudinger ligation.

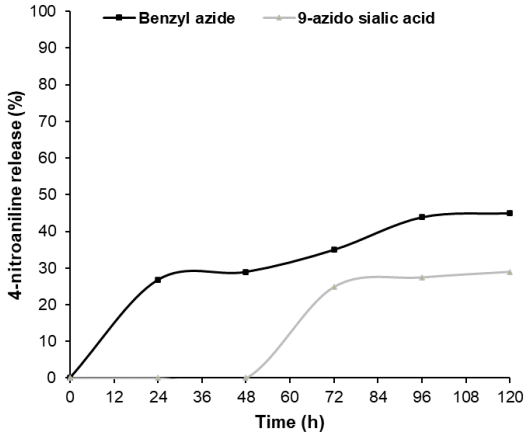


Figure 1. Release of 4-nitroaniline from the urea model prodrug **19** *via* the Staudinger ligation reaction monitored by HPLC as a function of time upon its incubation with benzyl azide or 9-azido sialic acid activators.

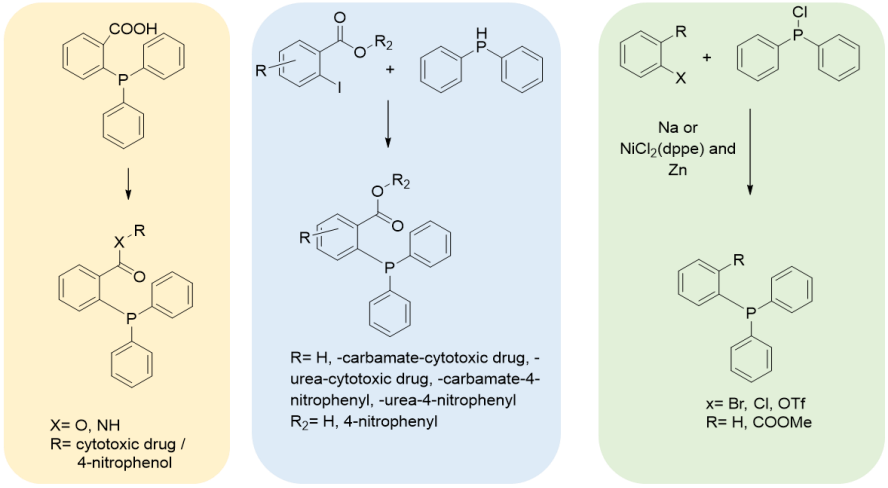
Discussion

Despite suggesting the feasibility of drug release form urea-linked triphenylphosphine-prodrugs by the proposed Staudinger ligation mechanism, limitations were evident. For example it was shown to be a slow process and complete consumption of the prodrug was not achieved by either azide activators (compared to the 90% release after 3 h reported by Azoulay *et al* release model [2]). Overall, this suggested that our proposed release mechanism may not be suitable for developing a prodrug activation strategy. The other major limitation was the synthetic procedures for accessing the urea- and the carbamate-linked triphenylphosphine-prodrugs. In this thesis, two synthetic approaches have been explored for the synthesis of the triphenylphosphine-prodrugs.

-*Approach 1*: DCC-mediated coupling of diphenylphosphanyl benzoic acid with a corresponding amine (doxorubicin and *N,N*-bis-(2-chloroethyl)benzene-1,4-diamine) **(Chapter 3 scheme 2)** to yield amide-linked triphenylphosphine-prodrugs. This has also been explored with a phenol (4-nitrophenol) to yield an ester-linked triphenylphosphine model prodrug **(Chapter 5 scheme 2).** This approach is considered advantageous due to its facile synthesis, higher yields, and the unlikelihood of oxidation of the triphenylphosphine reagent.[12,13]

-*Approach 2*: Substitution of a halogen with the diphenylphosphine moiety *via* a palladium-catalysed reaction **(Chapter 3 appendix, scheme 3)** subsequent to the attachment of the cytotoxic drug or 4-nitroaniline (for model prodrugs) to an iodo-substituted methyl benzoate. This approach is widely reported for the synthesis of triphenylphosphine compounds,[14–17] however, the high instability of the diphenylphosphine reagent to oxidation renders the reaction challenging. The unavailability of commercial 5-amino or 5-hydroxy derivatives of the diphenylphosphanyl benzoic acid reagent negated the use of the DCC-coupling approach for the urea- and carbamate-linked triphenylphosphine-prodrugs.

Other approaches have been reported for the synthesis of triphenylphosphine compounds without the use of the diphenylphosphine reagent where chloro-diphenylphosphine is used instead. The approach depends on the release of diphenylphosphine *in-situ* upon reaction of chloro-diphenylphosphine with sodium or zinc in the presence of a nickel catalyst (nickel-catalysed cross coupling). This strategy is considered advantageous as the chloro-diphenylphosphine is not pyrophoric and is not prone to air oxidation, unlike DPP.[18,19]



Approach 1

(DCC-mediated coupling of DPPBA with corresponding amine/phenol)

-Chapter 3 scheme 2

-Chapter 5 scheme 2

Approach 2

(Pd(II)-mediated coupling of DPP with corresponding *O*-iodobenzoates)

-Chapter 3 appendix

Approach 3

(Na and Ni-catalysed cross coupling of aryl halides/sulfonate esters with chloro-DPP)

-Ager *et al* 1997

-Jinbiao *et al* 2011

	Approach 1	**Approach 2**	**Approach 3**
Reaction conditions	DCC, 4-DMAP, DCM, N_2 atmosphere, RT, overnight.	DPP, anhydrous CH_3CN, anhydrous TEA, $Pd(OAc)_2$, N_2 atmosphere (Schlenk line), reflux, overnight	Zn, 1,2-bis (diphenylphosphino)ethane nickel(II) chloride, DMF, 100-110 °C.
Yield %	31-38%	25%	46-84%

Figure 2. Synthetic approaches for triphenylphosphine compounds.

Conclusion

A new Staudinger ligation-based release mechanism was investigated in which the drug is linked by a urea or carbamate linkage at the *para*-position of the diphenylphosphine moiety and *meta*-position of the ester electrophilic trap. Two model prodrugs were designed and their synthesis was attempted where a 4-nitroaniline was used instead of a cytotoxic moiety for the preliminary assessment of release. Due to the high instability of the reagents and products, the urea model prodrug was afforded in a relatively low yield (25%) and the synthesis of the carbamate model prodrug was not successful. HPLC-monitored release study of the 4-nitroaniline from the urea model prodrug upon incubation with benzyl azide and 9-azido sialic acid activators showed that the former afforded faster and higher release (44% *versus* 29%, respectively after 120 h) which may be attributed to the lower steric hindrance for the reaction. However, the incomplete activation of the urea model prodrug and the instability of the synthesis reagents and prodrugs imply that the proposed drug release mechanism may not be suitable for further development.

Experimental

Materials

The same materials and instruments were used as described earlier in **chapter 3** experimental section. Methyl 5-amino-2-iodobenzoate and methyl 5-hydroxy-2-iodobenzoate were purchased from BLD Pharm, Germany.

Synthesis

Methyl 2-iodo-5-(3-(4-nitrophenyl)ureido)benzoate (18) Compound **18** was synthesised using an adapted method from the literature.[3,5] 4-Nitrophenyl isocyanate **17** (88 mg, 0.53 mmol, 1.5 eq.) was dissolved in anhydrous DCM (5 mL) under a N_2 atmosphere. Methyl 5-amino-2-iodobenzoate **16** (100 mg, 0.36 mmol, 1 eq.) was added followed by TEA (100 µL, 0.7 mmol, 2 eq.). The reaction mixture was stirred overnight at room temperature resulting in formation of a yellow precipitate. The precipitate was filtered and washed with DCM (3 x 5 mL), then it was purified by column chromatography (ethyl acetate : hexane = 1 : 1 v/v) to yield the methyl 2-iodo-5-(3-(4-nitrophenyl)ureido)benzoate **18** as a yellow solid (47 mg, 30%). **^{1}H NMR** (DMSO-d_6, 400 MHz) δ 3.93 (3H, s, OC**H$_3$**), 7.41 (1H, dd, *J*= 8.6, 2.6 Hz, Ar-H), 7.77 (2H, d, *J* = 9.3 Hz, Ar-H), 7.97 (1H, d, *J* = 8.6 Hz, Ar-H), 8.06 (1H, d, *J* = 2.6 Hz, Ar-H), 8.26 (2H, d, *J* = 9.3 Hz, Ar-H), 9.34 (1H, s, NH), 9.61 (1H, s, NH). **^{13}C NMR** (DMSO-d_6, 100 MHz) δ 53.18 (O**C**H$_3$), 85.22 (**C**-I), 118.11 (Ar-C), 118.37 (Ar-CH), 123.02 (Ar-C), 125.53 (Ar-CH), 138.89 (Ar-C), 141.13 (Ar-C), 141.82 (C-NH), 142.69 (C-NH), 146.63 (C-NO$_2$), 152.05 (NH-**C**O-NH), 166.82 (**C**OCH$_3$).

Methyl 2-(diphenylphosphino)-5-(3-(4-nitrophenyl)ureido)benzoate (19) Compound **19** was synthesised using an adapted method from the literature.[14] In an oven-dried flask, methyl 2-iodo-5-(3-(4-nitrophenyl)ureido)benzoate **18** (150 mg, 0.3 mmol, 1 eq.) was dissolved in anhydrous degassed CH_3CN (10 mL), then anhydrous TEA (50 µL, 0.37 mmol, 1.2 eq.) and palladium diacetate (0.7 mg, 0.003 mmol, 0.01 eq.) were added sequentially. Air was removed by a vacuum pump then the Schlenk line was flushed three times with nitrogen. Then, diphenylphosphine (60 µL, 0.3 mmol, 1 eq.) was added dropwise. The reaction mixture was heated at reflux overnight. The solvent was removed under vacuum and the oily red residue was purified by column chromatography (ethyl acetate : hexane = 1 : 4 v/v) to yield the methyl 2-(diphenylphosphine)-5-(3-(4-nitrophenyl)ureido)benzoate **19** as a light yellow oil (42 mg, 25%).**^{1}H NMR** (DMSO-d_6, 400 MHz) δ 3.46 (3H, s, OC**H$_3$**), 7.31 - 7.39 (2H, m, Ar-H), 7.51 - 7.71 (10H, m, diphenylphosphine Ar-H), 7.78 (2H, d, *J* = 9.2 Hz, Ar-H), 8.09 – 8.12 (1H, m, Ar-H), 8.27 (2H, d, *J* = 9.2 Hz, Ar-H), 9.58 (1H, s, NH), 9.68 (1H, s, NH). **^{13}C NMR** (DMSO-d_6, 100 MHz) δ 52.39 (O**C**H$_3$), 118.37 (Ar-CH), 120.21 (Ar-CH), 125.21 (Ar-CH), 125.61 (Ar-CH), 128.93 (Ar-CH), 129.05 (Ar-CH), 131.61 (Ar-CH), 131.71 (Ar-CH), 132.07 (Ar-CH), 133.57 (Ar-CH), 134.62 (Ar-CP), 135.59 (Ar-CP), 137.44 (Ar-**C**CO), 141.87 (C-NH), 142.99 (C-NH), 146.30 (C-NO_2), 152.28 (NH-**C**O-NH), 167.91 (**C**OCH$_3$). **^{31}P NMR** (DMSO-d_6, 162 MHz) δ 27.43.

Methyl 2-iodo-5-(((4-nitrophenyl)carbamoyl)oxy)benzoate (21) Methyl 5-hydroxy-2-iodobenzoate **20** (380 mg, 1.3 mmol, 1 eq.) was dissolved in anhydrous THF under a N_2 atmosphere. 4-Nitrophenyl isocyanate **17** (330 mg, 2 mmol, 1.5 eq.) and TEA (250 µL, 1.7 mmol, 1.2 eq.) were added sequentially to the solution. The reaction mixture was stirred in an ice bath for 1h and then left to stir at room temperature overnight. The solution was concentrated under vacuum and the residue was dissolved in DCM (10 mL), then washed with saturated $NaHCO_3$ solution (15 mL). The solution was dried over $MgSO_4$, filtered, and concentrated under vacuum. The residue was then purified by recrystallisation from methanol to yield the methyl 2-iodo-5-(((4-nitrophenyl)carbamoyl)oxy)benzoate **21** as white crystals (83 mg, 14%).**^{1}H NMR** (DMSO-d_6, 400 MHz) δ 3.87 (3H, s, OC**H$_3$**), 7.35 (1H, d, *J*= 8.4 Hz, Ar-H), 7.71 (2H, d, *J* = 9.0 Hz, Ar-H), 7.88 (1H, d, *J* = 8.4 Hz, Ar-H), 8.00 (1H, s, Ar-H), 8.23 (2H, d, *J* = 9.0 Hz, Ar-H), 9.57 (1H, s, NH). **^{13}C NMR** (DMSO-d_6, 100 MHz) δ 52.99 (O**C**H$_3$), 80.61 (**C**-I), 117.95 (Ar-CH), 117.98 (Ar-CH), 121.12 (Ar-CH), 136.83 (Ar-CH), 141.80 (Ar-C-O), 141.86 (C-NO_2), 157.88 (O-**C**O-NH), 167.21 (**C**OCH$_3$).

HPLC-monitored release study

Methyl 2-(diphenylphosphino)-5-(3-(4-nitrophenyl)ureido)benzoate **19** (urea model prodrug) (0.25 mg/mL) (0.5 mM) in 2 mL of CH_3CN/H_2O (1:1 v/v) was reacted separately with two different azide triggers (1 mM) (0.3 mg/mL for 9-azido sialic acid) (0.1 mg/mL for benzyl azide) at 37C°. At set time

intervals, samples of 25 µL were withdrawn and analysed by HPLC. The flow rate was 1 mL/min, the mobile phase was 60% CH_3CN (1% CH_3COOH in CH_3CN) and 40% water (1% CH_3COOH in water) over 20 minutes, UV detector at λ =357 nm. The release profile curve for the 4-nitroaniline was produced by converting area under the curve (AUC) to concentration using the calibration curve equation. The calibration curve was obtained through preparation of a stock solution of 4-nitroaniline (1 mg/mL) in 1:1 mixture of water and CH_3CN and this was used to prepare a range of concentrations 0.0005 – 0.05 mg/mL.

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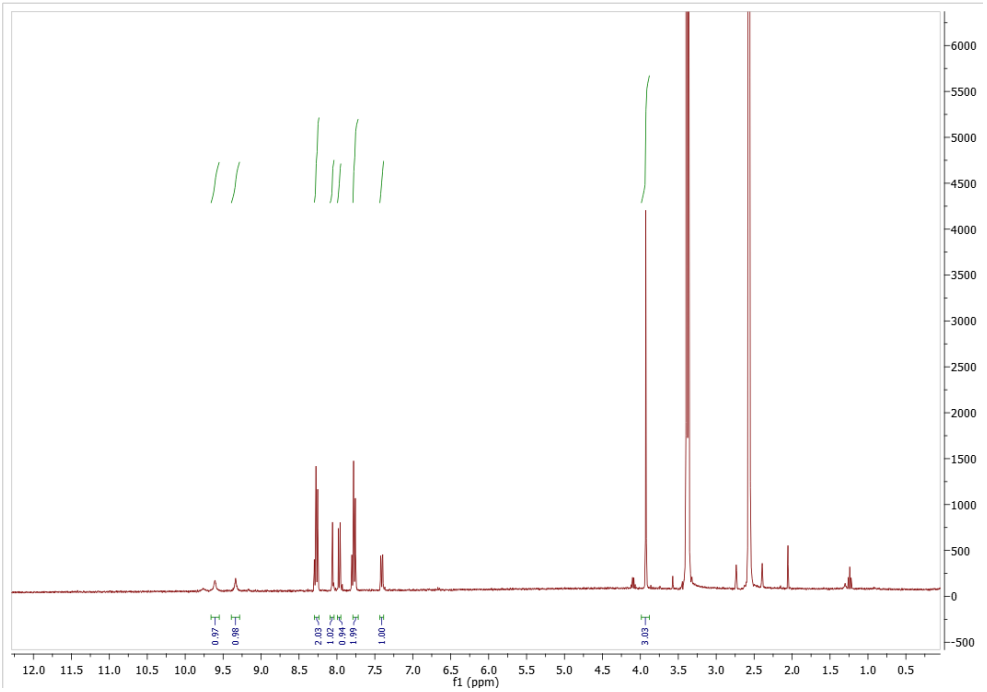
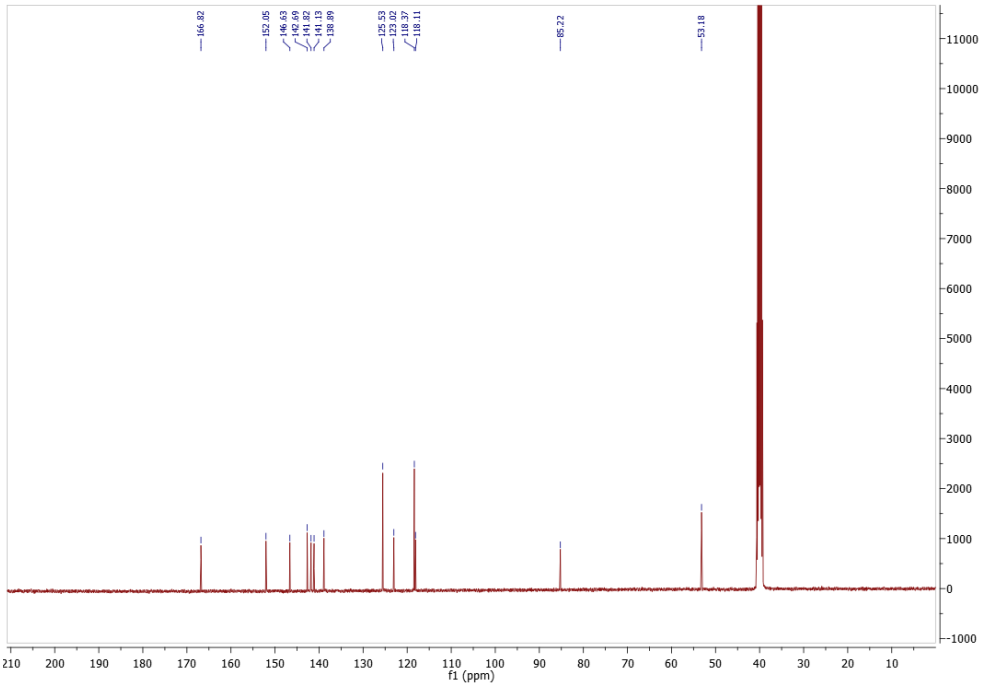
Supporting information

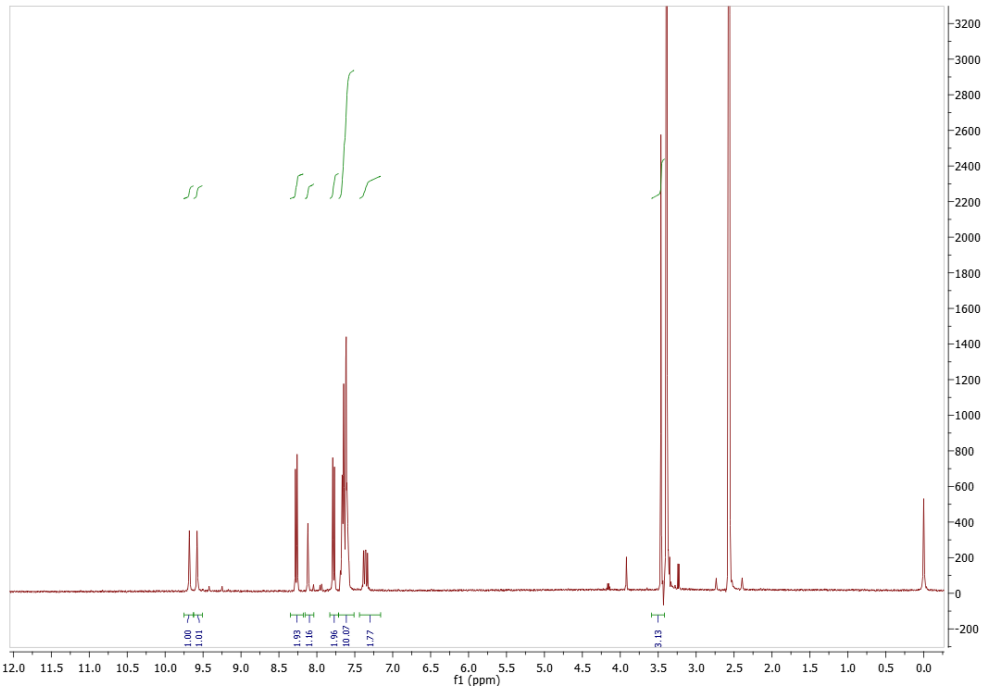
NMR spectra



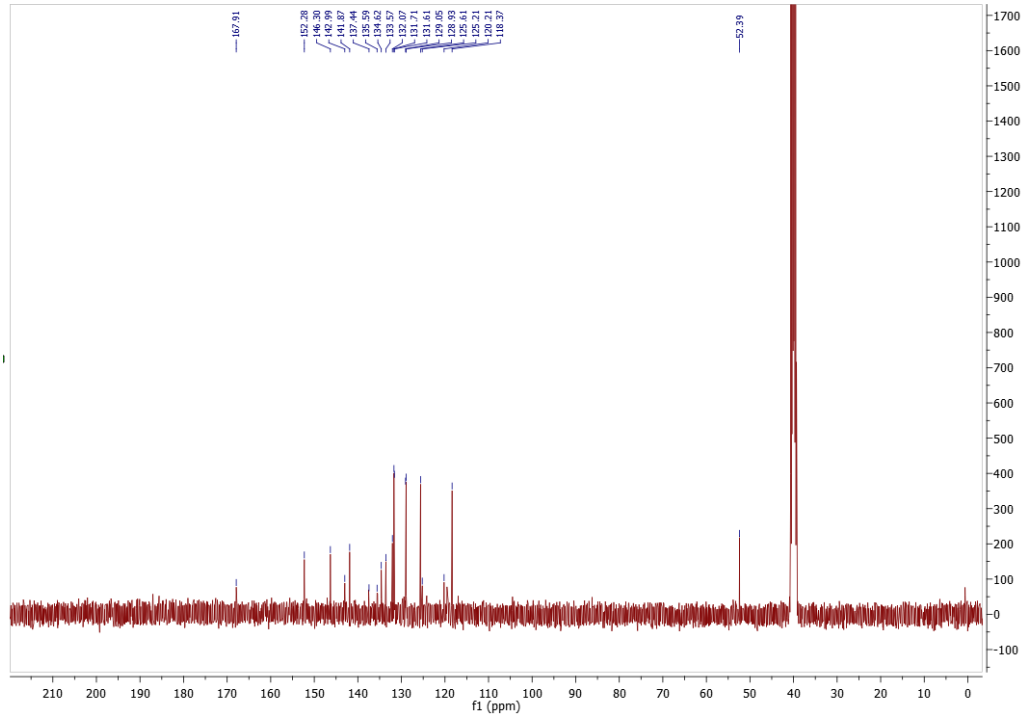
^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of compound **18**



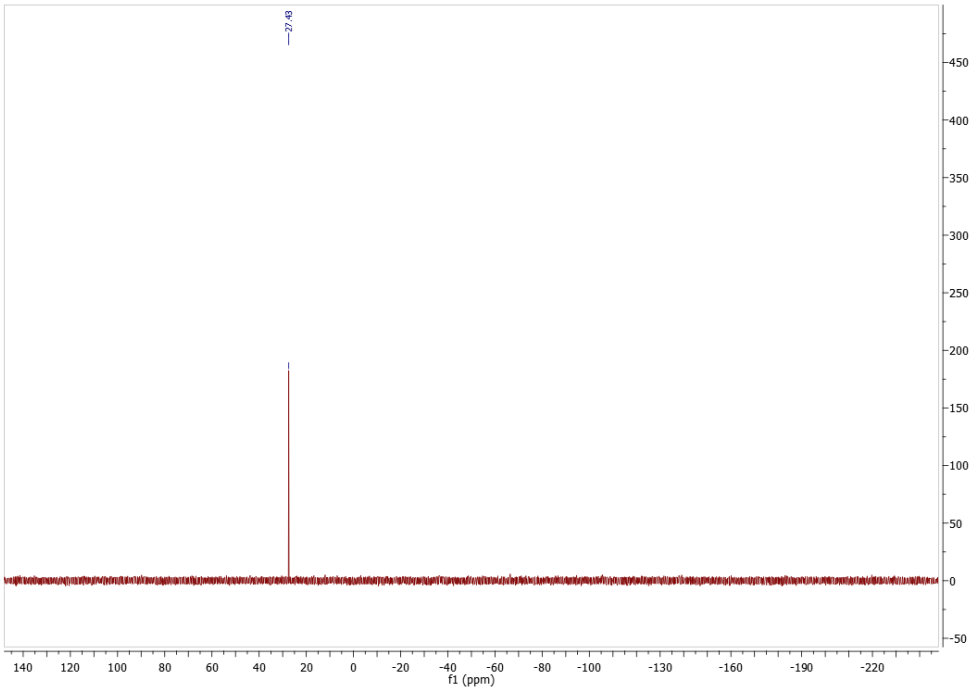
^{13}C NMR spectrum (DMSO-d_6, 100 MHz) of compound **18**



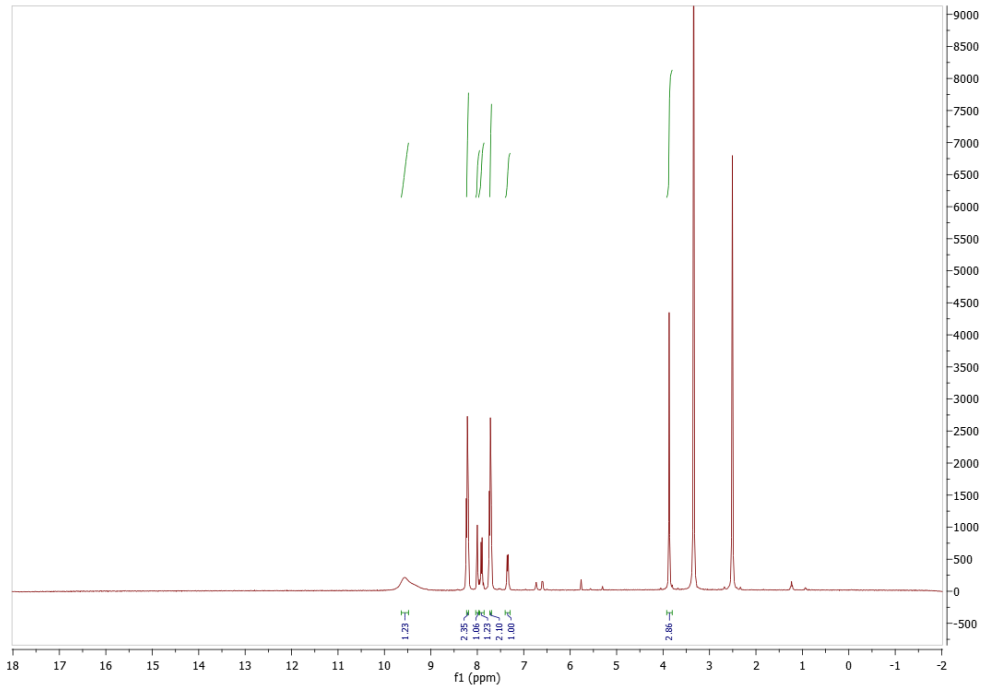
^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of compound **19**



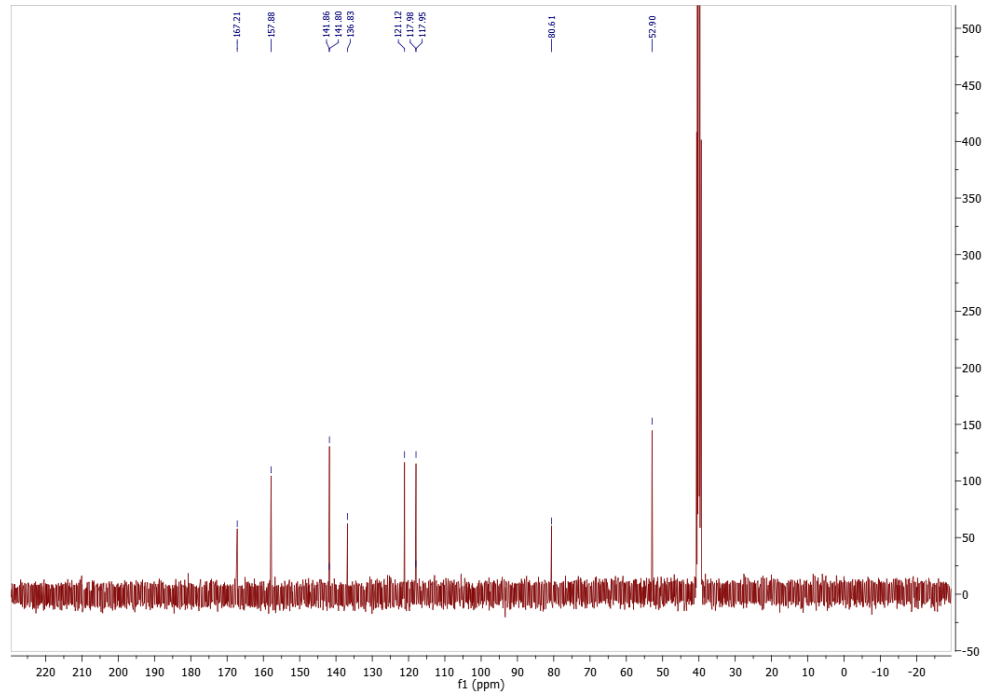
^{13}C NMR spectrum (DMSO-d_6, 100 MHz) of compound **19**



^{31}P NMR spectrum (DMSO-d_6, 162 MHz) of compound **19**

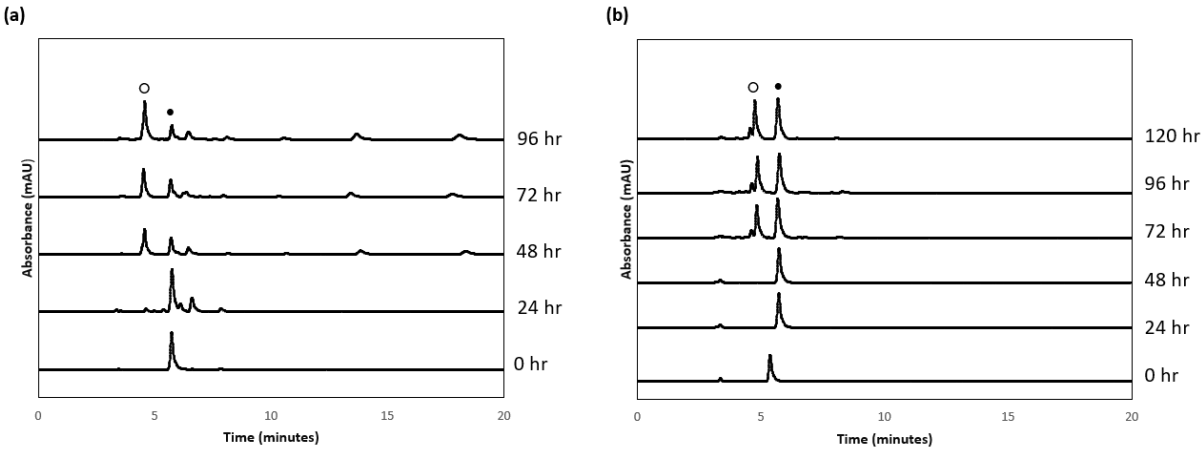


^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of compound **21**



^{13}C NMR spectrum (DMSO-d_6, 100 MHz) of compound **21**

HPLC chromatograms of 4-nitroaniline release



a) HPLC chromatograph of 4-nitroaniline release from urea model prodrug **19** by benzyl azide at 37 °C in aqueous CH_3CN (1:1 v/v) as a function of time. Legend: •: urea-linked triphenylphosphine model prodrug **19**; ○: 4-nitroaniline.

b) HPLC chromatograph of 4-nitroaniline release from urea model prodrug **19** by 9-azido sialic acid at 37 °C in aqueous CH_3CN (1:1 v/v) as a function of time. Legend: •: urea-linked triphenylphosphine model prodrug **19**; ○: 4-nitroaniline.

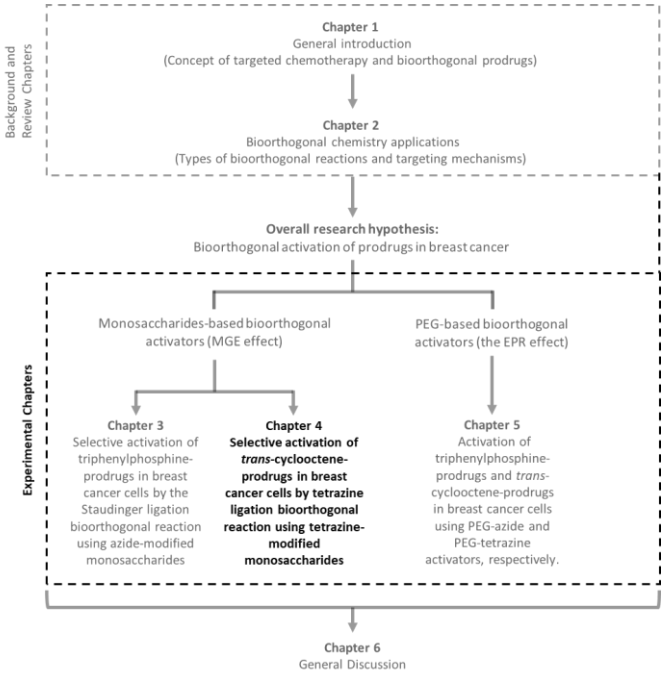
Chapter 4

Metabolic glycoengineering using tetrazine-modified monosaccharides for TCO-prodrugs' selective activation in breast cancer cells

Chapter summary: *The previous chapter evaluated MGE using azide-modified monosaccharides for selective prodrug activation by Staudinger ligation. This chapter develops this concept further and evaluates MGE using tetrazine-modified monosaccharides for selective prodrug activation by tetrazine ligation. This chapter is structured into 3 parts: the main paper, its associated supplementary information, and an appendix**.** In the **main paper**, the selective activation of trans cyclooctene (TCO)-prodrugs in MCF-7 breast cancer cells versus the non-cancerous L929 cells by the tetrazine ligation bioorthogonal reaction was evaluated. The synthesis of three tetrazine-modified monosaccharides and two TCO-prodrugs is described. The results showed the selective expression of tetrazine reporters on MCF-7 breast cancer cells via MGE (35-65 fold versus the non-cancerous L929 cells) which was confirmed by western blotting and confocal microscopy imaging. These tetrazine reporters serve as activators for TCO-prodrugs and in vitro studies showed the restoration of the activities of the parent cytotoxic drugs on MCF-7 breast cancer cells (~ 82-100% using 20 µM and ~ 32-77% using 10 µM of tetrazine-modified monosaccharides). **The supplementary information** provides the IC_{50} graphs and the NMR spectra of the compounds. **The appendix** details alternative synthetic approaches for accessing the Ac_4SiaTz compound.*

Bibliographic details: *M.M.A. Mitry, M. Dallas, S. Boateng, F. Greco, H.M.I. Osborn, Selective activation of prodrugs in breast cancer using metabolic glycoengineering and the tetrazine ligation bioorthogonal reaction, (Accepted for publication in Bioorg. Chem. DOI: 10.1016/j.bioorg.2024.107304).*

Author Contributions: *M.M.A. Mitry, F. Greco and H.M.I. Osborn designed, reviewed and supervised the study. M.M.A. Mitry carried out all the experimental work (synthesis and characterisation of compounds, western blotting and the confocal microscopy imaging assays under the supervision of S. Y. Boateng, the confocal microscopy imaging of the mouse-derived microglial BV2 cells under the supervision of M. Dallas, the in vitro activation studies, and data analysis). M.M.A. Mitry constructed figures and prepared the original draft of the manuscript. All authors edited the manuscript.*



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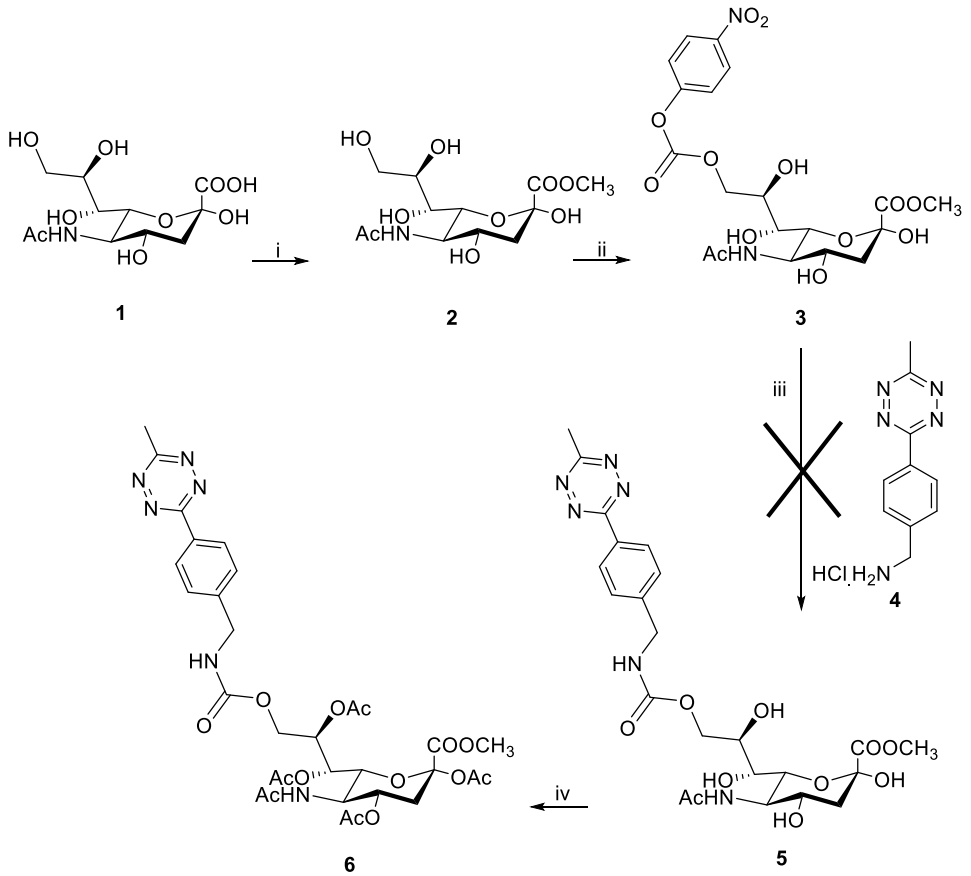
Appendix: Alternative synthetic approaches for accessing the Ac_4SiaTz compound

Introduction

Besides Man and Gal derivatives, sialic acid derivatives have been previously reported to intercept the biosynthesis of sialic acid and thereby can be used for MGE purposes.[1–5] As such, they were of interest to derivatise with azide and Tz moieties to influence expression of these activating moieties within cancer cells' surfaces' glycans by MGE. In **Chapter 3**, the synthesis of 9-azido sialic acid derivatives was detailed, and earlier in **Chapter 4**, the synthesis of 9-Tz sialic acid derivative was detailed. Both synthetic pathways utilized different protecting groups strategies. An alternative synthetic route for accessing the 9-Tz sialic acid derivative, Ac_4SiaTz **(Scheme 1),** was initially explored without employing the protecting group strategy detailed earlier in this chapter. This approach was devised with reference to the literature.[6–9]



(i) TFA, dry MeOH, RT, overnight, 86%; (ii) 4-nitrophenyl chloroformate, dry pyridine, DMAP, N_2 atmosphere, -20 °C, RT, overnight, 17%; (iii) DIPEA, DMF, N_2 atmosphere, 30 °C, 72 h; (iv) Acetic anhydride, pyridine, N_2 atmosphere, RT, overnight.

Scheme 1: Synthesis of Ac_4SiaTz without employing the protecting group strategy.

Results

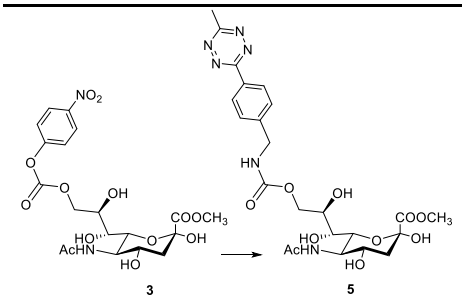
Synthesis of 9-*O*-(4-nitrophenoxy carbonyl)-Neu5Ac methyl ester (3)

The attempted synthesis of Ac_4SiaTz commenced by protecting the carboxylic acid group at position 1 to give the methyl ester derivative **2** in quantitative yield. This was followed by reacting the hydroxyl group at position 9 with 4-nitrophenyl chloroformate to introduce a more reactive 4-nitrophenyl carbonate group for subsequent reaction with an amine.[10] The reaction was conducted in dry pyridine to give the 9-*O*-(4-nitrophenoxy carbonyl) derivative **3** in 17% yield. This reaction required at least 2 equivalents of the 4-nitrophenyl chloroformate to proceed and also, it showed inconsistency in the yield of product where the reaction occurred at additional hydroxyl groups beside C9 as indicated by ^{1}H-NMR spectroscopic analysis. Disappearance of the methyl ester signal at δ 3.74 was also evident suggesting the hydrolysis of the ester group at C1 could be occurring, allowing attachment of a second 4-nitrophenyl carbonate group. This led to the alternative protecting group strategy (detailed in **Chapter 4 Scheme 2**) whereby only one free hydroxyl was present within the sialic acid derivative (Ac_4 9-hydroxy-sialic acid methyl ester) for the reaction.

Synthesis of 9-*O*-(6-methyl-1,2,4,5-tetrazine-3-benzyl-carbamoyl)-Neu5Ac methyl ester (5)

Despite the inconsistency in the yield of the previous step, the following reaction (**scheme 1 step iii**) was trialled with carbonate derivative **3** to test the feasibility of reacting the 4-nitrophenyl carbonate with an amine-tetrazine derivative **4** to afford the required tetrazine-carbamate **5**. Different conditions for reacting the 9-*O*-(4-nitrophenoxy carbonyl) derivative **3** with the tetrazine.methanamine HCl salt **4** were explored as summarised in **Table 1**.

Table 1. Summary of the reaction conditions used for the synthesis of compound 5

	Reaction conditions	Results	Ref
	$NaHCO_3$, DMF/H_2O, RT, 24-72 h.	No reaction as indicated by TLC and ^{1}H-NMR spectroscopic analysis.	[6]
	TEA, DMF, RT, 24-72 h.	No reaction as indicated by TLC and ^{1}H-NMR spectroscopic analysis.	[8]
	DIPEA, DMF, 30 °C, 72 h.	^{1}H-NMR spectrum consistent with correct structure but mass spectrometric analysis did not correlate with the expected structure.	[9]

Discussion

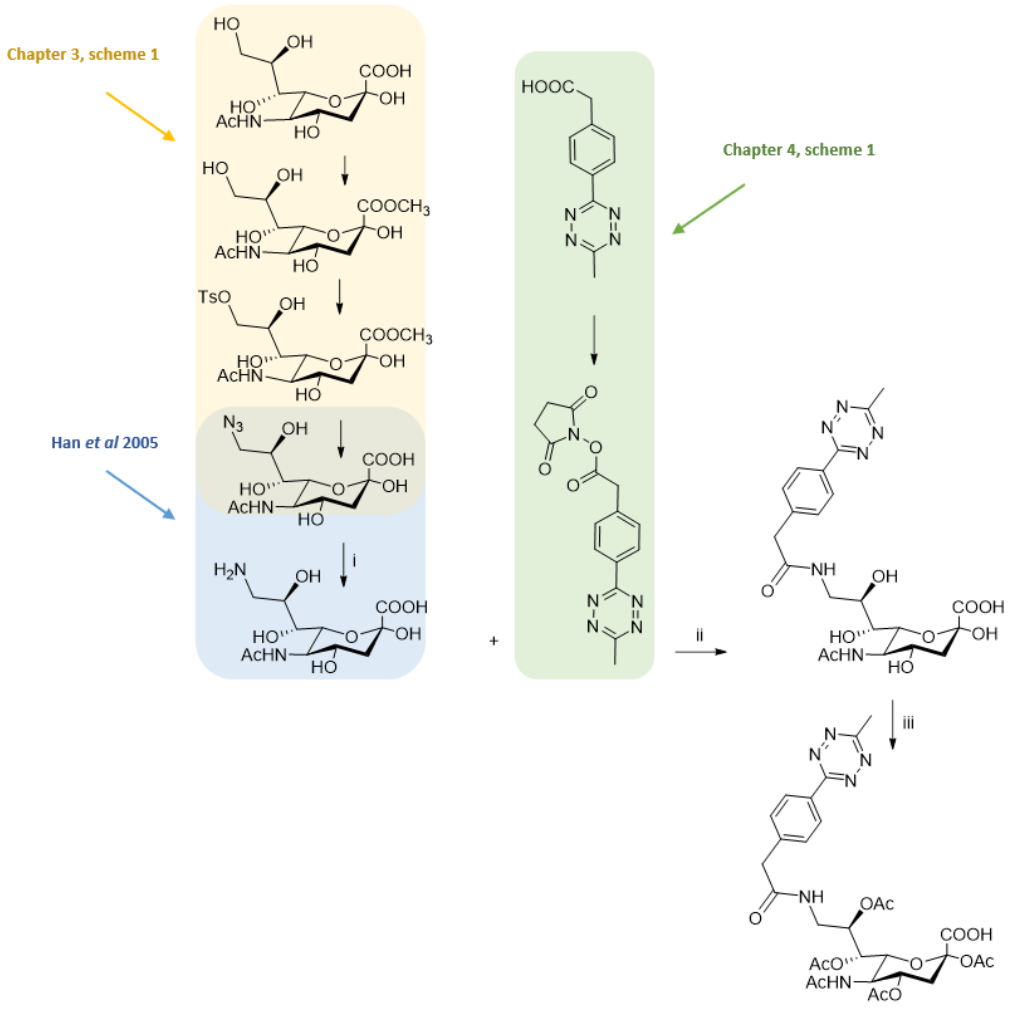
Due to the uncertainties with confirming the compounds' structures and the poor yield for accessing the 9-*O*-(4-nitrophenoxy carbonyl) derivative **3**, this synthetic scheme was not considered reliable and hence the protecting group approach presented in **Chapter 4 scheme 2** was used instead.

Utilizing a protecting group strategy is beneficial for selective reaction of compounds with the same functional group at different positions (e.g. hydroxyl groups in sialic acid) as it facilitates the regioselectivity of the required reaction. Two main approaches were employed in this thesis to allow the regioselective addition of the azide and Tz groups at position 9 of the sialic acid.

-*Approach 1*: this involved conversion of the primary alcohol at position 9 into a good leaving group, specifically a tosylate, to allow subsequent introduction of the key azide functional group **(Chapter 3, scheme 1)**.[11] When a similar strategy was used in an attempt to synthesize the Ac_4SiaTz derivative, through introduction of the 4-nitrophenyl leaving group at position 9, reduced reactivity and inconsistency in the reaction outcome were encountered. This was despite the feasibility of using 4-nitrophenyl chloroformate for the selective reaction with a primary hydroxyl in the presence of other secondary or tertiary hydroxyl groups being reported in the literature.[12,13]

-*Approach 2*: this involved the use of a *tert*-butyl dimethylsilyl ether protecting group at C9 prior to acetylation of the other hydroxyl groups in the molecule. This was then followed by deprotection (removal of the *tert*-butyl dimethylsilyl group). This yielded a sialic acid derivative bearing only one free hydroxyl group at position 9 that can be subsequently derivatised to install new functionalities at this position **(Chapter 4, scheme 2)**. Successful synthesis of the Ac_4-9-hydroxy-sialic acid methyl ester derivative was achieved, with improvements upon previous work from our research group.[14] Only one similar previous approach has been reported to synthesise this derivative.[15] However, that approach is more tedious as it involved an extra step of converting the OH group at C2 to a methoxy group.

Despite the success of approach 2 to yield the 9-Tz sialic acid derivative (Ac_4SiaTz), its low yield (14%) remains a limitation. Further improvements could be employed to increase the yield. One approach could involve conversion of the azide group at C9 in 9-azido sialic acid **(Chapter 3, scheme 1)** into a primary amino group through its hydrogenation in acidic conditions (pH 2).[11,16] Then the amino group at C9 could be reacted with methyltetrazine-NHS ester (synthesis reported in **Chapter 4, scheme 1**). This approach **(Fig. 1)** could result in the synthesis of a 9-Tz sialic acid derivative with improved yield as it does not involve the *tert*-butyl dimethylsilyl ether protecting group strategy nor the 4-nitrophenyl chloroformate reaction which is responsible for the low yield of formation of the Ac_4SiaTz compound.



(i) PdO, H_2, H_2O, acetic acid, pH 2, RT, 1h; (ii) TEA, MeOH, N_2 atmosphere, RT, overnight; (iii) Acetic anhydride, pyridine, N_2 atmosphere, RT, overnight.

Figure 1. Proposed Synthesis of an amide Ac_4SiaTz derivative for improved efficiency of synthesis.

Conclusion

In the attempt to synthesise the 9-Tz sialic acid derivative (i.e. Ac_4SiaTz), converting the hydroxyl group at the 9-position into a 4-nitrophenyl carbonate group showed high inconsistency in the yield. Despite the proposed preference for the reaction to proceed on the hydroxyl group at the 9-position because it is a primary alcohol, there was evidence of the reaction occurring at other hydroxyl groups in the sialic acid molecule. Therefore, using a protecting group strategy as detailed in chapter 4 to yield a sialic acid derivative bearing only one free hydroxyl group at position 9 is a more practical approach. Further improvement in the yield of Ac_4SiaTz compound could be achieved through reduction of azide in the 9-azido sialic acid derivative to amine. This would afford a 9-amino sialic acid derivative that could react with methyltetrazine-NHS ester to yield an amide-linked 9-Tz sialic acid derivative.

Experimental

Materials

The same materials and instruments were used as described earlier in **Chapter 4** experimental section.

Synthesis

5-Acetamido-9-*O*-(4-nitrophenoxy carbonyl)-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid methyl ester (3) Neu5Ac methyl ester **2** (200 mg, 0.62 mmol, 1 eq.) was dissolved in pyridine (8 mL). The solution was cooled in ice to -20°C and then 4-nitrophenyl chloroformate (250 mg, 1.24 mmol, 2 eq.) was dissolved in pyridine (2 mL) under a N_2 atmosphere and was added gradually to the solution of **2** under a N_2 atmosphere followed by DMAP (75 mg, 0.62 mmol, 1 eq.). The reaction mixture was left to warm to room temperature and stirred overnight. The pyridine was co-removed with toluene under vacuum, then the residue was purified by column chromatography (ethyl acetate : MeOH = 20:1) to yield the 9-*O*-(4-nitrophenoxy carbonyl)-Neu5Ac methyl ester **3** as a yellow solid (52 mg, 17%). **^{1}H NMR** (D_2O, 400 MHz) δ 1.78-1.87 (1H, m, H_3), 1.96 (3H, s, NHCOC**H$_3$**), 2.23 (1H, dd, *J* = 13.0, 4.9 Hz, $H_{3'}$), 3.46 (1H, d, *J* = 9.2 Hz, H_7), 3.53 (1H, dd, *J* = 11.8, 6.3 Hz, H_5), 3.60-3.68 (1H, m, H_8), 3.74 (3H, s, COOC**H$_3$**), 3.79-3.90 (2H, m, H_4, H_6), 3.97 (2H, dd, *J* = 13.1, 7.7 Hz, H_9, $H_{9'}$), 6.79 (2H, d, *J* = 7.8 Hz, ArH), 7.91 (2H, d, *J* = 7.8 Hz, ArH). **^{13}C NMR** (D_2O, 100 MHz) δ 22.06 (NHCO**C**H$_3$), 39.29 (CO**C**H$_3$), 52.03 (C3), 53.48 (C5), 63.13 (C4), 66.64 (C9), 68.18 (C7), 70.10 (C8), 70.33 (C6), 95.32 (C2), 106.73 (phenyl C), 138.07 (phenyl C), 149.26 (carbonate C=O), 157.52 (amide C=O), 171.41 (ester C=O).

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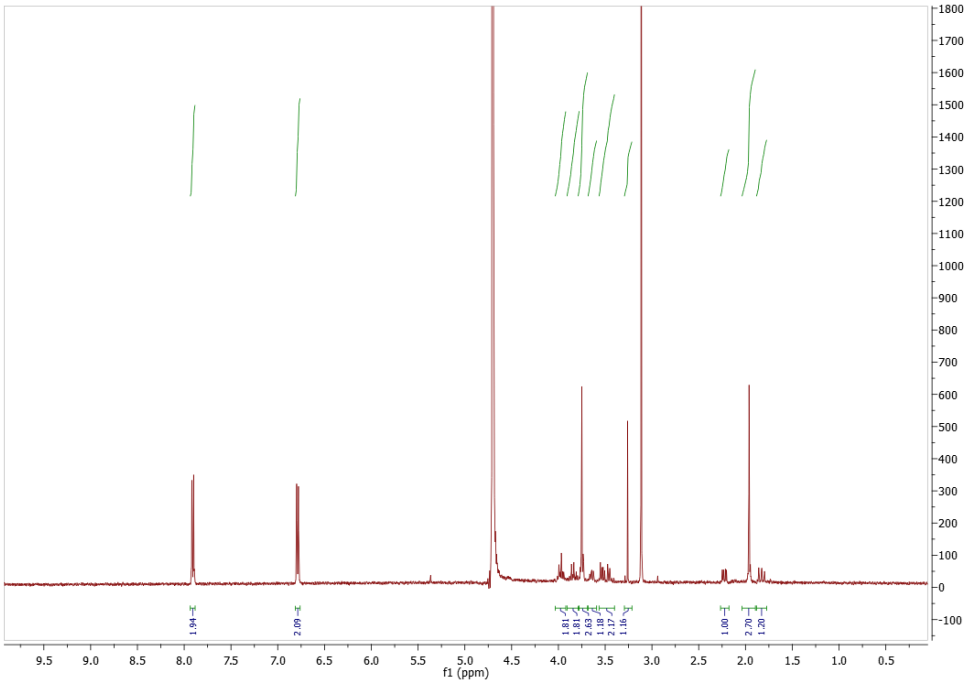
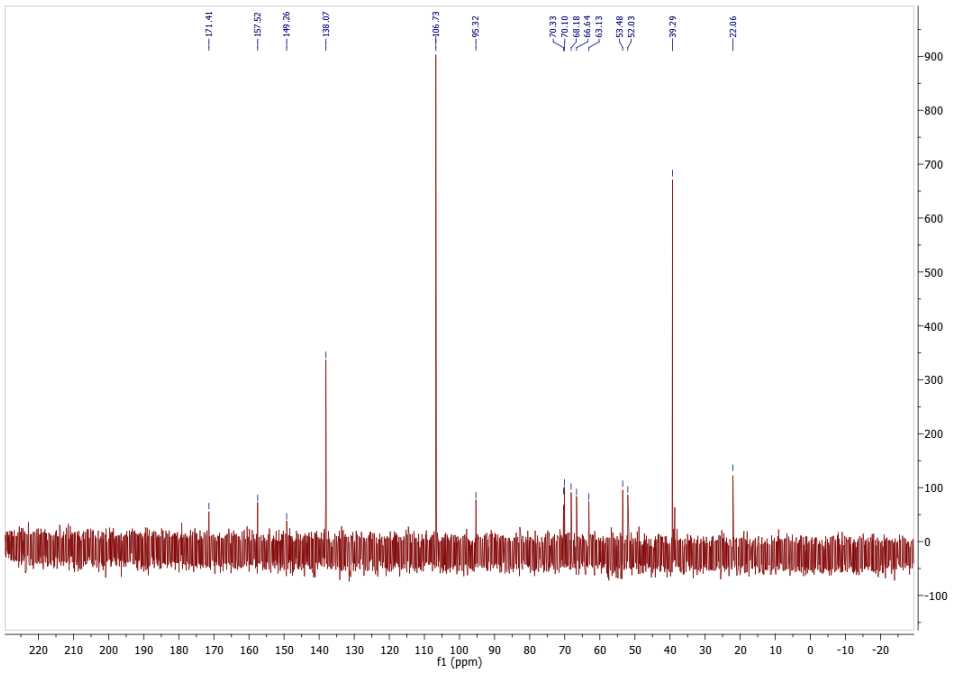
Supporting information

NMR spectra



^{1}H NMR spectrum (400 MHz, D_2O) of compound **3**



^{13}C NMR spectrum (100 MHz, D_2O) of compound **3**

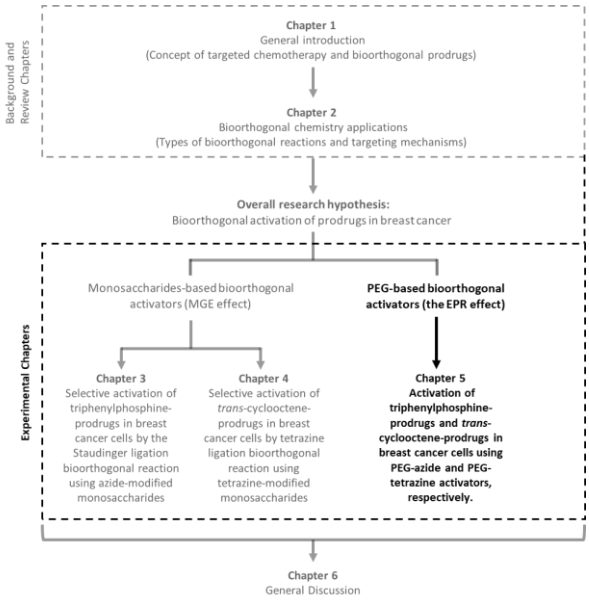
Chapter 5

Activation of triphenylphosphine-prodrugs and *trans*-cyclooctene-prodrugs in breast cancer cells using PEG-azide and PEG-tetrazine, respectively.

Chapter summary: *The previous two chapters evaluated the utilisation of monosaccharide-based activators for selective labelling and prodrug activation in breast cancer cells. This chapter evaluates the utilisation of complementary polymer-based activators for prodrug activation in breast cancer cells. This chapter is structured into 3 parts: the main paper, its associated supplementary information, and an appendix**.** In the **main paper**, the feasibility of using PEG-based polymer activators for bioorthogonal activation of prodrugs in MCF-7 and MDA-MB-231 breast cancer cells was evaluated. HPLC release monitoring studies showed that the size of the polymer affected the release rate by the Staudinger ligation from a triphenylphosphine model ester prodrug bearing a 4-nitrophenol moiety (83% release versus 44% release by 10 and 20 KDa PEG-azide, respectively). Complete release of doxorubicin from triphenylphosphine-doxorubicin prodrug was shown upon incubation with a 10 KDa PEG-azide activator. Complete release of 4-nitrophenol and doxorubicin from a TCO model carbonate prodrug and a TCO-doxorubicin prodrug, respectively were shown upon incubation with a 10 KDa PEG-tetrazine activator after 24 hours and 20 minutes, respectively. In vitro prodrug activation studies on MCF-7 and MDA-MB-231 breast cancer cells showed the restoration of parent drugs cytotoxicity (~ 68-76% in the case of PEG-azide activator for triphenylphosphine-prodrugs and 100% in the case of PEG-tetrazine for TCO-prodrugs). **The supplementary information** provides the IC_{50} graphs and the NMR spectra of the compounds. **The appendix** details results from an in vitro scratch assay to determine the effect of prodrug activation on the migration of MDA-MB-231 cells.*

Bibliographic details: *M.M.A. Mitry, F. Greco, H.M.I. Osborn, Polymeric PEG-based bioorthogonal triggers for prodrug activation in breast cancer, (Manuscript in preparation for submission to Polym. Chem.).*

Author Contributions: *F. Greco and H.M.I. Osborn designed, reviewed and supervised the study. M.M.A. Mitry designed the compounds. M.M.A. Mitry carried out all the experimental work (synthesis and characterisation of compounds, the HPLC release studies, the serum stability study, the in vitro activation studies, and data analysis). M.M.A. Mitry constructed figures and prepared the original draft of the manuscript. All authors edited the manuscript.*



Polymeric PEG-based bioorthogonal triggers for prodrug activation in breast cancer

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Keywords

Poly(ethylene glycol); Enhanced permeability and retention; Bioorthogonal chemistry; Staudinger ligation; Tetrazine ligation; Breast cancer.

Abstract

Prodrug strategies have been introduced to address various drug delivery challenges including systemic toxicity of chemotherapeutics. Bioorthogonal chemistry has positively impacted prodrug activation strategies through the introduction of biocompatible reactions that enable on-demand prodrug activation and active drug release. In addition to on-demand activation, on-target activation is also essential to achieve site specific activation of prodrugs. Polymers such as poly(ethylene glycol) (PEG) are known to target solid tumours by passive targeting *via* the enhancer permeability and retention (EPR) effect. In this paper, the feasibility of derivatising long PEG chains to afford bioorthogonal activators (PEG-azide and PEG-tetrazine) for prodrug activation *via* the Staudinger ligation and the tetrazine ligation reactions, respectively, is evaluated. The molecular weight of the PEG used in the activator and the type of linkage between the drug and prodrug moiety were shown to significantly affect the rate of prodrug activation and hence the rate of drug release. *In vitro* cytotoxicity studies on breast cancer cells (MCF-7 and MDA-MB-231) showed ~68-76% restoration of the parent drug's cytotoxicity for the Staudinger ligation-based prodrug activation and 100% restoration of the parent drug's cytotoxicity for the tetrazine ligation-based prodrug activation strategy.

1. Introduction

Selective targeting of therapeutic agents to tumour cells is a powerful strategy to increase the effectiveness of cancer therapy. One approach for achieving this is the on-site activation of chemotherapeutic prodrugs through enzymatic catalysis. In theory, this approach can minimise the systemic adverse effects of the chemotherapies by reducing exposure of healthy cells to the toxic drugs. However, off-site hydrolysis and non-specific enzymatic activation can limit the selectivity of this approach.[1–3] Attention has therefore focused on developing selective prodrug activation approaches that combine *in situ* prodrug activation with targeting mechanisms that deliver the prodrug, or a component that can activate the prodrug, specifically to the tumour.

Bioorthogonal chemistry has emerged as a promising platform for on-demand prodrug activation, as it comprises chemical reactions that can proceed under physiological conditions without interfering with biological processes.[4,5] The selectivity, specificity, and considerably fast kinetics of these reactions allow precise control of the activation of the prodrugs.[6–8] Many bioorthogonal reactions have been reported to have high potential for selective prodrug activation such as the Staudinger ligation between an azide and a triphenylphosphine,[9] and the tetrazine ligation between a *trans*-cyclooctene (TCO) and a tetrazine (Tz).[10] The Staudinger ligation is mainly used for ligation applications due its relatively slow kinetics, and a small number of reports have demonstrated its potential for prodrug activation.[11–13] The tetrazine ligation is well known for its fast click-to-release reaction kinetics at low concentrations, however, many reports have demonstrated that the reactivity of the Tz moiety, and consequently the reaction kinetics, can be fine-tuned by altering the properties e.g. size/electron density of the Tz moiety.[14–16]

Complementary targeting approaches are engaged to selectively deliver the prodrug or the activator to tumours to maximise the impact of the on-target prodrug activation.[7] These targeting approaches include metabolic glycoengineering (MGE),[11] active targeting,[17–19] and passive targeting.[20] Passive targeting is a promising approach to target solid tumour as it relies on the enhanced permeability and retention (EPR) effect. EPR is a phenomenon that explains the preferential accumulation of nanosystems in tumours due to the enhanced release of vascular permeability factors, which increases the blood vessels permeability in tumours.[21,22] This mechanism allows the passive delivery and accumulation of relatively large molecules such as polymers, liposomes and proteins hence rendering the EPR effect a valuable cancer-targeting mechanism.[22–24]

In this paper, we propose the derivatization of poly(ethylene glycol) (PEG) of suitable molecular weight to afford activators that can be incorporated within a passive targeting bioorthogonal prodrug activation strategy for breast cancer underpinned by the EPR effect **(Fig. 1)**. PEG was selected as the polymeric component due its documented aqueous solubility, high safety profile (almost inert reactivity with blood components), low antigenic and immunogenic properties, good pharmacokinetics, and approval by the FDA for use in humans.[25–28] Short PEG chains (< 10 KDa) already proved effective in bioorthogonal applications where they were utilised in coating carbon nanotubes and gold nanoparticles for bioorthogonal synthetic, diagnostic or therapeutic purposes.[29–33] Longer PEG chains (≥ 10 KDa) are reported to be suitable for use in cancer targeting by the EPR effect due to their good accumulation levels in solid tumours.[34–37] Therefore, herein, the feasibility of using PEG derivatives of larger molecular weights (≥ 10 KDa) in bioorthogonal Staudinger ligation- and tetrazine ligation-based prodrug activation is probed. PEG polymers derivatised with azide or Tz moieties were firstly tested for their feasibility to activate complementary

prodrugs *via* the Staudinger ligation or Tz ligation biorthogonal reactions, respectively, through HPLC-monitored release studies. Then, prodrug activation and restoration of the antiproliferative activity of the active drugs were confirmed *in vitro* using breast cancer cells.

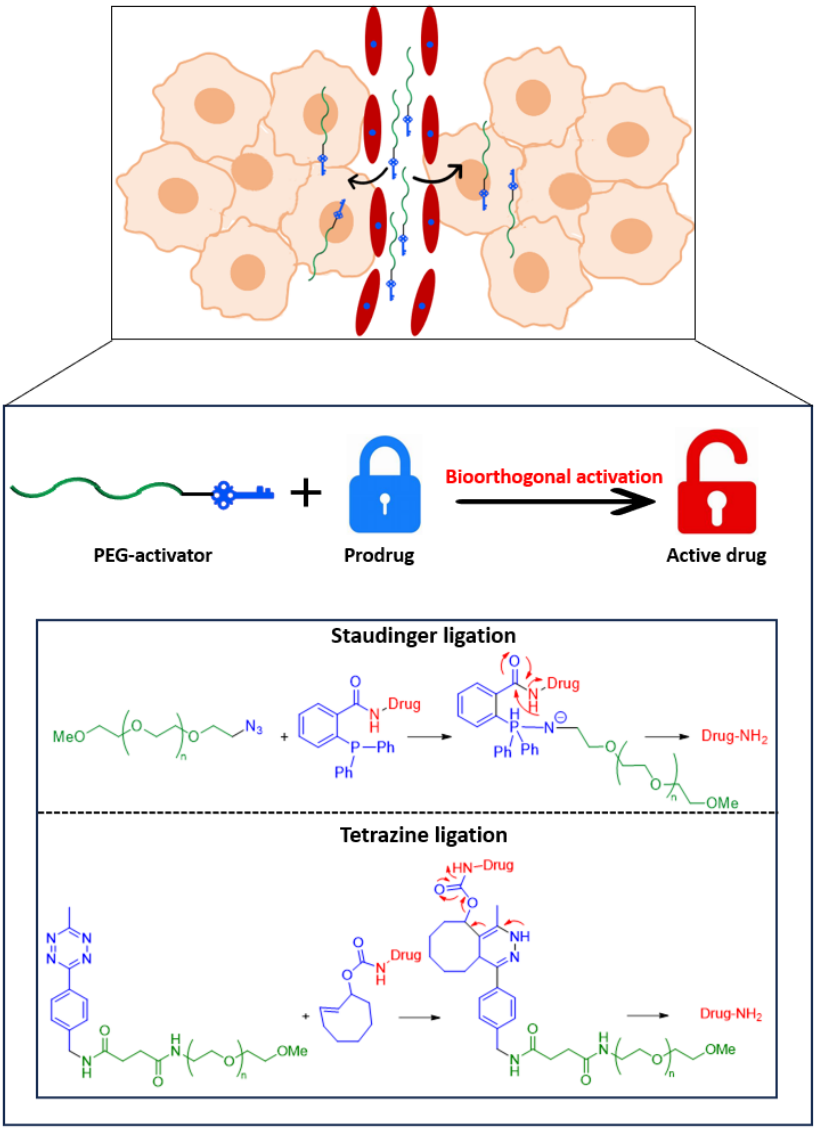


Figure 1. Schematic illustration of prodrug activation by the Staudinger ligation and tetrazine ligation bioorthogonal reactions between prodrugs and PEG-activators for selective delivery to solid tumours through the EPR effect.

2. Results and discussion

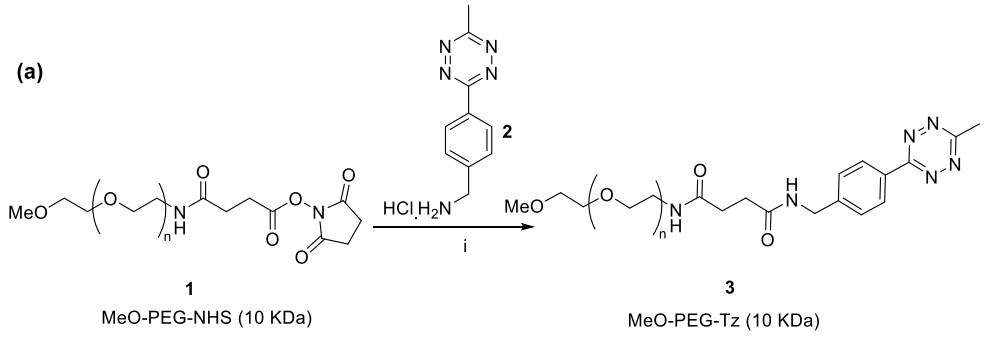
2.1. Chemical synthesis and proof-of-concept

PEG-activators

To test the feasibility of using the polymer triggers to activate prodrugs by the Staudinger ligation and tetrazine ligation reactions, two polymeric activators were employed, specifically, a PEG-azide and a PEG-Tz, respectively. Despite the biostability of the PEG polymer, it is critical to take into consideration the molecular weight of the PEG used according to the purpose. PEGs with low molecular weights (< 400 Da) are reported to produce toxic hydroxy acids and diacid metabolites due to their ease of

metabolism *in vivo*,[26,38] while PEGs with high molecular weights (≥ 40 KDa) tend to accumulate in the liver.[25] Therefore, PEGs with MW between 10-40 KDa are considered more suitable to use for selective tumour delivery by passive targeting and the EPR effect.[34–37]

To design the PEG-Tz activator, the structure of the employed Tz moiety to be conjugated to the PEG was also considered. Electron withdrawing groups as substituents on the tetrazine ring are shown to increase the reaction rate but decrease the serum stability of the Tz moiety, while electron donating groups have the opposite effects.[15] Therefore, a tetrazine ring substituted with a 3-benzyl group and a 6-methyl group is proposed to have a good balance between the reaction kinetics and the serum stability and hence was selected for conjugation to the PEG. The synthesis of the PEG-Tz activator **3** was carried out as shown in **scheme 1** [39] using MeO-PEG-NHS (10 KDa) to afford the conjugate in 74%. The conjugate was purified by dialysis and characterised using ^{1}H and ^{13}C NMR spectroscopy. The average Tz content in the conjugate, as determined *via* ^{1}H-NMR spectroscopic analysis, was 1.78% w/w, and the amount of free Tz was determined to be < 0.2% using RP-HPLC. The commercially available methoxy-azido-PEG was used as the PEG-azide activator, the structure for this is shown in **scheme 1b**.

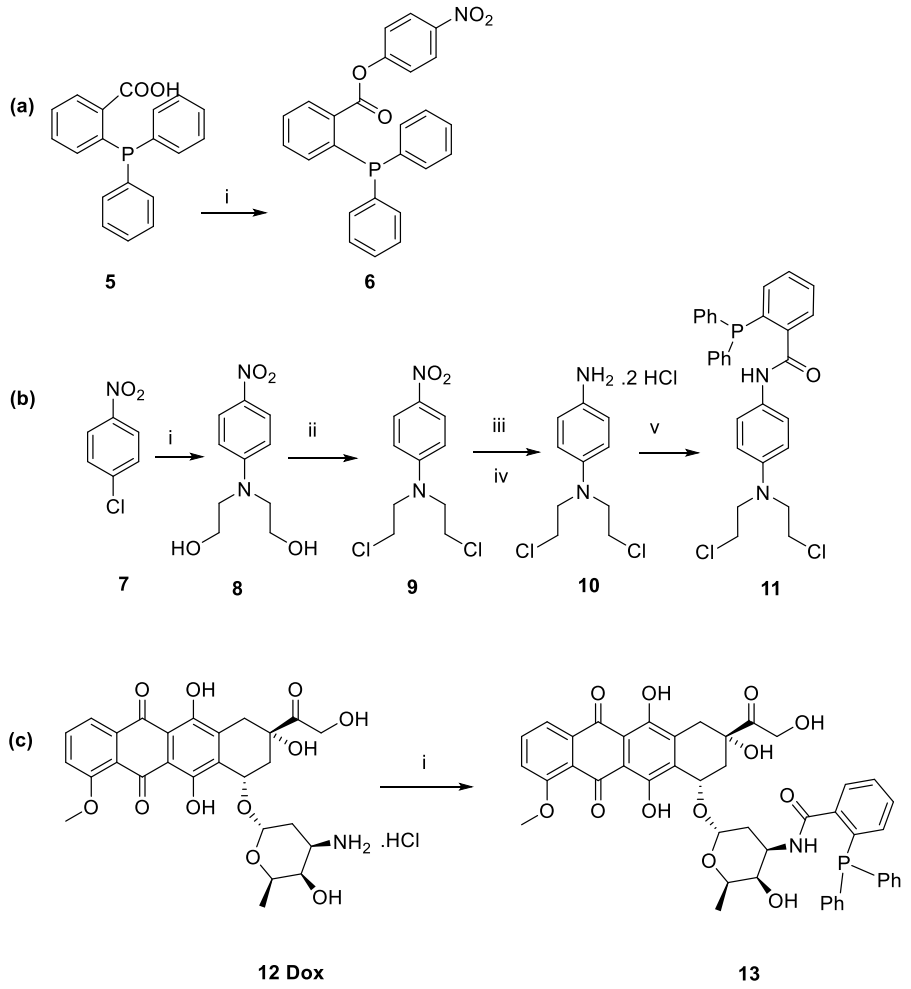


Scheme 1. (a) Synthesis of PEG-tetrazine activator **3**. Tz loading: 1.78% W/W, free Tz < 0.2% of total Tz. (b) Chemical structure of PEG-azide activator **4**.

Triphenylphosphine model ester prodrug and doxorubicin and N-mustard amide prodrugs

A model prodrug was initially used where a 4-nitrophenol moiety was used as a substitute for the cytotoxic drug moiety. 4-Nitrophenol was linked by an ester bond to the triphenylphosphine to yield a triphenylphosphine model ester prodrug **6** for the Staudinger ligation prodrug activation approach**.** The synthesis was carried out as shown in **scheme 2a**[9] through DCC coupling-mediated Steglich esterification of diphenylphosphanyl benzoic acid **5** with 4-nitrophenol to yield the model ester prodrug **6** in 38%. The compound was purified by column chromatography and characterised using ^{1}H

and ^{13}C NMR spectroscopy, and mass spectrometry. For prodrugs containing cytotoxic components, doxorubicin and nitrogen mustard were selected to be derivatised as bioorthogonal cleavable prodrugs as they are effective against breast cancer, but suffer from low selectivity. The prodrugs were designed to comprise the cytotoxic drug attached to the triphenylphosphine masking moiety in a way that would render the prodrugs non-toxic. Triphenylphosphine-*N*-mustard prodrug **11** and triphenylphosphine-doxorubicin prodrug **13** were synthesised according to our reported procedures as shown in **scheme 2b and 2c.**[11]



(a) i) 4-nitrophenol, DCC, DMAP, DCM, N_2 atmosphere, RT, 24 h, 38%. (b) i) Diethanol amine, DMSO, reflux 140 °C, 24 h, 69%; ii) MsCl, pyridine, N_2 atmosphere, 0 °C, 30 minutes then reflux at 80 °C 1 h, 59%; iii) RaNi, hydrazine monohydrate, DCM/MeOH, RT, 2.5 h; iv) dry HCl in ether, DCM, 68%; v) DPPBA, DCC, DMAP, N_2 atmosphere, RT, 24 h, 31%. (c) i) DPPBA, DCC, DMAP, N_2 atmosphere, RT, 24 h, 39%.

Scheme 2. (a) Synthesis of triphenylphosphine model ester prodrug **6**. (b) Synthesis of triphenylphosphine-*N*-mustard prodrug **11**. (c) Synthesis of triphenylphosphine-doxorubicin prodrug **13**

A RP-HPLC-monitored release study was then conducted to test the feasibility of using PEG-azide to activate the triphenylphosphine-prodrugs.

Activation of triphenylphosphine model ester prodrug by PEG-azide activators via the Staudinger ligation: Two molecular weights of PEG-azide (10 and 20 KDa) were tested alongside a small molecule azide activator (i.e. benzyl azide) as a comparator to ascertain whether the sterics of the polymer would negatively impact the activation reaction. The release of 4-nitrophenol from the triphenylphosphine model ester prodrug **6** using three different azide activators was monitored by HPLC over 24 h in aqueous condition (H_2O:CH_3CN, 1:1 v/v) at 37 °C. After 24 h, 4-nitrophenol was completely released (100%) upon reaction with the small molecule benzyl azide activator, 83% of 4-nitrophenol was released upon reaction with PEG-azide (10 KDa) and 44% of 4-nitrophenol was released upon reaction with PEG-azide (20 KDa) **(Fig. 2a)**. These results clearly indicate that the size of the activator affects the rate of release and are suggestive that different molecular weights can be selected and further developed depending on the desired application and kinetics of release (i.e. using a PEG of higher molecular weight for slow or sustained release). As the 10 KDa size showed faster activation rate compared with the 20 KDa PEG-azide, and considered suitable molecular weight for use within drug release strategies based on the EPR effect, it was selected for subsequent release studies and *in vitro* biological studies.

Activation of triphenylphosphine-doxorubicin prodrug by PEG-azide activator via the Staudinger ligation: To test the feasibility of the PEG-azide for activation of the prodrugs with the cytotoxic component, the triphenylphosphine-doxorubicin prodrug **13** was incubated with the selected PEG-azide **4** activator (10 KDa) and the release of doxorubicin was monitored. The triphenylphosphine-doxorubicin prodrug **13** was selected for the release study as the *N*-mustard moiety that would be released from the triphenylphosphine-*N*-mustard prodrug **11** is unstable making its monitoring difficult. Using the same conditions as trialled previously (i.e. H_2O: CH_3CN, 1:1 v/v) at 37 °C, doxorubicin was completely released (100%) after 24 h **(Fig. 2b)**. Compared to the 4-nitrophenol release from the model ester triphenylphosphine-prodrug **6** with and without the activator, these results may suggest that the amide prodrug might be a better model, in terms of release and stability, than the ester prodrug for the PEG-azide activator (10 KDa) to activate the triphenylphosphine-prodrugs by the Staudinger ligation reaction.

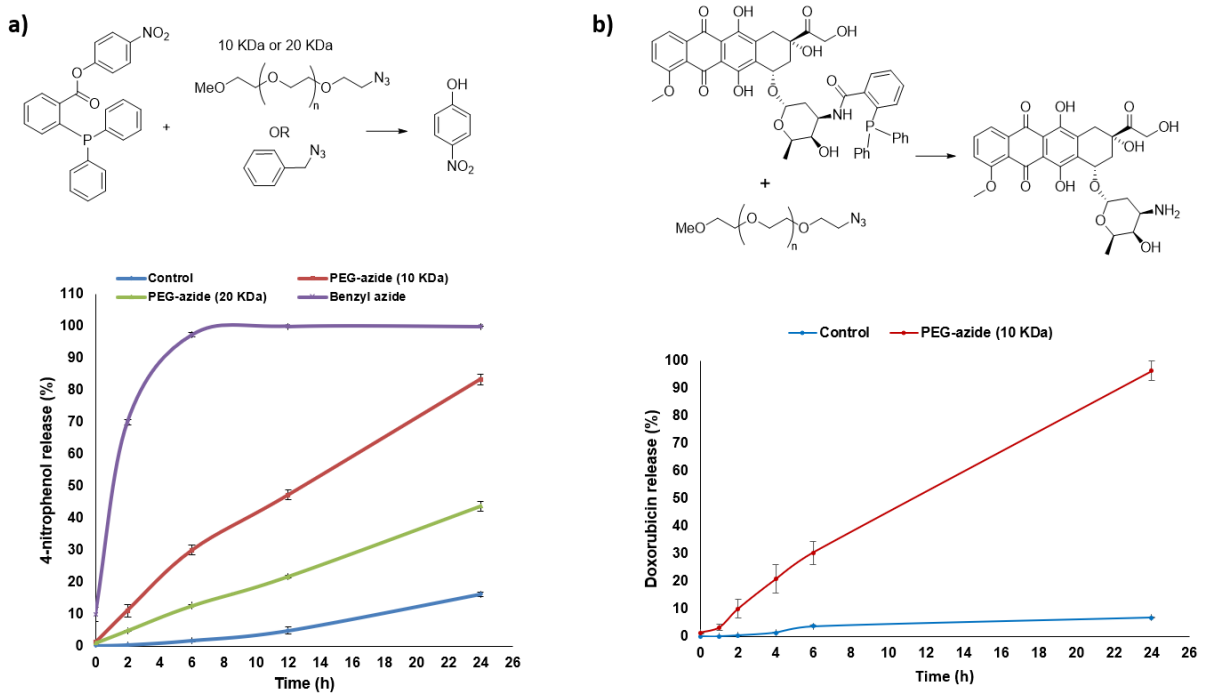
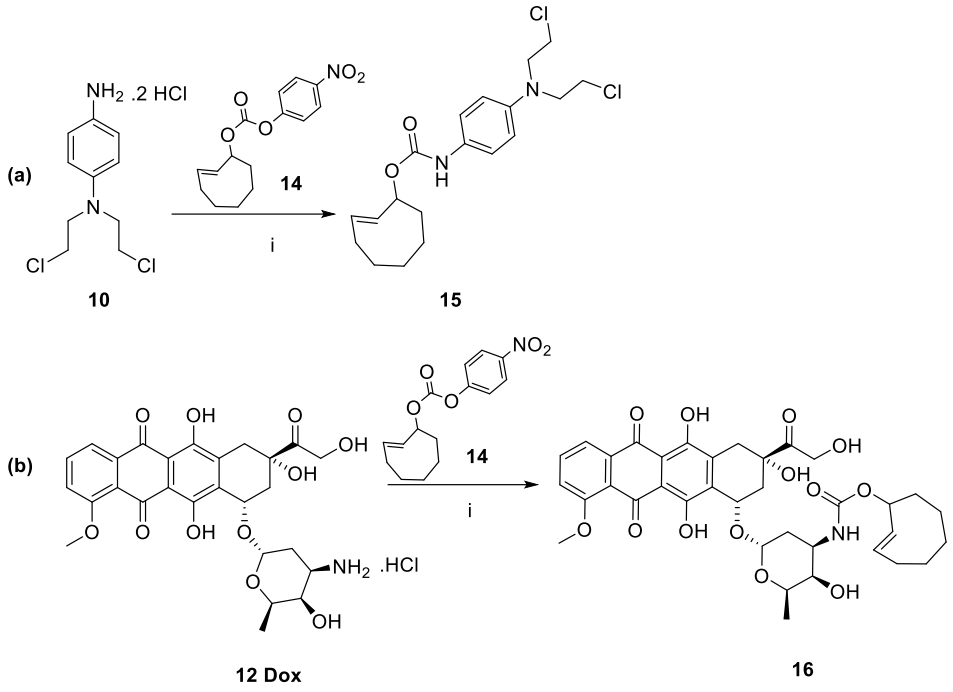


Figure 2. (a) Effect of polymer molecular weight on release rate of 4-nitrophenol from model triphenylphosphine-prodrug **6** *via* the Staudinger ligation reaction, release profile was monitored by HPLC as a function of time upon incubation of **6** with activators PEG-azide (10 KDa) or PEG-azide (20 KDa) or benzyl azide or without activators (control). (b) Release profile summary of doxorubicin **12** from triphenylphosphine-Dox prodrug **13** *via* the Staudinger ligation reaction monitored by HPLC as a function of time upon its incubation with activator PEG-azide (10 KDa) **4** or without **4** (control). Data are presented as mean ± SEM (n=3).

TCO model carbonate prodrug and doxorubicin and N-mustard carbamate prodrugs

A commercially available TCO compound **14** was used as a model prodrug where the 4-nitrophenol was linked by a carbonate bond to the TCO (structure shown in **scheme 3)**. A novel TCO-*N*-mustard prodrug **15** along with the previously reported TCO-doxorubicin prodrug **16** [10] were also synthesised according to our reported procedures (in **Chapter 4**) as shown in **scheme 3a and 3b.**[40]



i) TEA, DMF, N_2 atmosphere, RT, 72 h, 45% and 50%

Scheme 3. Synthesis of TCO-*N*-mustard prodrug **15** and TCO-*N*-doxorubicin prodrug **16**.

Then, to test the feasibility of the PEG-Tz to activate the TCO-prodrugs, a RP-HPLC-monitored release study was conducted.

Activation of TCO-model carbonate prodrug by PEG-Tz activator via the tetrazine ligation: The release of 4-nitrophenol from the 4-nitrophenyl carbonate TCO model prodrug **14** was monitored by HPLC after its incubation with PEG-Tz (10 KDa) activator **3** under the same previously used aqueous conditions (H_2O: CH_3CN, 1:1 v/v) at 37 °C. After 24 h, 4-nitrophenol was completely released (100%) **(Fig. 3a)**.

Activation of TCO-doxorubicin prodrug by PEG-Tz activator via tetrazine ligation: To test the feasibility of the PEG-Tz for activation of the prodrugs with the cytotoxic component, the TCO-doxorubicin prodrug **16** was incubated with PEG-Tz **3** activator (10 KDa) and the release of doxorubicin was monitored using the same previously used aqueous conditions (H_2O: CH_3CN, 1:1 v/v) at 37 °C. Doxorubicin was completely released (100%) after 20 min of incubation **(Fig. 3b)**. Compared to the 4-nitrophenol release from the model carbonate TCO-prodrug, these results suggest that the carbamate linkage for the cytotoxic moiety at the allyl position of the TCO has faster release rate than the carbonate linkage for the PEG-Tz activator to activate the TCO-prodrugs by the tetrazine ligation reaction.

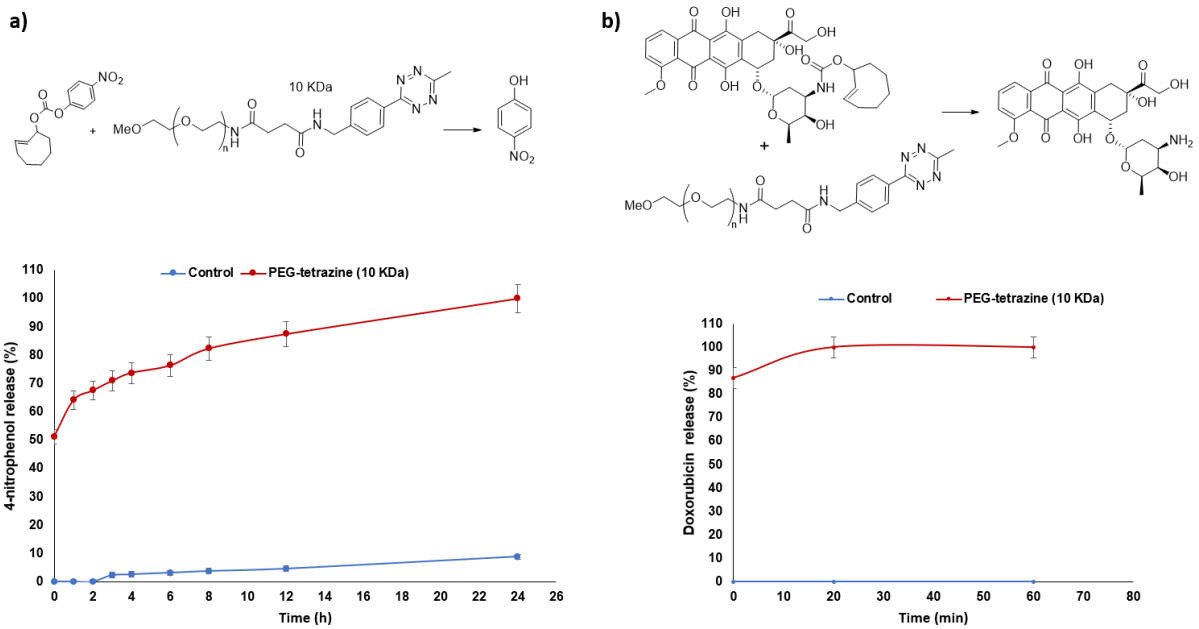


Figure 3. (a) Release profile summary of 4-nitrophenol from model carbonate TCO-prodrug **14** *via* the tetrazine ligation reaction monitored by HPLC as a function of time upon its incubation with activator PEG-Tz (10 KDa) **3** or without **3** (control). (b) Release profile summary of doxorubicin **12** from TCO-Dox prodrug **16** *via* the tetrazine ligation reaction monitored by HPLC as a function of time upon its incubation with activator PEG-Tz (10 KDa) **3** or without **3** (control). Data are presented as mean ± SEM (n=3).

Taken together, these HPLC release studies suggest the feasibility of using the 10 KDa Mw PEG-based activators in conjunction with triphenylphosphine- or TCO-prodrugs for bioorthogonal activation. Studies were therefore progressed to *in vitro* testing in relevant cell models.

2.2. *In vitro* prodrug activation

When selecting an appropriate tumour type in which to test our strategy further, it was important to select one that is documented as being suitable for passive targeting through the EPR effect. Pancreatic, colorectal and breast cancers have been reported to display the highest accumulation ratio of nanosystems in the tumour *versus* normal tissues[41] and since doxorubicin and *N*-mustards are effective on breast cancer,[42,43] MCF-7 and MDA-MB-231 breast cancer cell lines were selected to determine the effectiveness of prodrug activation by the PEG derivatives *in vitro*.

The IC_{50} of the triphenylphosphine-Dox prodrug **13** was found to be more than 20-fold higher (4.6 µM and 9.5 µM) than that of the active Dox **12** (0.2 µM and 0.4 µM) in MCF-7 and MDA-MB-231 breast cancer cells, respectively. The IC_{50} of the TCO-Dox prodrug **16** was found to be around 10-fold higher (2.4 µM and 3.5 µM) than that of the active Dox **12** (0.2 µM and 0.4 µM) in MCF-7 and MDA-MB-231 breast cancer cells, respectively **(table 1)**. This suggests that the triphenylphosphine and the TCO moieties are effective for masking the cytotoxic activity of doxorubicin. For the *N*-mustard-based prodrugs, only the IC_{50} of the prodrugs were assessed due to the instability of the active *N*-mustard drug.

To evaluate prodrug activation by the Staudinger ligation reaction, the MCF-7 and MDA-MB-231 breast cancer cells were first pre-treated with the PEG-azide activator **4**, and then with the triphenylphosphine-*N*-mustard prodrug **11** or the triphenylphosphine-Dox prodrug **13.** The IC_{50} values **(table 1**, **Fig. 4**, and **Fig. *S1a* and *S2a)*** indicate successful prodrug activation with good levels of restoration of the active doxorubicin's IC_{50} (1.6 µM and 2.6 µM versus 0.2 µM and 0.4 µM) in MCF-7 (*$p < 0.05$) and MDA-MB-231 (***$p < 0.001$) breast cancer cells, respectively. The IC_{50} for the triphenylphosphine-*N*-mustard prodrug **11** was found to be 20.8 µM and 19.7 µM in MCF-7 and MDA-MB-231 breast cancer cells, respectively and after testing the prodrug activation as previously described with the doxorubicin prodrug, these IC_{50} values decreased to 4.7 µM and 4.3 µM, respectively (***$p < 0.001$) indicating the successful prodrug activation by the Staudinger ligation reaction **(table 1**, **Fig. 4**, and **Fig. *S1b* and *S2b*).**

Next, to evaluate the prodrug activation by the tetrazine ligation reaction, the MCF-7 and MDA-MB-231 breast cancer cells were first pre-treated with the PEG-Tz activator **3**, and then with the TCO-*N*-mustard prodrug **15** or the TCO-Dox prodrug **16.** The IC_{50} values **(table 1**, **Fig. 4**, and **Fig. *S1c* and *S2c)*** indicate the complete restoration of the doxorubicin activity on both cell lines (non-significant $p > 0.05$). With the TCO-*N*-mustard prodrug **15**, the IC_{50} was found to be 23.1 µM and 30.7 µM in MCF-7 and MDA-MB-231 breast cancer cells, respectively and after the pre-treatment with the PEG-Tz activator **3,** the IC_{50} values decreased to 0.7 µM and 2.4 µM, respectively (***$p < 0.001$) indicating the successful prodrug activation by the tetrazine ligation reaction **(table 1**, **Fig. 4**, and **Fig. *S1d* and *S2d*)**.

To further test the safety of the prodrugs **11**, **13**, **15** and **16** on non-cancerous cells, the IC_{50} of the triphenylphosphine-Dox prodrug **13** and TCO-Dox prodrug **16** were determined on L929 fibroblast cells and were found to be 11.3 µM and 6.4 µM, respectively (2 folds higher than their IC_{50} on MCF-7 cells). The IC_{50} for the triphenylphosphine-*N*-mustard prodrug **11** and the TCO-*N*-mustard prodrug **15** on L929 cells were found to be 31.4 µM and 38 µM, respectively (~2 folds higher than their IC_{50} on MCF-7 cells). Moreover, the IC_{50} of the PEG-azide **4** and the PEG-Tz **3** on L929 cells were >150 µM. These results suggest the decreased potency of the prodrugs on non-cancerous cells compared to cancerous cells as well as the PEG activators safety on normal cells **(Fig. *S3*)**.

Table 1: IC_{50} values (μM) for compounds 11, 13, 15 and 16 activation determined in the MCF-7 and MDA-MB-231 cells using the MTT assay. Data indicate mean ± SEM (n = 3)

Compounds	**IC_{50} (μM)**	
	MCF-7	**MDA-MB-231**
PEG-N_3 (10 KDa) **4**	>150	>150
Doxorubicin **12**	0.2 ± 0.03	0.4 ± 0.009
Triphenylphosphine-Dox prodrug **13**	4.6 ± 0.19	9.5 ± 0.12
Triphenylphosphine-Dox prodrug **13** + PEG-N_3 **4**	1.6 ± 0.48	2.6 ± 0.03
Triphenylphosphine-*N*-mustard prodrug **11**	20.8 ± 1.27	19.7 ± 0.78
Triphenylphosphine-*N*-mustard prodrug **11** + PEG-N_3 **4**	4.7 ± 0.32	4.3 ± 0.18
PEG-Tz (10 KDa) **3**	>150	>150
Doxorubicin **12**	0.2 ± 0.03	0.4 ± 0.009
TCO-Dox prodrug **16**	2.4 ± 0.03	3.5 ± 0.44
TCO-Dox prodrug **16** + PEG-Tz **3**	0.2 ± 0.02	0.43 ± 0.11
TCO-*N*-mustard prodrug **15**	23.1 ± 0.65	30.7 ± 0.96
TCO-*N*-mustard prodrug **15** + PEG-Tz **3**	0.7 ± 1.02	2.4 ± 0.26

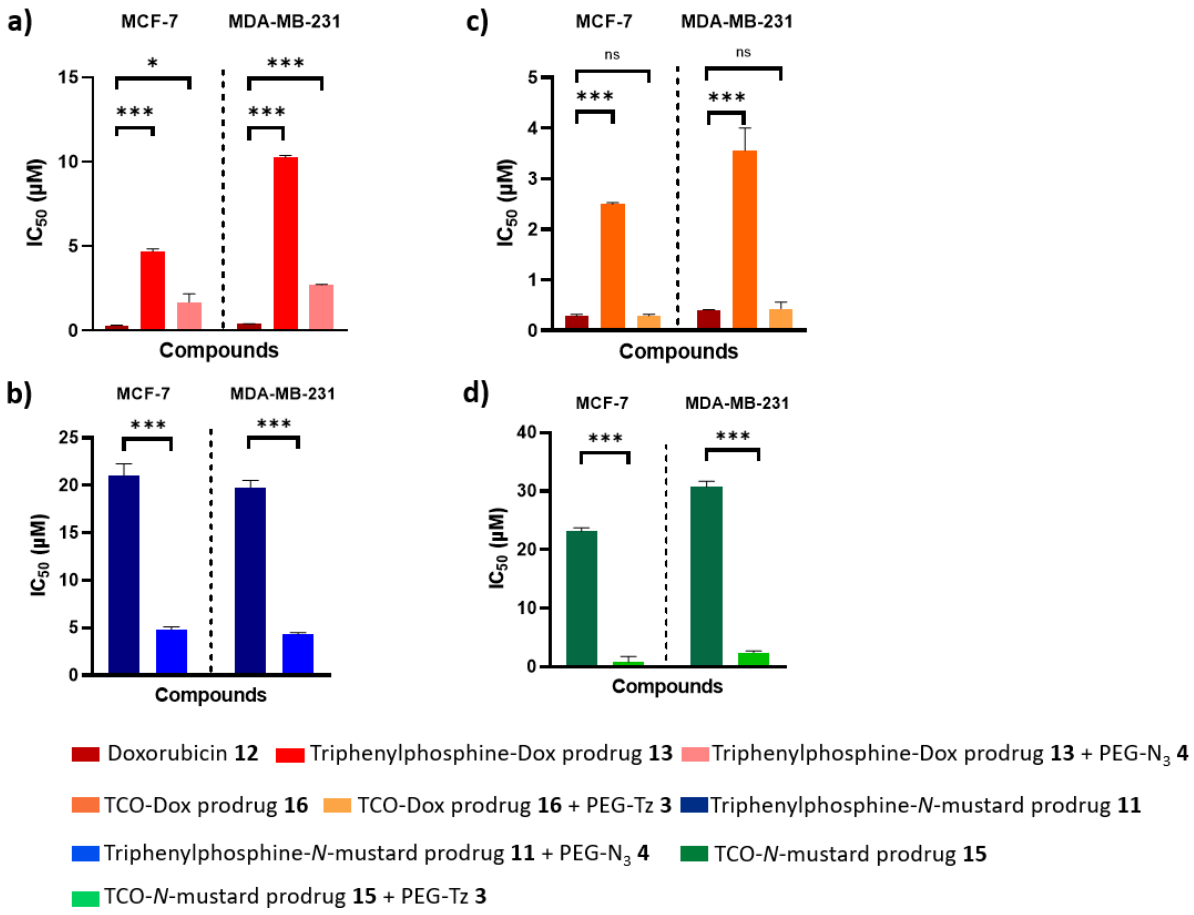


Figure 4. Statistical significance of the prodrugs IC_{50} with or without activator.(a) Determined IC_{50} of Doxorubicin **12** and its triphenylphosphine-prodrug **13** against MCF-7 and MDA-MB-231 cells and IC_{50} of the prodrug **13** after activation by PEG-azide **4**. (b) Determined IC_{50} of triphenylphosphine-*N*-mustard prodrug **11** and the prodrug **11** after activation by PEG-azide **4** against MCF-7 and MDA-MB-231 cells. c) Determined IC_{50} of Doxorubicin **12** and its TCO-prodrug **16** against MCF-7 and MDA-MB-231 cells and IC_{50} of the prodrug **16** after activation by PEG-Tz **3**. D) Determined IC_{50} of TCO-*N*-mustard prodrug **15** and the prodrug **15** after activation by PEG-Tz **3** against MCF-7 and MDA-MB-231 cells. Data are presented as mean ± SEM (n=3). ns represents no significance ($p > 0.05$), * indicates difference at the $p < 0.05$, *** indicates difference at the $p < 0.001$ significance level.

In summary, the TCO-Tz activation system can be considered to be more advantageous than the azide-triphenylphosphine system, in terms of rate of activation and restoration of active drug cytotoxicity. However, the lower toxicity of the triphenylphosphine-prodrugs, as demonstrated by their higher IC_{50} values on both of the breast cancer cell lines compared with the TCO-prodrugs, would allow higher concentrations to be used. This could potentially further enhance the Staudinger ligation activation rate in future research.

2.3. Fluorescence spectra of drug-DNA complexes

To confirm the activation of the doxorubicin-prodrugs **13** and **16** by the PEG activators, the DNA intercalation abilities of the prodrugs, and the active doxorubicin, were also tested.[44] Doxorubicin is an anthracycline drug that intercalates with DNA and supresses the action of the topoisomerase II enzyme and thereby inhibits the cancer cell replication.[45,46] Therefore if doxorubicin's mechanism

of action is diminished upon forming the prodrug, intercalation with DNA may be reduced. An aqueous solution of DNA (calf thymus) (1mg/mL) was added to separate samples of doxorubicin **12**, TCO-Dox prodrug **16** alone, and TCO-Dox prodrug **16** in the presence of the PEG-Tz **3**. In a parallel experiment, the DNA aqueous solution was added to separate samples of doxorubicin **12**, triphenylphosphine-Dox prodrug **13** alone, and triphenylphosphine-Dox prodrug **13** which was previously incubated with PEG-azide **4** for 24 h at 37 °C. The fluorescence of both prodrugs decreased to a comparable level to that of the free doxorubicin, hence demonstrating restoration of the doxorubicin's DNA intercalation activity upon activation by the PEG-activators **(Fig. 5).**

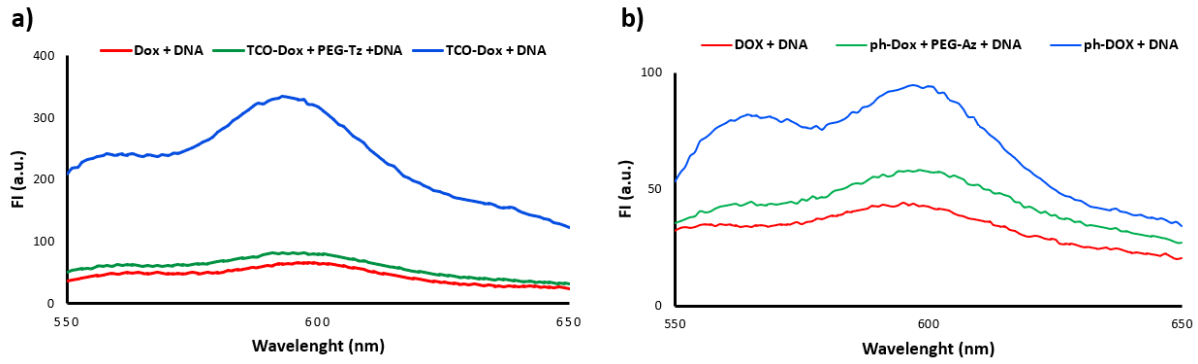


Figure 5. Fluorescence spectra of DNA solution incubated with a) Dox **12**, TCO-Dox **16** and TCO-Dox **16** + PEG-Tz **3**. B) Dox **12**, triphenylphosphine-Dox **13** and triphenylphosphine-Dox **13** + PEG-N_3 **4**.

2.4. Serum stability of PEG-Tz

Since Tzs are reported to have limited stability *in vivo,* [15,47,48] the stability of PEG-Tz **3** under physiologically relevant conditions was tested. PEG-Tz **3** was incubated with 10% BSA in PBS and in mouse serum at 37 °C and the amount of the remaining intact Tz was measured by HPLC over 24 h.[10,39,49] PEG-Tz **3** showed excellent stability in 10% BSA in PBS (99 ± 0.4% remaining intact after 24 h incubation) and good stability in mouse serum (56 ± 3.6% remaining intact after 24 h incubation) **(Fig. 6)**. Overall, this suggests that conjugation of Tz to the 10 KDa PEG polymer improved its serum stability in comparison with other reported Tz activators that completely lose their stability in serum over the same period of time.[49]

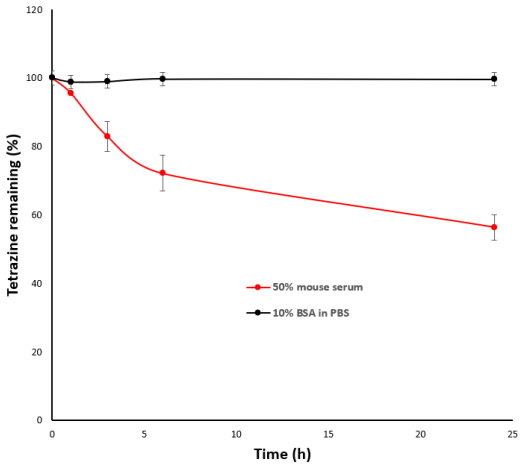


Figure 6. Stability study of PEG-Tz **3** in 10% BSA in PBS and in 50% mouse serum/PBS at 37 °C monitored over 24 h at λ=520 nm. Data represented as mean ± SEM (n=3).

3. Conclusion

A bioorthogonal prodrug activation strategy has been developed for breast cancer using PEG-based activators. Two triphenylphosphine-prodrugs and two TCO-prodrugs containing the N-mustard and doxorubicin cytotoxic moieties were also developed. HPLC-monitored release studies demonstrated that activation of the prodrug was dependent on the molecular weight of the PEG-activator and was slower for the Staudinger ligation compared with the Tz ligation (24 h versus 20 min for complete release). The feasibility of using PEG-activators for prodrug activation was further validated in vitro in MCF-7 and MDA-MB-231 breast cancer cell lines. Very good levels of drug activity were restored upon incubation of PEG-azide with the triphenylphosphine-prodrugs (~68-76% restoration of cytotoxicity) and full restoration of activity was achieved upon the incubation of PEG-Tz with the TCO-prodrugs (100% restoration of cytotoxicity). These results suggest the feasibility of using PEG-based activators for bioorthogonal prodrug activation in breast cancer. Their size (10 KDa) renders them suitable for further in vivo application for selective cancer targeting through accumulation in solid tumours via the EPR effect and passive targeting.

4. Experimental section

4.1. Materials

All chemicals and solvents were purchased from Sigma Aldrich, UK unless otherwise specified. MeO-PEG-NHS (MW 11153 Da) was purchased from Iris Biotech, Germany. (4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine.HCl was purchased from BLD Pharm, Germany. Compound **6** was purified by flash column chromatography using Silica gel 60 (particle size 40-63 µm). ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded in deuterated chloroform ($CDCl_3$) using a Bruker DPX 400 (400 MHz) spectrometer. Human breast adenocarcinoma epithelial cell line (MCF-7), the triple negative breast

cancer cell line (MDA-MB-231), and mouse fibroblasts (L929) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Fetal bovine serum (FBS) and trypsin were purchased from Gibco, UK.

4.2. Chemical synthesis

Compounds **8**, **9**, **10**, **11** and **13** were prepared and characterised according to previously reported procedures.[11] TCO-*N*-mustard prodrug **15** and TCO-doxorubicin prodrug **16** were prepared according to procedures described **Chapter 4 scheme 3a,b**.

MeO-PEG-tetrazine (3) (4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine hydrochloride (20 mg, 0.08 mmol, 4 eq.) was dissolved in anhydrous chloroform (4 mL). To the solution, *N,N*-diisopropyl-ethylamine (DIPEA) (29 µL, 168.29 µmol, 10 eq.) was added. MeO-PEG-NHS (MW 11153, 200 mg, 17.93 µmol, 1 eq.) was then added in portions. The reaction was stirred for 3 days, under an inert atmosphere and protected from light. The reaction mixture was condensed by evaporation under vacuum before it was poured dropwise on ice-cooled diethyl ether (30 mL). The precipitate was left on ice for 1 h and then it was filtered to obtain a pink product. The product was left to dry under vacuum. Then, the pink product was dissolved in deionised H_2O (~15 mL) and dialysed against deionised H_2O (5 L) using regenerated cellulose membrane (MWCO 3500 Da). The H_2O was changed 3 times over 24 h. The solution was then collected and freeze-dried to yield the MeO-PEG-tetrazine **3** as a pink powder (150 mg, 74%). The amount of free tetrazine (< 0.2%) and the content of tetrazine (1.78% wt/wt) were determined using RP-HPLC and ^{1}H NMR, respectively. **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 2.57 – 2.74 (4H, m, CO-C**H$_2$**-C**H$_2$**-CO), 3.09 (3H, s, C**H$_3$**), 3.38 (3H, s, OC**H$_3$**), 3.42 - 3.72 (441H, m, PEG unit), 3.78 – 3.86 (4H, m, C**H$_2$**-C**H$_2$**-OCH_3), 4.54 (2H, d, *J* = 6.0 Hz, C**H$_2$**-benzyl), 7.50 (2H, d, *J* = 8.5 Hz, Ar-H), 8.54 (2H, d, *J* = 8.4 Hz, Ar-H). **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 21.14 (**C**H_3), 31.59 (CO-**C**H_2-CH_2-CO), 31.84 (CO-CH_2-**C**H_2-CO), 39.34 (PEG unit), 43.11 (**C**H_2-benzyl), 59.02 (O**C**H_3), 69.69 (**C**H_2-CH_2-OCH_3), 70.54 (PEG unit), 71.91 (**C**H_2-OCH_3), 128.12 (Ar-CH), 128.31 (Ar-CH), 130.65 (Ar-C), 143.68 (Ar-C), 167.19 (Ar-C), 172.36 (C=O), 172.53 (C=O).

4-Nitrophenyl 2-(diphenylphosphanyl) benzoate (6)[9] 2-Diphenylphosphanyl benzoic acid **5** (0.37 g, 1.20 mmol, 1eq.) was dissolved in anhydrous DCM (50 mL) under an inert atmosphere. DCC (0.25 g, 1.20 mmol, 1 eq.) and DMAP (0.07 g, 0.060 mmol, 0.5 eq.) were added to the reaction mixture which was kept under an inert atmosphere. The reaction mixture was stirred at room temperature for 30 min and then 4-nitrophenol (0.15 g, 1.20 mmol, 1 eq.) was added. The mixture was stirred overnight at room temperature. The solvent was removed under vacuum and ice-cold acetone was added to the residue to precipitate the urea by-product to be removed by filtration. After removal of acetone under vacuum, the crude product was purified using flash column chromatography (hexane/ethyl acetate,

9:1 v/v) to yield the 4-nitrophenyl 2-(diphenylphosphanyl) benzoate **6** as a pale yellow solid (0.16 g, 38%). m.p. 125-126 °C. **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 6.98-7.05 (1H, m, Ar-H), 7.06-7.13 (2H, m, 4-nitrophenyl ring Ar-H), 7.24-7.40 (10H, m, diphenyl rings Ar-H), 7.44-7. 54 (2H, m, Ar-H), 8.17-8.22 (2H, m, 4-nitrophenyl ring Ar-H), 8.22-8.28 (1H, m, Ar-H). **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 122.49 (Ar-CH), 125.09 (Ar-CH), 128.44 (Ar-CH), 128.65 (Ar-CH), 128.72 (Ar-CH), 129.00 (Ar-CH), 131.51 (Ar-CH), 133.07 (Ar-CH), 133.95 (Ar-CH), 134.16 (Ar-CH), 134.59 (Ar-CP), 137.15 (Ar-CP), 137.32 (Ar-C-NO_2), 145.32 (Ar C-O), 155.28 (C=O). **^{31}P NMR** ($CDCl_3$, 162 MHz) δ -3.63. **IR ν_{max}** [cm^{-1}] (powder) 3042 (C=C-H), 1737 (C=O). ***m/z* (FTMS+ESI)** M^{+1} ($C_{25}H_{19}NO_4P$) requires 428.1046. Found 428.1043. HPLC analysis: CH_3CN (1% CH_3COOH) – H_2O (70:30 v/v), 99.05% purity.

4.3. HPLC release studies

Release reaction studies were measured by RP-HPLC, Hewlett-Packed Series 1100 system, with an ACE C18 reverse phase column (250 × 4.6 mm, 5 µm particle size, 300 Å pore size). UV-Vis spectrophotometers (Cary 300 Bio UV-Visible spectrophotometer and Jenway-7315 spectrophotometer) were used to record ultraviolet absorbance of the target compounds.

4-Nitrophenyl 2-(diphenylphosphanyl)benzoate **6** (0.043 mg/mL) (0.1 mM) in 3 mL of CH_3CN/H_2O (1:1 v/v) was reacted with PEG-azide 10 KDa or 20 KDa or benzyl azide (0.2 mM) at 37 °C and 4-nitrophenyl carbonate TCO **14** (0.03 mg/mL) (0.1 mM) in 3 mL of CH_3CN/H_2O (1:1 v/v) was reacted with PEG-Tz **3** (0.2 mM) at 37 °C. At different time intervals, samples of 25 µL were withdrawn and analysed by HPLC. The flow rate was 1 mL/min, the mobile phase was 70% CH_3CN (1% CH_3COOH in CH_3CN) and 30% H_2O (1% CH_3COOH in H_2O) over 20 min in case of **6** with azide activators and the mobile phase was 20% of CH_3CN increasing to 70% over 25 min, returning to 20% for 5 min in the case of **14** with **3**, UV detector at λ =310 nm and 254 nm together. The release profile curve for 4-nitrophenol was produced by converting AUC to concentration using the calibration curve equation. The calibration curve was obtained through preparation of a stock solution of 4-nitrophenol (1 mg/mL) in CH_3CN: H_2O (1:1 v/v) and was used to prepare a range of concentrations 0.01-1 mg/mL.

Triphenylphosphine-Dox prodrug **13** (0. 08 mg/mL) (0.1 mM) in 3 mL of CH_3CN/H_2O (1:1 v/v) was reacted with PEG-azide 10 KDa **4** (0.2 mM) at 37 °C and TCO-Dox prodrug **16** (0.07 mg/mL) (0.1 mM) in 3 mL of CH_3CN/H_2O (1:1 v/v) was reacted with PEG-Tz **3** (0.2 mM) at 37C°. At different time intervals, samples of 25 µL were withdrawn and analysed by HPLC by a gradient elution method using an aqueous gradient in CH_3CN. The flow rate was 1 mL/min, the mobile phase was 20% of CH_3CN increasing to 70% over 25 min, returning to 20% for 5 min (UV detector at λ =233 nm). The release profile curve for doxorubicin was produced by converting AUC to concentration using the calibration curve equation. The calibration curve was obtained through preparation of a stock solution of

doxorubicin (1 mg/mL) in CH_3CN: H_2O (1:1 v/v) and was used to prepare a range of concentrations 0.01-0.5 mg/mL.

4.4. *In vitro* prodrug activation

MCF-7 cells were cultured in RPMI-1640 medium supplemented with 5% FBS. MDA-MB-231 and mouse fibroblasts were cultured in Dulbecco's modified eagle's medium (1g/L glucose and 5g/L, respectively with L-glutamine) supplemented with 10% FBS.

The *in vitro* prodrug activation and antiproliferative activity of compounds **11, 13, 15** and **16** were determined using the MTT assay. MCF-7 cells or MDA-MB-231 cells were seeded on 96-well plates (4×10^4 cells/mL) and (2×10^4 cells/mL), respectively and incubated at 37 °C for 24 h. Cells were then treated with a range of concentration of the prodrugs **11, 13, 15** and **16** (0.001-10 µM) and respective two equivalents range of concentration of the PEG activators **3** and **4** (0.002-20 µM) and incubated for 67 h. After incubation, 20 µL of MTT in PBS solution (0.5 mg/mL) were added in each well and further incubated for 5 h. The resulting formazan crystals were then dissolved in DMSO (100 µL) and incubated for 30 min after carefully removing the treatment-containing media and MTT solution. The absorbance was recorded at 570 nm by microplate reader (infiniteF50 TECAN). The cells without treatment were used as the control. Assays were performed in three replicates, and the statistical mean and standard error of mean were used to estimate the cell viability. IC_{50} (inhibitory concentration to induce 50% cell death) values were determined using GraphPad Prism 8.0.2 according to the fitted data.

4.5. Fluorescence spectra of drug-DNA complexes

A solution of TCO-Dox prodrug **16** was mixed with a solution of PEG-Tz **3** and used instantaneously. A solution of triphenylphosphine-Dox prodrug **13** was mixed with a solution of PEG-azide **4** and incubated for 24 h at 37°C. The molar ratio of prodrugs to PEG-activator was 1: 2 and the final concentration of the prodrugs was 10 µM. Subsequently, the mixture was added to an equal volume of DNA (deoxyribonucleic acid sodium salt from calf thymus) aqueous solution (1 mg/mL), and then the fluorescence spectrum of Dox was recorded (excitation wavelength was 488 nm and emission wavelength was 550–650 nm). Similarly, the Dox **12** or TCO-Dox **16** or triphenylphosphine-Dox **13** was added into DNA aqueous solution, and the fluorescence spectra were recorded.

4.6. Stability of PEG-Tz

The stability of PEG-Tz **3** was evaluated in 10% BSA in PBS and in 50% mouse serum/PBS over time. Stock solutions of **3** were prepared in PBS at a concentration of 5 mM of tetrazine according to the loading %, the stock solution was diluted to 0.5 mM in 50% mouse serum/PBS or 10% BSA in PBS. After incubation for various time points (0, 1, 3, 6 and 24 h), the samples were mixed with 250 µL of cold CH_3CN for extraction. The samples were then centrifuged at 1560 g for 5 min and the clear supernatant was analysed by HPLC at λ=520 nm (n=3).

4.7. Statistical analysis

Data were presented as mean ± standard error of mean. Statistical analysis was carried out for active Dox **12** against Dox prodrugs **13** and **16** and Dox prodrugs **13** and **16** after addition of PEG-azide **4** and PEG-Tz **3**, respectively on MCF-7 cells and MDA-MB-231 cells, and for *N*-mustard prodrugs **11** and **15** against *N*-mustard prodrugs **11** and **15** after addition of PEG-azide **4** and PEG-Tz **3**, respectively on MCF-7 cells and MDA-MB-231 cells by one-way ANOVA followed by Bonferroni *post hoc* test using GraphPad Prism 8.0.2 software and statistical significance was set at $p < 0.05$ (specifically, * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$).

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Supporting Information

(To be published with the article)

Polymeric PEG-based bioorthogonal triggers for prodrug activation in breast cancer

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Table of contents

IC_{50} of Dox prodrugs 13 and 16 and *N*-mustard prodrugs 11 and 15 against MCF-7 cells and MDA-MB-231 cells……. **187**

IC_{50} of Dox prodrugs 13 and 16 and *N*-mustard prodrugs 11 and 15 against L929 cells… **188**

^{1}H, ^{13}C, and ^{31}P NMR spectra and mass spectra…… **189**

HPLC chromatographs of 4-nitrophenol and Dox release profiles……. **192**

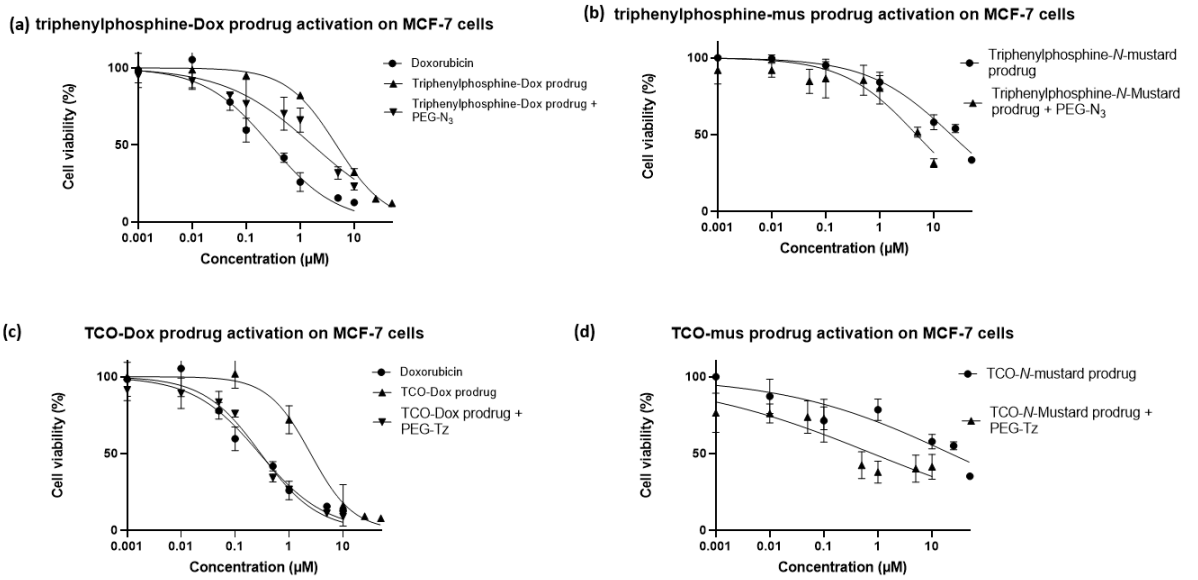


Figure *S*1: a) Cytotoxicity profile of Dox **12**, triphenylphosphine-Dox prodrug **13** and prodrug **13** after activation by PEG-azide **4** against MCF-7 cells. b) Cytotoxicity profile of triphenylphosphine-*N*-mustard prodrug **11** and prodrug **11** after activation by PEG-azide **4** against MCF-7 cells. c) Cytotoxicity profile of Dox **12,** TCO-Dox prodrug **16** and prodrug **16** after activation by PEG-Tz **3** against MCF-7 cells. d) Cytotoxicity profile of TCO-*N*-mustard prodrug **15** and prodrug **15** after activation by PEG-Tz **3** against MCF-7 cells. Data are presented as mean ± SEM (n=3).

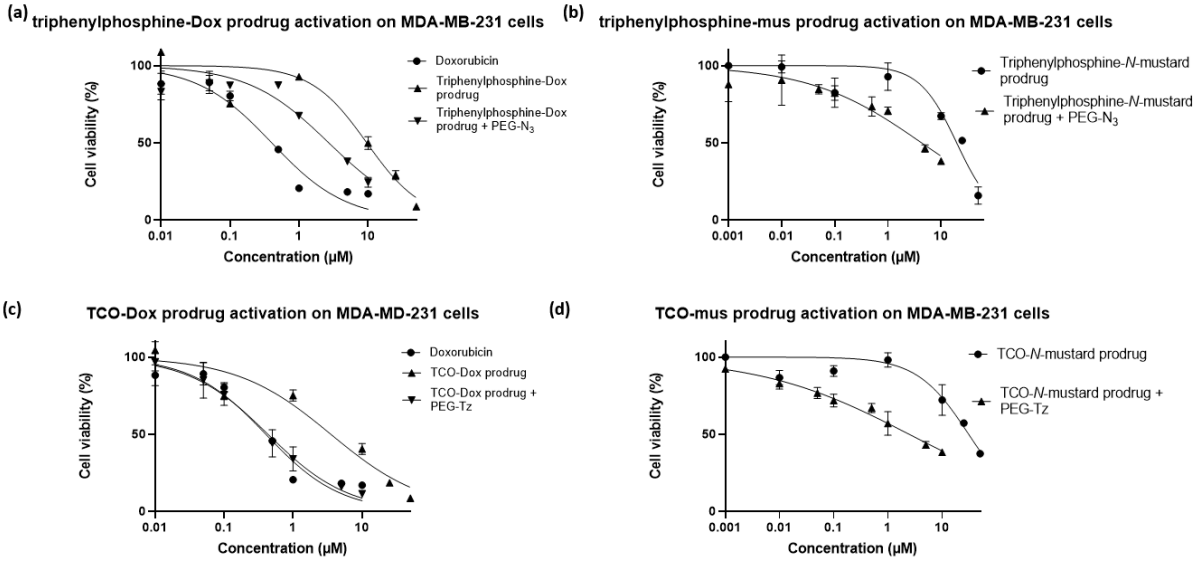


Figure *S*2: a) Cytotoxicity profile of Dox **12**, triphenylphosphine-Dox prodrug **13** and prodrug **13** after activation by PEG-azide **4** against MDA-MB-231 cells. b) Cytotoxicity profile of triphenylphosphine-*N*-mustard prodrug **11** and prodrug **11** after activation by PEG-azide **4** against MDA-MB-231 cells. c) Cytotoxicity profile of Dox **12,** TCO-Dox prodrug **16** and prodrug **16** after activation by PEG-Tz **3** against MDA-MB-231 cells. d) Cytotoxicity profile of TCO-*N*-mustard prodrug **15** and prodrug **15** after activation by PEG-Tz **3** against MDA-MB-231 cells. Data are presented as mean ± SEM (n=3).

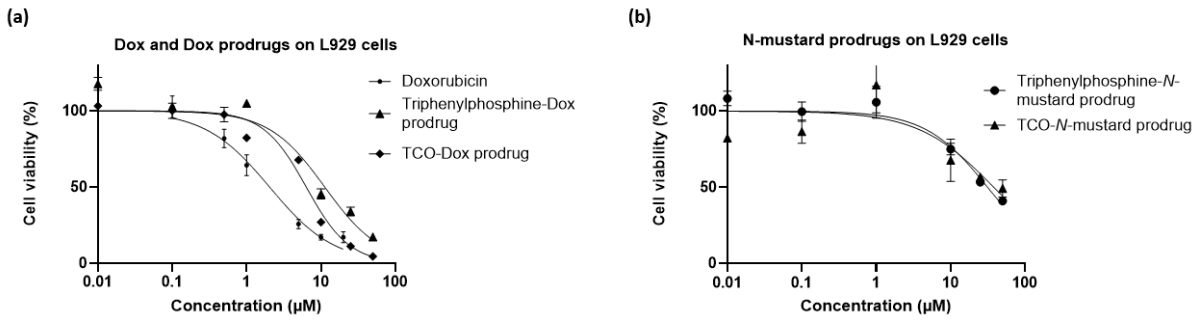
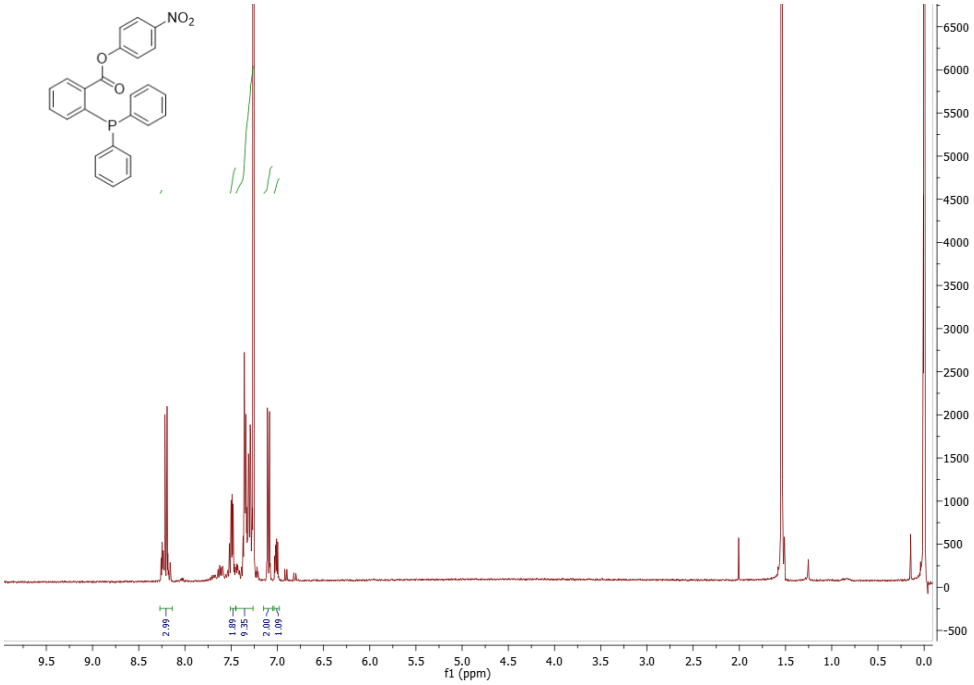
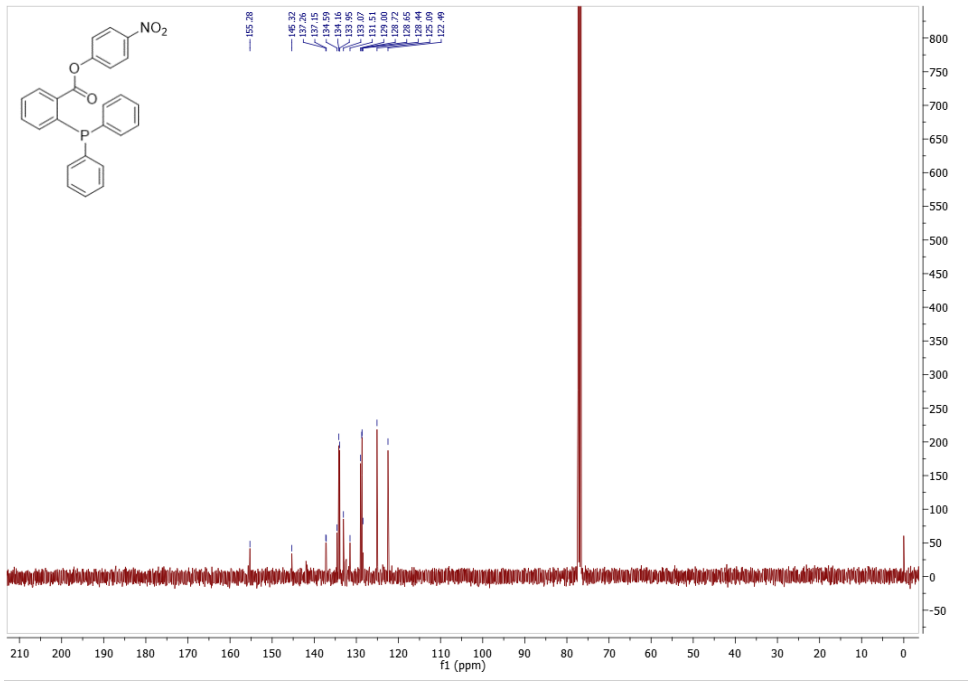


Figure *S*3: a) Cytotoxicity profile of Dox **12**, triphenylphosphine-Dox prodrug **13** and TCO-Dox prodrug **16** against L929 cells. b) Cytotoxicity profile of triphenylphosphine-*N*-mustard prodrug **11** and TCO-*N*-mustard prodrug **15** against L929 cells. Data are presented as mean ± SEM (n=3).

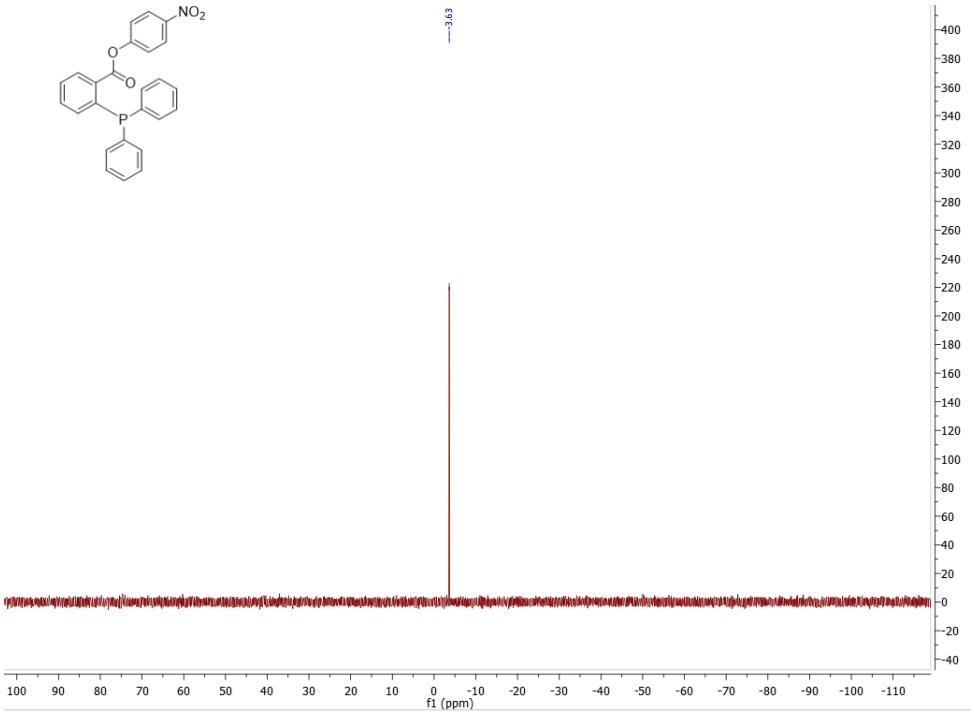
^{1}H and ^{13}C NMR Spectra



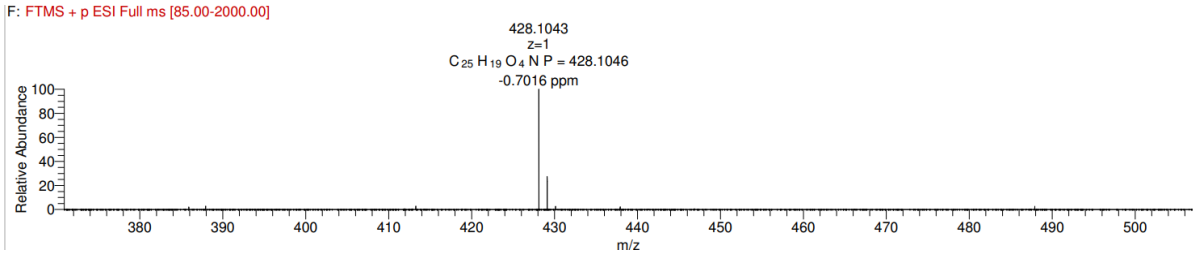
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **6**



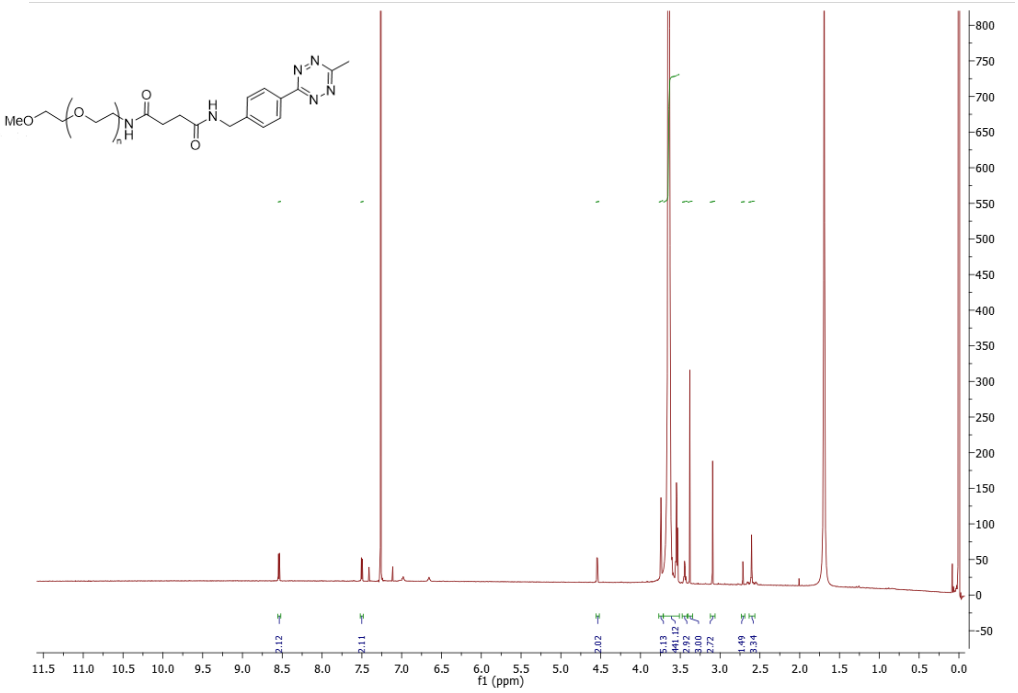
^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound **6**



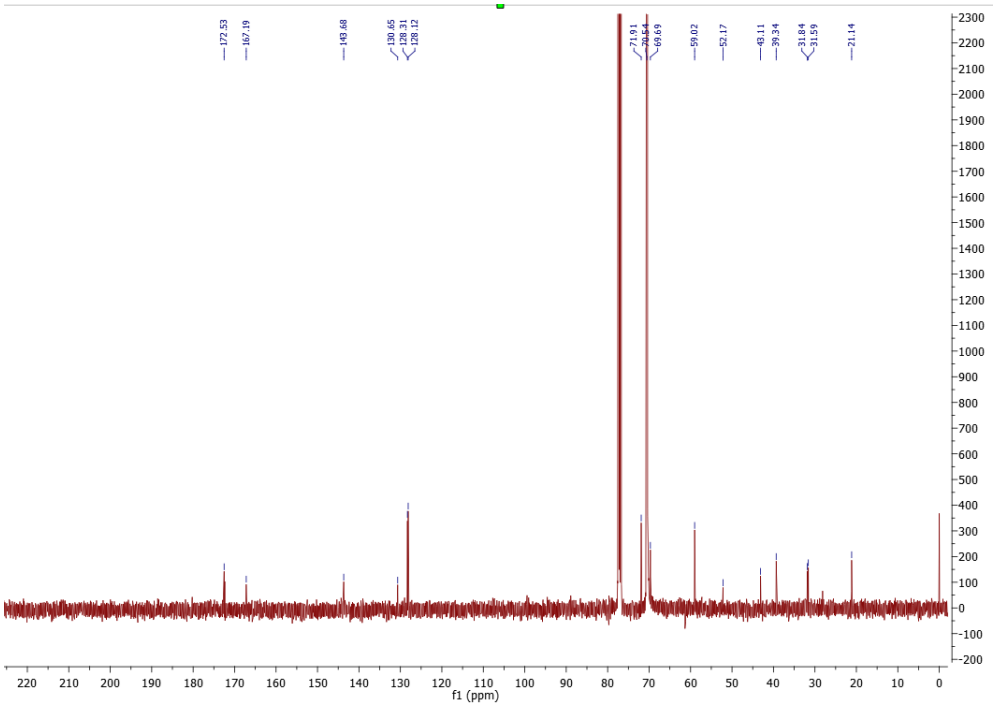
^{31}P NMR spectrum (162 MHz, $CDCl_3$) of compound **6**



Mass spectrum of compound **6**

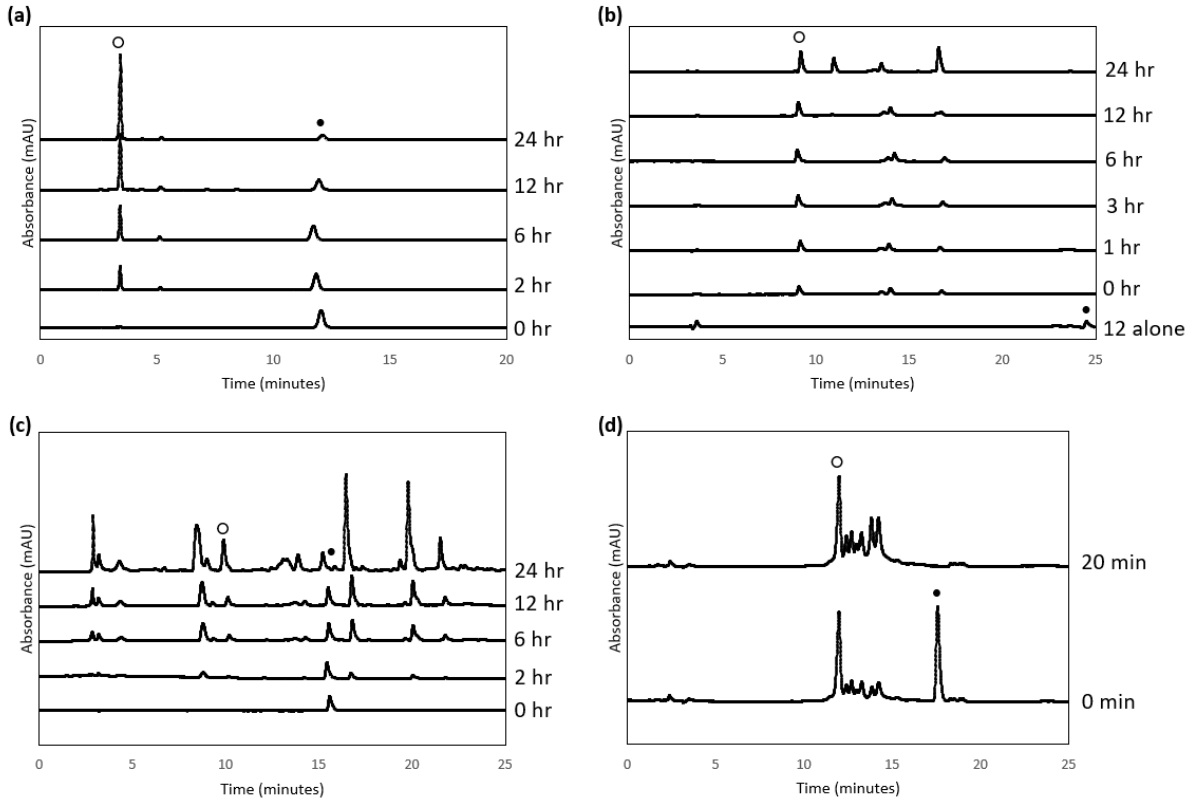


^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **3**



^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound **3**

HPLC chromatograms of release profile of 4-nitrophenol and Doxorubicin



a) HPLC chromatograph of 4-nitrophenol release from triphenylphosphine ester model prodrug **6** by PEG-azide (10 KDa) **4** at 37 °C in aqueous CH_3CN (1:1 v/v) as a function of time. Legend: •: triphenylphosphine ester model prodrug **6**; ○: 4-nitrophenol.

b) HPLC chromatograph of 4-nitrophenol release from TCO carbonate model prodrug **14** by PEG-Tz (10 KDa) **3** at 37 °C in aqueous CH_3CN (1:1 v/v) as a function of time. Legend: •: TCO carbonate model prodrug **14**; ○: 4-nitrophenol.

c) HPLC chromatograph of doxorubicin **12** release from triphenylphosphine-Dox prodrug **13** by PEG-azide (10 KDa) **4** at 37 °C in aqueous CH_3CN (1:1 v/v) as a function of time. Legend: •: triphenylphosphine-Dox prodrug **13**; ○: doxorubicin **12**.

d) HPLC chromatograph of doxorubicin **12** release from TCO-Dox prodrug **16** by PEG-Tz **3** at 37 °C in aqueous CH_3CN (1:1 v/v) as a function of time. Legend: •: TCO-Dox prodrug **16**; ○: doxorubicin **12**

Appendix: *In vitro* wound-healing scratch assay to test the effect of the activated prodrug on the migration of MDA-MB-231 cells.

Introduction

Cancer cell migration is essential for tumour invasion and accounts for the spread from primary to secondary sites *via* metastases.[1] Indeed breast cancer-related deaths are mainly due to its metastases to secondary sites (most commonly to the bone, lung, brain, and liver).[2] Many studies have used the *in vitro* wound closure scratch assay to demonstrate the ability of doxorubicin and *N*-mustard to decrease the migration of breast cancer cells, and thus inhibit metastases.[3–6] In this context, the ability of our synthesised TCO-prodrugs (i.e. TCO-doxorubicin and TCO-*N*-mustard) to regain their anti-migratory effect after activation by PEG-Tz activator was evaluated on MDA-MB-231 breast cancer cells (as a model for metastatic breast cancer) by an *in vitro* 24 h scratch assay. This was performed as an additional assay to confirm the feasibility of the PEG-Tz to activate the TCO-prodrugs and restore their biological activity.

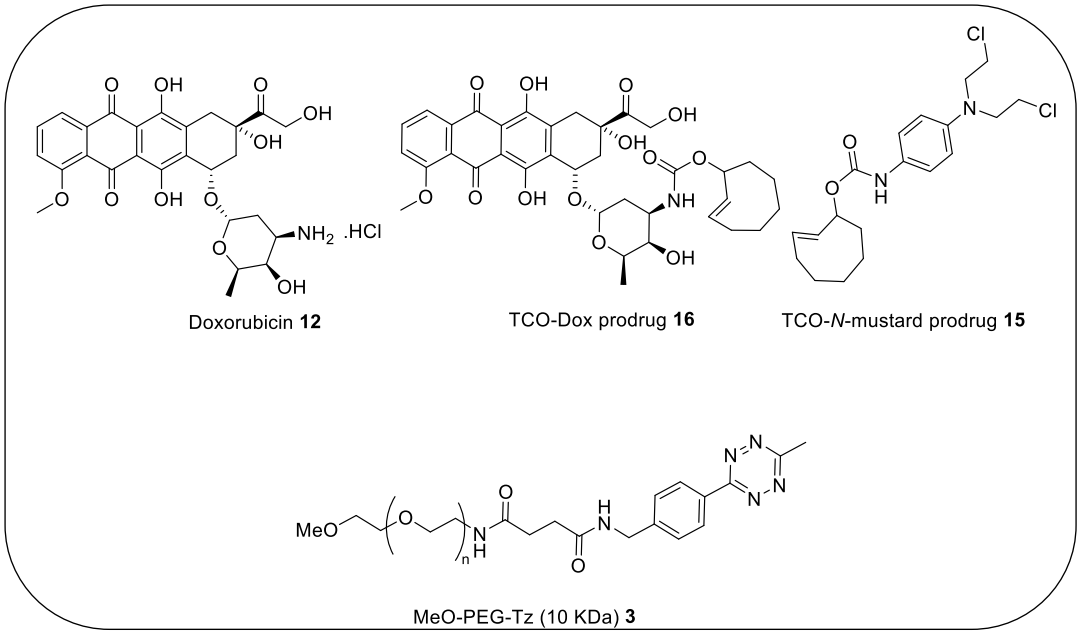


Figure 1. Chemical structures of doxorubicin, TCO-prodrugs, and PEG-Tz activator used in this study.

Results

Sub-cytotoxic concentrations (0.1 µM) of doxorubicin, TCO-doxorubicin prodrug, and TCO-*N*-mustard prodrug were used in the scratch assay as these concentrations did not significantly affect the viability of the MDA-MB-231 cells (> 80 % cell viability) as shown by the MTT cell viability assay described previously in this chapter (**Chapter 5, supp data Fig. *s*2c, *s*2d**, also, see below **Fig. 2a**). For the PEG-Tz activator, no change in cell viability was observed up to 100 µM concentration. A concentration of 0.2 µM (i.e. 2 equivalents compared to the prodrug) was therefore considered appropriate for activation of the prodrugs in the scratch assay without causing any cytotoxic effects.

At the concentrations stated above, doxorubicin inhibited the migration by 67 ± 4.4% (relative to control, $^{**}p < 0.01$) on MDA-MB-231 breast cancer cells, while the TCO-doxorubicin prodrug did not show any significant migration inhibition (88 ± 3.7% relative to control, $p > 0.05$) **(Fig. 2b)**. This supports our hypothesis that converting the active drug to a TCO prodrug masks its initial activity. The migration inhibition of the TCO-doxorubicin prodrug after the addition of the PEG-Tz activator was found to be 70 ± 2.3% (relative to control, $^{**}p < 0.01$) which confirms activation of the prodrug activation and restoration of doxorubicin's anti-migration activity **(Fig. 2b)**. As noted earlier, it was not possible to determine the *in vitro* profile of the *N*-mustard drug due to its instability. The TCO-*N*-mustard prodrug did not show any significant migration inhibition (81 ± 7.7% relative to control, $p > 0.05$) and this changed to 66 ± 3.7% (relative to control, $^{**}p < 0.01$) after the addition of the PEG-Tz activator **(Fig. 2b)** which indicates the prodrug activation.

The PEG-Tz activator and the native unconjugated PEG did not show any significant migration inhibition (79 ± 4.2% and 85 ± 10.7%, respectively relative to control, $p > 0.05$) **(Fig. 2b)** which also suggests that the Tz moiety does not have an anti-migration activity alone as a bioorthogonal activator.

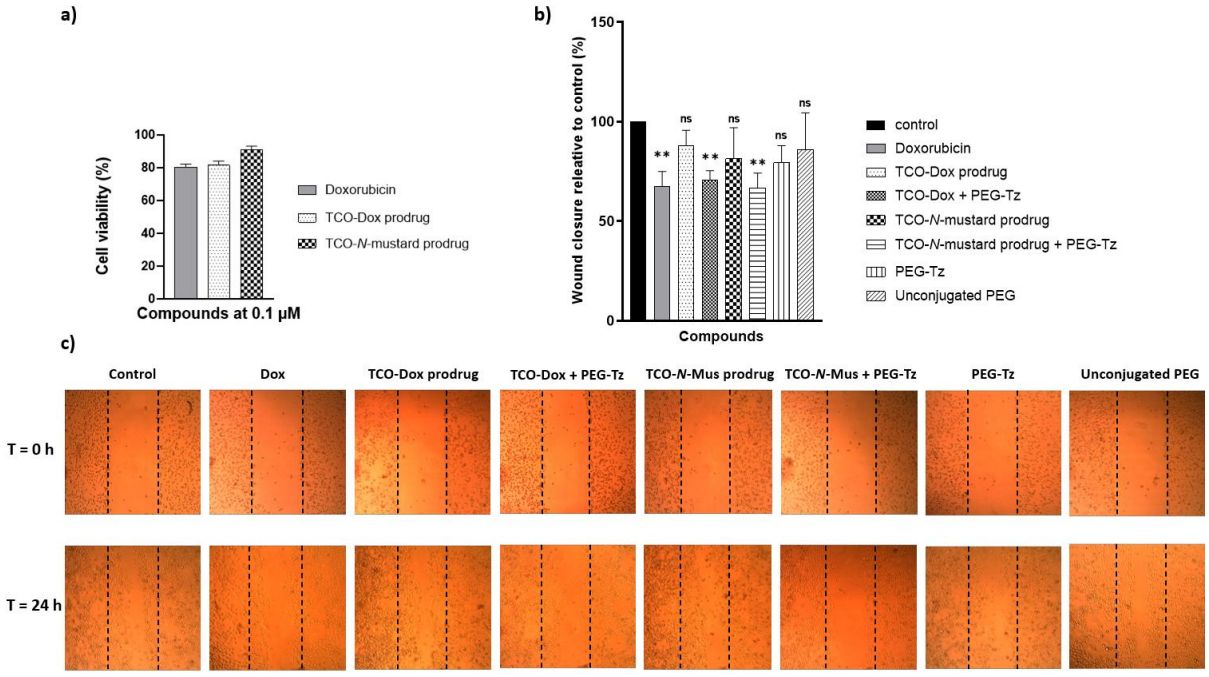


Figure 2. Biocompatibility and *in vitro* wound closure (migration) inhibition activity on MDA-MB-231 cells. (a) Cell viability of MDA-MB-231 cells in the presence of 0.1 µM of tested compounds, data represented as mean ± SEM, n=3. (b) Wound closure percentage relative to control at T = 24 h post treatments, data represented as mean ± SEM, n=3. Statistical significance was estimated between control and all groups by one-way ANOVA followed by Bonferroni's *post hoc* test using GraphPad Prism 8.0.2 software ($^{**}p < 0.01$, ns= non-significant $p > 0.05$). (c) Representative images of the scratch and the cell migration of MDA-MB-231 cells at 0 h and 24 h post treatment at 4x magnification, images were analyzed using Image*J* software.

Discussion

A sub-cytotoxic concentration (0.1 μM) was chosen for this assay to ensure that any anti-migration effects observed on the MDA-MB-231 cells are not due to cytotoxicity. This concentration aligns with literature data for assessing the inhibition of migration through a wound healing assay on MDA-MB-231 cells.[4,6]

The results above demonstrate that the anti-migratory properties of the free drugs can be removed upon formation of prodrugs, as required. They also demonstrate the feasibility of using the PEG-Tz activator to restore the original anti-migration activity of the parent drugs.

However, in this study, only one concentration of the prodrugs (0.1 μM) and only one size of the PEG-Tz activator (10 KDa) were used. Further studies are required using different sub-cytotoxic concentrations of the prodrugs to determine if the anti-migration activity would be concentration dependant or not. This would also determine if the PEG size would affect the prodrug activation and/or the anti-migration of the PEG-Tz activator.

Conclusion

Converting doxorubicin and *N*-mustard chemotherapies into TCO-prodrugs decreased their anti-migratory activity on MDA-MB-231 breast cancer cells, which was restored upon activation of the prodrugs by the PEG-Tz *via* tetrazine ligation bioorthogonal reaction. Further studies using a sub-cytotoxic concentration range of compounds, different PEG-Tz molecular weights, and other cell migration assays (e.g. cell exclusion zone/barrier assay) are required to confirm the potential of this prodrug activation approach for inhibition of the migration of breast cancer cells and thereby decrease metastases.

Experimental

In vitro cell migration assay (wound-healing/scratch assay)

MDA-MB-231 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (1g/L glucose, with L-glutamine, Gibco™, UK) supplemented with 10 % FBS (Gibco™, UK). The cells were seeded in 12-well plates at 1 x 10^5 cells/well (1 mL/well) and incubated at 37 °C until 70 - 80% confluency (48 h). Then, the media was aspirated and replaced with serum starvation media (0% FBS in DMEM) for 24 h to inactivate proliferation. Afterwards, using a 200 μL pipette tip, a scratch was performed vertically on the cells monolayer. Cells were washed gently with PBS twice and treated with 1 mL of medium containing either doxorubicin (0.1 μM) or PEG-Tz (10 KDa) (0.2 μM) or TCO-doxorubicin prodrug (0.1 μM) or TCO-*N*-mustard prodrug (0.1 μM) or free PEG (0.2 μM) or TCO-doxorubicin prodrug (0.1 μM) + PEG-Tz (0.2 μM) or TCO-*N*-mustard prodrug (0.1 μM) + PEG-Tz (0.2 μM). Images of the scratch were taken immediately (T = 0 h), then the cells were incubated and at the standard culture conditions

before taking the images at 24 h post scratch (T = 24 h). The assay was performed in triplicate. The area of the scratch not covered by the cells was quantified using Image*J* software. % of wound closure was quantified according to the following equation:

$$100 \times (\text{Area of scratch at } T_0 - \text{Area of scratch at } T_{24}) / \text{Area of scratch at } T_0$$

The wound closure percentage data for the treatments were presented relative to the control.

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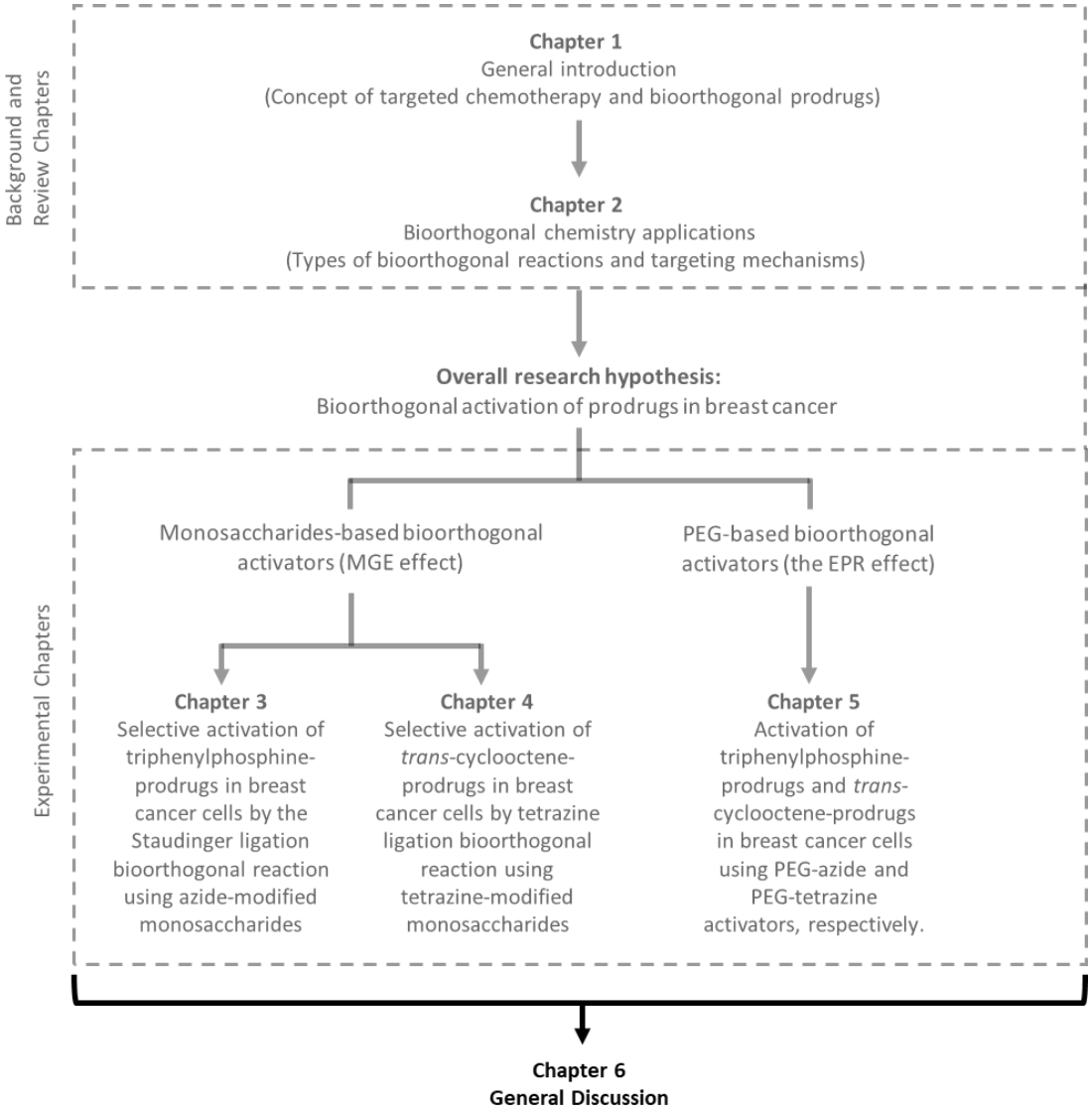
Chapter 6

General discussion

Chapter summary: *This chapter summarises the key findings and provides a critical evaluation of the work presented in this thesis. Also, potential areas for future research are suggested.*



6.1. Introduction

Bioorthogonal chemistry has become an important tool in the field of biomedical science since it was first introduced by Carolyn Bertozzi in 2003.[1] Bertozzi was very recently awarded the Nobel Prize in Chemistry (2022) jointly with Morten Meldal and K. Barry Sharpless "for the development of click chemistry and bioorthogonal chemistry".[1,2] The vast majority of applications for bioorthogonal reactions have sought to improve cancer diagnosis and treatment.[3] However, the translation of these strategies from bench to clinical settings is very challenging, with only one biorthogonal-based treatment strategy currently undergoing clinical trials (the CAPAC™ platform).[4,5]

In this chapter, a general discussion of the work demonstrated in this thesis is presented. In section **6.2.** the key findings of each chapter are summarised, in section **6.3.** a critical evaluation of the research findings is outlined, and finally, in section **6.4.** potential future areas for investigation are proposed.

6.2. Key findings of the current work

The overarching aim of this work was to improve the selectivity of chemotherapeutic approaches for treating breast cancer. The devised strategy consisted of two main components, specifically: a) prodrugs with a functional group that can be activated to effect drug release *via* a bioorthogonal reaction; b) activators that localise the required complementary bioorthogonal functional group at the tumour *via* MGE (**Chapter 3** and **4**) or the EPR effect (**Chapter 5**). Breast cancer was chosen as a relevant model, and MGE, and passive targeting by the EPR effect, were selected as breast cancer-targeting mechanisms due to their suitability for targeting solid tumours. By virtue of the aim and objectives, a comprehensive body of synthetic work was required for the design and synthesis of the prodrugs, functionalised monosaccharides, and PEG derivatives.

Bioorthogonal reactions and their in vivo applications (chapter 2) [3]
This chapter critically appraised the *in vivo* applications of bioorthogonal reactions to identify the current progress and challenges when translating bioorthogonal reactions to *in vivo* and consequently clinical settings. When choosing a bioorthogonal reaction for a therapeutic or diagnostic application, it is crucial to consider the reaction type and the targeting mechanism applied. High selectivity and fast kinetics of the bioorthogonal reaction under physiological conditions are key parameters to consider when using bioorthogonal reactions *in vivo*. The complexity of chemically modifying the targeting moieties to incorporate bioorthogonal components, alongside the physiological stability and requirement for low toxicity of these components, have also been identified as key challenges for *in vivo* applications. The feasibility of using the Staudinger ligation and tetrazine ligation for prodrug activation was demonstrated with a focus on mechanistic detail. MGE was shown to be a facile

targeting mechanism utilising chemically-modified monosaccharides, and passive targeting was also shown to be the most convenient targeting mechanism for delivering bioorthogonal components in solid tumours which manifested in their incorporation in prodrug strategies herein.

Selective delivery of azide activators to breast cancer cells by MGE for activation of triphenylphosphine-prodrugs by the Staudinger ligation reaction (Chapter 3) [6]

A two-component bioorthogonal prodrug activation system was developed in which two novel triphenylphosphine-prodrugs, and two novel azide-modified sialic acid monosaccharides, were designed and synthesised through multistep synthesis. Attaching a triphenylphosphine moiety through an amide linkage to chemotherapies (i.e. *N*-mustard and doxorubicin) converted them into less cytotoxic prodrugs with their cytotoxicity on MCF-7 breast cancer cells and the non-cancerous L929 fibroblasts decreasing by 20 and 6 fold, respectively. 9-Azido-sialic acid and its 2,4,7,8 tetra-acetylated derivative Ac_4-9-azido sialic acid, along with the commercially available Ac_4ManNAz were used to deliver the azide activator to breast cancer cells through MGE. Western blot analysis and confocal microscopy imaging showed that the azido-sialic acid derivatives were 17-fold more selective towards the MCF-7 cells than the L929 cells, whereas the Ac_4ManNAz showed only 5-fold more selectivity towards the MCF-7 cells over the L929 cells. Upon addition of the triphenylphosphine-prodrugs to the azide-engineered MCF-7 cells, restoration of the parent drug's cytotoxicity was achieved (up to 100%). This work represents the first study to combine MGE, for selective delivery of azide activators to breast cancer cells, with the Staudinger ligation reaction, for activation of triphenylphosphine-prodrugs.

The multistep synthesis of urea- and carbamate-linked triphenylphosphine-prodrugs for activation by a different Staudinger-initiated activation mechanism was also investigated (detailed in appendix to **Chapter 3**). However, due to the instability of the intermediate compounds and reaction reagents, only one urea model prodrug bearing a 4-nitroaniline residue was successfully synthesised. An HPLC-monitored release study demonstrated a very slow release rate for this, suggesting this alternative prodrug activation mechanism was unsuitable for further progression.

Selective delivery of tetrazine activators to breast cancer cells by MGE for activation of TCO-prodrugs by the tetrazine ligation reaction (Chapter 4)

Previous studies have highlighted the excellent kinetics (k_2 up to 10^4 $M^{-1}s^{-1}$) for the click-and-release tetrazine ligation reaction for activation of TCO-prodrugs, making it the most used bioorthogonal reaction for prodrug activation.[7–11] The feasibility of using MGE as a new selective targeting mechanism to deliver the Tz activators to breast cancer cells was herein investigated. Three novel Tz-modified monosaccharides were designed and synthesised using multistep pathways, and they all showed high levels of selectivity towards the MCF-7 breast cancer cells (35-65 folds) over the non-

cancerous L929 fibroblasts. In addition, lower concentrations (10 and 20 µM compared to 50 µM that is usually used) of the chemically-modified monosaccharides proved effective for the MGE strategy. Two TCO prodrugs (including the novel TCO-*N*-mustard) were synthesised, and these showed activation and restoration of activity (up to 100%) on the Tz-engineered MCF-7 breast cancer cells. No activation occurred on L929 cells confirming the selectivity of the prodrug activation approach. A serum stability study for the Tz-modified monosaccharides over 24 hours revealed their stability (i.e. intact nature) for up to 4 h and complete loss of stability after 24 h. Serum stability study for the TCO-prodrugs revealed the good stability of the TCO-*N*-mustard prodrug for up to 12 h and the lower stability of the TCO-doxorubicin prodrug (up to 2 h and complete loss of stability after 6 h). However, further *in vivo* studies would be required to ensure that these time intervals are sufficient to deliver the Tz-modified monosaccharides to the breast cancer cells for MGE and to deliver the TCO-prodrugs for activation.

Evaluation of the feasibility of using polymeric PEG-based activators for bioorthogonal prodrug activation in breast cancer (Chapter 5)

Passive targeting has been reported to be highly impactful for delivering bioorthogonal components to tumour sites in bioorthogonal cancer-related applications (see **Chapter 2.5.** summary and outlook for more details). This chapter tested the feasibility of using PEG-based polymer activators for bioorthogonal activation of prodrugs in MCF-7 and MDA-MB-231 breast cancer cell lines. Two commercially available PEG-azide conjugates (10 and 20 KDa) and a novel synthesised PEG-Tz conjugate (10 KDa) were tested for their feasibility as bioorthogonal triggers for activation of the triphenylphosphine- and TCO-prodrugs. For the Staudinger ligation reaction, the size of the polymer highly affected the release rate where the 10 KDa derivative resulted in approximately 2-fold greater release of 4-nitrophenol (83% *versus* 44% release after 24 h) from a synthesised triphenylphosphine model ester prodrug. The 10 KDa PEG-azide trigger also affected complete release of doxorubicin from a triphenylphosphine-doxorubicin amide prodrug after 24 h suggesting that the amide linkage of the triphenylphosphine moiety is more effective for release than the ester linkage. For the tetrazine ligation reaction, the PEG-Tz (10 KDa) activator resulted in complete release of 4-nitrophenol from a TCO-model carbonate prodrug after 24 h. Indeed complete release of doxorubicin from the TCO-doxorubicin carbamate prodrug was observed after 20 min suggesting that the carbamate linkage is more effective than the carbonate for the release kinetics. These findings are in agreement with literature reports that the rates of bioorthogonal reactions can vary due to the size, electron density, and steric properties of the trigger along with the nature of the reaction solvent.[12,13]

Cytotoxicity studies on breast cancer cells (MCF-7 and MDA-MB-231) revealed the restoration of the parent drug's cytotoxicity after activation of the prodrug (up to 76% and 100% for Staudinger ligation- and tetrazine ligation-based release, respectively). Moreover, a DNA fluorescence assay showed

restoration of doxorubicin's DNA intercalation. A serum stability study revealed that PEG-Tz was stable in serum for up to 24 h which is higher than that observed with the Tz-modified monosaccharides, which could be attributed to the bigger size of the PEG-Tz. However, further *in vivo* studies are required to investigate the extent of the EPR effect to selectively deliver the bioorthogonal PEG activators to breast tumours *in vivo*.

6.3. Critical evaluation

6.3.1. Suitability of the Staudinger ligation and tetrazine ligation reactions for prodrug activation

On-demand activation of prodrugs requires the bio-reversible derivatisation of drugs to produce prodrug moieties that are stable but readily and selectively cleavable at the desired site of action.[14–16] Stability of the reaction parties and efficiency of reaction when they come into proximity are the critical requirements to develop *in vivo* candidates for on-demand prodrug activation strategies.[17–19] The kinetics and selectivity of the activation reactions are also important factors to consider and study. In this thesis, two bioorthogonal reactions, the Staudinger ligation reaction and the tetrazine ligation reaction, were studied in the context of those requirements.

Parameters affecting the Staudinger reaction-based prodrug activation

The Staudinger ligation reaction is mainly reported in the literature for ligation applications including peptide and protein synthesis, cell imaging, and studying cell surface glycans.[20–25] The high selectivity and recognition between azides and phosphine moieties that occur in mild aqueous conditions are the key factors for its advantageous ligation in biological systems.[26] However, very few attempts have been implemented to develop the Staudinger reaction for prodrug activation (summarised in **Fig. 1** and **table 1**).[6,27,28]

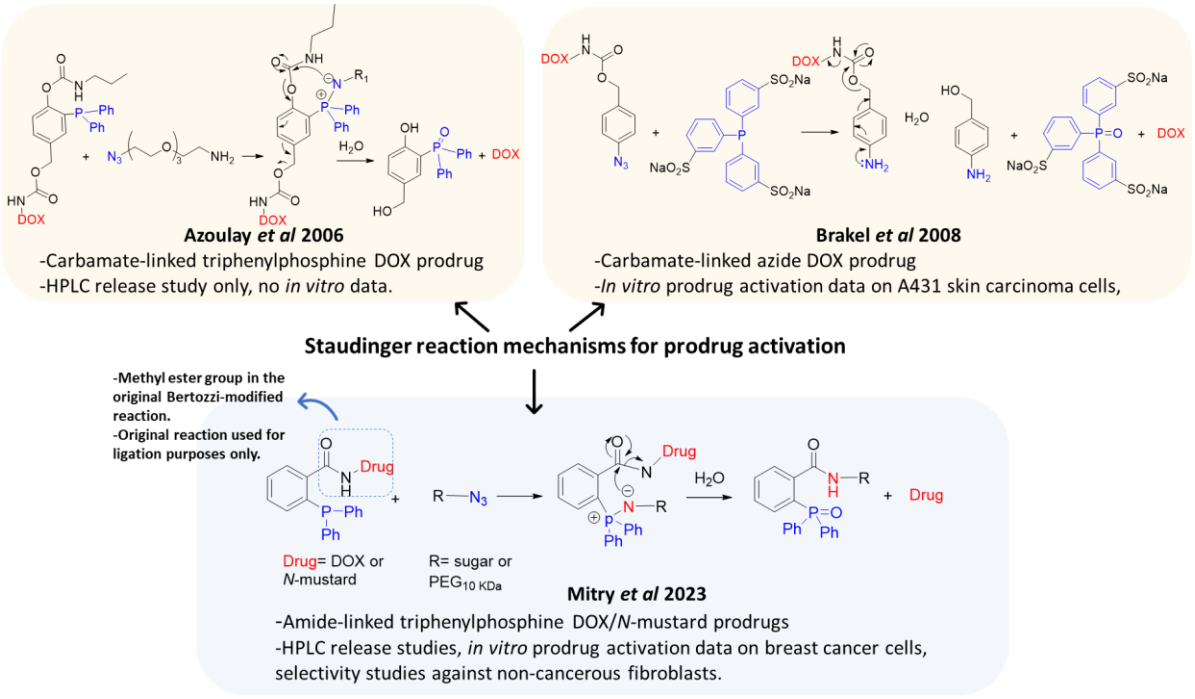


Figure 1. Staudinger reaction mechanisms for prodrug activation.

Table 1: Summary of parameters considered for Staudinger reaction-based prodrug activation

	***In vitro* activation**		**Stability**	**Engaged targeting approach**	**Key points in terms of tested parameters**
Azoulay *et al* [27]	**Activation**	**Concentrations used**	ND*	ND*	**Activation:** Very good activation level
	HPLC-monitored release studies showed 90% drug release within 3 h.	-Prodrug (5 mM or 2.8 mM) -Activator (10 mM or 5.2 mM)			**Concentration:** High concentrations of prodrug and activator are needed for sufficient release which limits its translation to cellular systems
					Selectivity: No targeting approach was tested meaning this could limit the selectivity of the approach and its suitability for *in vivo* application.
Brakel *et al* [28]	**Activation**	**Concentrations used**	Complete oxidation of the triphenylphosphine activator within 4 h hence requiring multiple dosages.	ND*	**Activation:** Very good activation level
	-HPLC-monitored release studies showed only 15% drug release within 4 h.	-Prodrug (10 µM) -Activator (20 µM)			**Concentration:** The high instability of the triphenylphosphine activator forced a multiple-daily dosing scheme with high concentrations. This challenges the further translation of this approach to *in vivo* settings.
	-*In vitro* cell proliferation studies on skin carcinoma cells showed full restoration of drug cytotoxicity.	5 doses (60 µM each) of the activator over 72 h.			**Selectivity:** No targeting approach was tested meaning this could limit the selectivity of the approach and its suitability for *in vivo* application.
Mitry *et al* chapter 3 [6] **And chapter 5**	**Activation**	**Concentrations used**	Stability of the triphenylphosphine-prodrugs in aqueous medium up to 48 h.	-MGE -Passive targeting by the EPR effect	**Activation:** Very good activation level over a long duration which would align with a sustained release approach for clinical application.
	-HPLC-monitored release studies showed 44 - 100% release according to the size of the azide activator within 24 h.	-Prodrug (100 µM) -Activator (200 µM)			**Concentration:** The stability of the triphenylphosphine prodrugs allows the use of micromolar concentrations which makes this approach suitable for progression to *in vivo* settings.
	-*In vitro* cell proliferation studies on breast cancer cells showed ~ 66-100% restoration of cytotoxicity according to the targeting mechanism used.	-Prodrug (0.001-10 µM) -Activator 0.002-20 µM (in case of PEG-azide) and 50 µM pre-treatment (in case of azide-monosaccharides)			**Selectivity:** The targeting approaches enhance the selectivity which makes this approach suitable for progression to *in vivo* settings.

*ND = not determined

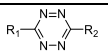
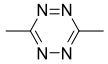
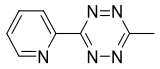
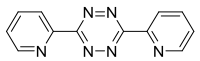
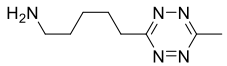
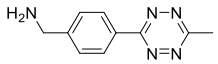
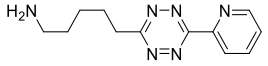
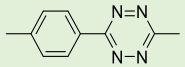
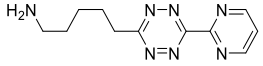
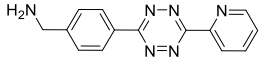
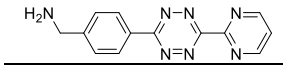
Green highlighting represents favoured findings, red highlighting represents challenging findings.

From **table 1**, it can be argued that there are two critical parameters that particularly affect the applicability of the Staudinger reaction (for prodrug activation). These are the concentrations of the reaction moieties, and the stability of the triphenylphosphine moieties. The Staudinger ligation is a second-order reaction with relatively slow kinetics (k_2 ~ 10^{-2} - 10^{-3} M^{-1} s^{-1}) which achieves reasonable activation levels by increasing the concentrations of the two reactants.[12,29] The instability of the triphenylphosphine moiety, and its tendency to oxidise, potentially restrict the quantitative control of its concentration at the desired site of action.[26] The approaches presented here in **Chapter 3** and **Chapter 5** (**table 1, entry 3**) show good prodrug activation levels *in vitro* achieved with reasonable reactant concentrations, and also show a good level of triphenylphosphine stability which promote its suitability for *in vivo* testing.

Parameters affecting the tetrazine ligation-based prodrug activation

The tetrazine ligation is currently the most utilised bioorthogonal reaction in prodrug activation applications due to its fast click-and-release kinetics (k_2 up to 10^4 M^{-1} s^{-1}).[13] The main parameters that affect prodrug activation are the reactivity and stability of the Tz moieties, with the type of substituents on the Tz ring being a key factor affecting both parameters. **Table 2** summarises the effect of different Tz substituents on the release kinetics and serum stability of the systems.[7,13,30] Tz moieties used for bond cleavage and prodrug activation usually comprise substituents at the 3- and 6-positions of the Tz ring (R_1 and R_2). Incorporation of an electron withdrawing group (EWG) accelerates the first step in the reaction (addition step) but decreases the rate of the decaging (elimination) step. On the other hand, while the incorporation of an electron donating group (EDG) is essential for the decaging step, it decreases the rate of the addition step hence decreasing the overall drug release rate. The second parameter that is affected by the different Tz substituents is serum stability. Tz moieties substituted with EDGs (e.g. methyl and pentyl) are shown to have long serum stability (i.e. the Tz moiety remains intact), however, they have slower reaction kinetics. On the other hand, Tz substituted with EWG (pyridines and pyrimidines) are shown to have poor serum stability, despite their faster reaction kinetics **(table 2)**.[13] Therefore, it can be implied that an unsymmetrical Tz activator bearing an EWG at one position and a small EDG such as an alkyl group at the other could contribute to a good balance between fast kinetics and good serum stability.[30,31] The synthesised Tz activators in this thesis (**Chapter 4** and **Chapter 5**) (highlighted entry 7 in **table 2**) showed excellent release which was demonstrated by the *in vitro* prodrug activation with full restoration of cytotoxicity in the majority of tested cases (i.e. PEG-Tz, 20 µM Ac_4ManNTz and Ac_4GalNTz) and good serum stability (especially with the PEG-Tz > 56% intact after 24 h).

Table 2: Summary of reported kinetics for reaction of Tz moieties with TCO at 20 °C/37 °C along with their serum stability.[13,30]

Tz moiety	R_1 and R_2 	k_2 (M^{-1} S^{-1})*	Serum stability %	Release % with TCO-Dox	Ref
	R_1=R_2=methyl	0.54 ± 0.06	100 (at 4 h)	79 (10 equivalents of Tz activator)	[7]
	R_1=2-pyridine, R_2=methyl	5.94 ± 0.24	96 (at 4 h)	55 (10 equivalents of Tz activator)	[7]
	R_1=R_2=2-pyridine	57.7 ± 5.0	75 (at 4 h)	7 (10 equivalents of Tz activator)	[7]
	R_1=pentan-1-amine, R_2=methyl	210 ± 20	96 (at 10 h)	ND	[13,30]
	R_1=4-tolylmethanamine, R_2=methyl	820 ± 70	96 (at 10 h)	ND	[13,30]
	R_1=pentan-1-amine, R_2=2-pyridine	2300 ± 300	85 (at 10 h)	ND	[13,30]
	R_1=4-benzyl, R_2=methyl	ND	PEG-Tz→72 (at 6 h), 56 (at 24 h) Tz-monosaccharides →63-81 (at 4 h)	-100 (2 equivalents of PEG-Tz) -28-100 (according to Tz expressed amount after MGE, which depends on the Tz-monosaccharide used)	**Chapter 4** and **Chapter 5**
	R_1=pentan-1-amine, R_2=2-pyrimidine	4400 ± 300	40 (at 10 h)	ND	[13,30]
	R_1=4-tolylmethanamine, R_2=2-pyridine	5300 ± 400	ND	ND	[13,30]
	R_1=4-tolylmethanamine, R_2=2-pyrimidine	22000 ± 2000	23 (at 10 h)	ND	[13,30]

*Kinetics of addition step (first step) as it is the rate-limiting step for the reaction.

ND = not determined

Generally, bioorthogonal reactions with fast kinetics are more favoured for *in vivo* applications as they achieve efficient and sufficient drug activation and payload release within an appropriate time interval. Reactions with fast kinetics and/or high concentrations of reactants have shorter prodrug activation half-lives which enables fast activation.[32] For example:

→ Reactions with a low rate constant ($k_2 = 10^{-2}$ M^{-1} S^{-1}) have prodrug activation $t_{1/2}$ (~ 28000 h) with micromolar prodrug and activator concentrations (1 µM).

→ Reactions with a moderate rate constant ($k_2 = 10^{1}$ M^{-1} S^{-1}) have prodrug activation $t_{1/2}$ (~ 28 h) with micromolar prodrug and activator concentrations (1 µM).

→ Reactions with a high rate constant ($k_2 = 10^{4}$ M^{-1} S^{-1}) have prodrug activation $t_{1/2}$ (~ 100 sec) with micromolar prodrug and activator concentrations (1 µM).

Therefore, fast kinetic-reactions have more potential for proceeding to *in vivo* settings, while for slow kinetic-reactions, the reactants can be subjected to many adverse factors such as clearance and instability before the activation is complete. However, it can be argued that slow-release kinetics can also be considered for *in vivo* applications where a sustained-release mode of activation is desired.

The choice between using a slow-release reaction (i.e. Staudinger reaction) or a fast-release reaction (i.e. tetrazine ligation) for further *in vivo* applications is mainly dependent on the intended purpose of prodrug activation (fast release or sustained/slow release). **Table 3** summarises the final conclusions drawn for the Staudinger ligation and tetrazine ligation activation approaches in this thesis, and also outlines ways to improve these strategies further for possible subsequent *in vivo* applications.

Table 3: Summary of conclusions for the Staudinger ligation and tetrazine ligation activation approaches in this thesis and ways to improve these strategies further for possible *in vivo* applications

	Staudinger ligation		**Tetrazine ligation**	
	Conclusion	Strategies to improve	Conclusion	Strategies to improve
Stability	Triphenylphosphine moiety is generally prone to oxidation in different aqueous media.	Pharmacokinetic studies are needed to understand the prodrug's fate in biological systems. This is crucial to determine the extent of the prodrug's stability and the amount remaining intact for activation. Using TCO for reaction with azides instead of phosphines could be better for further *in vivo* applications to avoid the oxidation and poor solubility of triphenylphosphines (further discussed in **section 6.4.2.**).	Serum stability of the Tz moiety is highly affected by its substituents and the size of the Tz activator.	Tz-monosaccharides can be encapsulated into nanoparticles (with efficient release at tumour site) to enhance their stability until they reach the desired site of action for MGE. Other Tz moieties with fast kinetics and high serum stability (e.g. entries 5 and 6 in **table 2**) could be incorporated within monosaccharides and PEG activators.
Prodrug activation	Restoration of drug cytotoxicity is highly affected by the size and concentration of the activator.	Ac_4ManNAz achieved the best activation but its selectivity remains a challenge. Encapsulation into nanoparticles could enhance its selectivity to tumour sites over normal cells.	Restoration of drug cytotoxicity was not affected by the activator size but by its concentration.	Determination of the highest tolerated concentration of the Tz activator intended for use (monosaccharides or PEG) before proceeding to *in vivo* prodrug activation applications.

In addition to considering the parameters affecting prodrug activation by either the Staudinger or tetrazine ligations for *in vivo* applications, parameters affecting the efficiency of the selected targeting mechanism should also be considered (generally discussed in **section 6.3.2.**)

6.3.2. Suitability of MGE, and passive targeting by the EPR effect, for targeted prodrug activation

Despite the advances introduced by using bioorthogonal reactions for prodrug activation, a targeting mechanism to achieve the selective delivery of one or more of the components to the target site is crucial. Tumour targeting for diagnostic or therapeutic purposes is often challenged by small differences between biomarkers in normal and tumour tissues, as well as tumour heterogeneity (between cells within the same tumour and between different tumours).[33,34] Hence selecting a targeting mechanism that can address these challenges is required. **Table 4** summarises how MGE and the EPR effect can address and alleviate these limitations.

Table 4: Advantages of MGE and the EPR effect for overcoming tumour heterogeneity and biomarker limitations

	MGE	**Passive targeting by the EPR effect**
Tumour heterogeneity	MGE is based on interception of the biosynthesis of overexpressed TACAs which terminates in sialic acid in certain cancer types[35–37]. Reports show that the level of chemical tags expression by MGE can significantly change according to different factors (explained in table 5) rather than tumour heterogeneity.	The EPR effect is based on the physical high permeability of tumour vasculature and preferred retention of macromolecules in solid tumour tissues. EPR effect does not depend on individual distribution to tumour cells which can be affected by heterogeneity.
Low differences in biomarkers between normal and tumour tissues	Chemically-modified monosaccharides used for MGE are reported to have a differential partition between various cells or tissue types even in non-cancer models.[38,39] In tumours, this differential partition is magnified and shifts in the direction of cancer cells due to their high metabolic rates favouring incorporation of the non-natural monosaccharides in the overexpressed TACAs.[40–42]	EPR is a physical phenomenon that does not depend directly on biomarkers being more abundant in tumour tissues.

EPR: enhanced permeability and retention; MGE: metabolic glycoengineering; TACA: tumour-associated carbohydrate antigen

To the best of our knowledge, only one recent reported study and the studies reported in this thesis have proposed combining MGE with bioorthogonal prodrug activation.[6,43] Therefore, when considering parameters that may influence the expression of the chemical prodrug activators *via* MGE, reference can only be made to the expression of reporters used for studying cell surface glycans and diagnostic cell imaging which have been more extensively studied.[21,24,25,44,45] **Table 5** demonstrates that the level of MGE is highly influenced by exposure time (72-h exposure time achieve

the highest expression level), concentration of monosaccharides (20 µM and 50 µM achieve the highest expression level), and the type of monosaccharides used (acetylated mannosamine derivatives achieve the highest expression level). Considerations that need to be taken into account for translating these studies to *in vivo* settings are also discussed in **table 5**.

Table 5: Effect of different parameters on level of expression of the chemical tags by MGE and considerations for future developments.

	Level of expression of chemical tags by MGE	Considerations	Ref
Exposure time	The longer the incubation of the chemically-modified monosaccharides with the cells intended for MGE, the higher the level of expression achieved (> 2-fold higher with 72 h) **72 h > 48 h > 24 h**	This time interval should be carefully considered before proceeding to *in vivo* prodrug activation to optimise the time interval between administration of the activator and prodrug.	[46–49]
Concentration of mono-saccharides	The higher the concentration of the chemically-modified monosaccharides, the higher the level of expression achieved. 5 µM achieves little to no expression, while 20 µM and 50 µM achieve the highest (> 1.2-fold than 5 µM and 10 µM). **50 µM > 20 µM > 10 µM > 5 µM**	The cytotoxicity of the monosaccharide derivative should be taken into consideration to ensure that the concentration used is tolerated by cells.	[50–54] and chapter 4
Type of mono-saccharide	Acetylated mannosamine derivatives are shown to achieve the highest expression level (~ 1.2-1.5-fold) within different cancer cells types (e.g. lung, breast, brain, and stroma cells), followed by galactosamine derivatives, glucosamine derivatives, and sialic acid derivatives. **Ac_4ManN-X > Ac_4GalN-X > Ac_4GlcN-X > Ac_4Sia-X**	Despite achieving the lowest expression levels, sialic acid derivatives are shown to have promising tumour selectivity among considered monosaccharides. Hence, for prodrug activation in cancer applications, a balance between selectivity and level of expression should be considered.	[6,48,50, 54–57] and chapter 4

Green highlighting represents favoured conditions, yellow highlighting represents important considerations.

As discussed earlier in **table 1**, this thesis presents the first study to engage a targeting mechanism with the Staudinger ligation for prodrug activation, however, Tz ligation has been reported for a small range of targeting techniques for selective prodrug activation (mainly active and passive targeting). **Table 6** provides a comparison between these targeting techniques highlighting their effectiveness in terms of selectivity and drug release, along with their limitations. The potential advantages of the targeting approaches presented in this thesis over these studies are discussed afterwards.

Table 6: Comparison between the reported targeting strategies for prodrug activation using the TCO-Tz bioorthogonal reaction

Targeting moiety	Complementary moiety	Targeting strategy	Tumour type	Effectiveness (selectivity and drug release)	Challenges	Ref
CC49-TCO-MMAE (antibody-prodrug conjugate)	PEG_{11}-Tz	Active targeting	Adenocarcinoma and ovarian cancer	High concentration of released drug at tumour site (~ 300 nM) which is about 100-fold higher than that found in plasma and liver.	High dosage of Tz activator is needed (0.335 mmol/kg) which can be a challenge for clinical translation due to potential toxicity.	[58]
CC49-TCO-DOX (antibody-prodrug conjugate)	PEG_{10}-Tz Dextran-Tz	Active targeting	Adenocarcinoma	Good concentration of released drug at tumour site (~ 51% Dox released) which is about 70-fold higher than that found in control model.	High dosage of Tz activator is needed (0.335 mmol/kg) which can be a challenge for clinical translation due to potential toxicity. A clearing agent for the antibody-prodrug conjugate is needed to remove it from blood to prevent undesired prodrug activation in blood.	[59]
Trastuzumab-pH sensitive hydrazone linker-TCO-DOX (antibody-prodrug conjugate)	Tz	Active targeting	Breast cancer	48% and 80% tumour suppression with 20 and 30 mg/kg antibody-prodrug conjugate dosage.	High dosage of Tz activator is needed (50 equivalents). Also, no quantification of released Dox in healthy tissues was conducted which questions the approach's selectivity.	[60]
Enzymatic supramolecular self-assembly Tz to form hydrogel	TCO-Dox	Passive targeting	Metastatic cervical cancer	Released Dox concentration > 10-fold in tumour than liver and blood.	Rapid accumulation and clearance of the Tz activator from tumour (24 h) forced a narrow administration interval between the Tz and the TCO-Dox (2 h).	[61]

Green highlighting represents favoured findings, red highlighting represents challenging findings.

CC49: anti-TAG72 antibody; Dox: doxorubicin; MMAE: Monomethyl Auristatin E; TCO: *tans*-cyclooctene; Tz: tetrazine

Table 6 illustrates how some strategies have been developed to enhance the selectivity of the Tz ligation prodrug activation approach. It can be concluded that using active targeting to accumulate the prodrug usually requires high concentrations of the Tz activator which challenges the clinical translation of this mechanism. Therefore, conjugating the Tz to a targeting moiety and developing a non-toxic Tz activator (for example a Tz-modified monosaccharide or PEG-Tz) that will explicitly target the cancer cell is an attractive and more clinically practical alternative. Further suggestions to increase the *in vivo* tumour selectivity of MGE and passive targeting for the prodrug activation approach presented in this thesis include:

1- Combining both targeting approaches, for example encapsulating the monosaccharide precursors into nanoparticles for selective delivery based on the EPR effect, to increase the cancer-selective MGE labelling process.[55,62,63]
2- Cancer-selective MGE labelling can also be increased by conjugating the monosaccharide precursors to a substrate that can be cleaved specifically to afford the required monosaccharide by overexpressed enzymes in cancer cells.[46,64]
3- Intratumoral injection (IT) of the monosaccharide precursors for MGE prior to the systemic injection of the prodrugs.[50,65]

The reported *in vivo* tumour selectivity of the above mentioned strategies are summarised in **table 7.** IT injection of the monosaccharide precursors has been reported to achieve the highest tumour selectivity (according to the tumour type).

Table 7: Summary of the reported strategies to increase the MGE selectivity *in vivo*

Strategy/ injection route	Monosaccharide/ formulation	Complementary moiety	Tumour	Tumour selectivity	Ref
Encapsulated sugars (IV)	Ac_4ManNAz-loaded liposomes	DBCO-modified liposomes	Lung	3.4-fold	[62]
	Ac_4ManNAz-loaded chitosan NPs	DBCO-modified chitosan NPs	Lung	1.5-fold	[66]
Enzyme-cleavable sugars (IV/IT)	DCL-AAM	DBCO-CY5/ Dox	Colon	1.5-fold	[67]
	Cathepsin B-activatable Ac_3ManNAz	DBCO-CY5.5	Colon	3-fold	[68]
Sugars (IT)	Ac_4ManNAz	BCN-modified NPs	Glioblastoma	1.6-fold	[50]
	Ac_4ManNAz	DBCO-modified liposomes	Lung	2-fold	[69]
	Ac_4ManNAz	DBCO-CY5 dye	Colon	5-fold	[67]

BCN: bicyclononyne; CY5: cyanine 5; DBCO: dibenzocyclooctyne; DCL-AAM: histone deacetylase/cathepsin L-responsive acetylated azidomannose ;NP: nanoparticle.

Based on the present evidence, it is not possible to determine whether MGE or the EPR effect would achieve tumour selectivity without *in vivo* testing. While the *in vitro* testing in breast cancer cells showed promising MGE selectivity, the same selectivity level is not guaranteed *in vivo* where a complex integration of physiological and genetic factors can undermine the targeting approach.

Since the area of using bioorthogonal reactions for prodrug activation is in its infancy, *in vivo* experiments would need to establish pre-clinical models to gain better understanding of the opportunities and challenges of bioorthogonal-based prodrug activation strategies for future clinical applications.[70,71]

6.4. Possible future work

Based on the findings from this project, three major areas of possible future investigation are identified:

1) Further modifications to the Tz compounds used in the prodrug activation strategy presented in this thesis could aid in improving serum stability **(Section 6.4.1)**.

2)Targeting TACAs via MGE has shown potential as a novel targeting mechanism in breast cancer **(Chapters 3 and 4)**. Engaging other bioorthogonal reactions (such as azide-TCO cycloaddition) with MGE for selective activation of prodrugs in breast cancer cells is suggested as a new approach for selective bioorthogonal prodrug activation **(Section 6.4.2)**.

3) Targeting TACAs via MGE could be further investigated for targeting other cancer types that are characterised by overexpression of TACAs **(Section 6.4.3)**.

6.4.1. Further modification of the Tz compounds to improve stability

As discussed earlier, the serum stability of the Tz compounds plays a critical role in the potential use of these compounds *in vivo* for prodrug activation, Tz activators in this thesis showed good serum stability, however this could be further improved. An alternative strategy could be explored where the tetrazine ligation click-to-release prodrug activation is still utilised, but the drug payload is conjugated to the Tz moiety instead of the TCO, shifting the TCO to be the reaction activator **(Fig. 2)**. This release mechanism was introduced by Robillard *et al* in 2020,[72] the cycloaddition step in this system **(Fig. 2b)** was faster than the original one **(Fig. 2a)** (k_2 = 420 ± 49 – 23800 ± 400 M^{-1} s^{-1} compared to 57.7 ± 5 M^{-1} s^{-1}). This strategy could overcome the low serum stability of the Tz-monosaccharides, as TCO-monosaccharides will be used instead for MGE, while the Tz-prodrugs can be subsequently administered for activation.

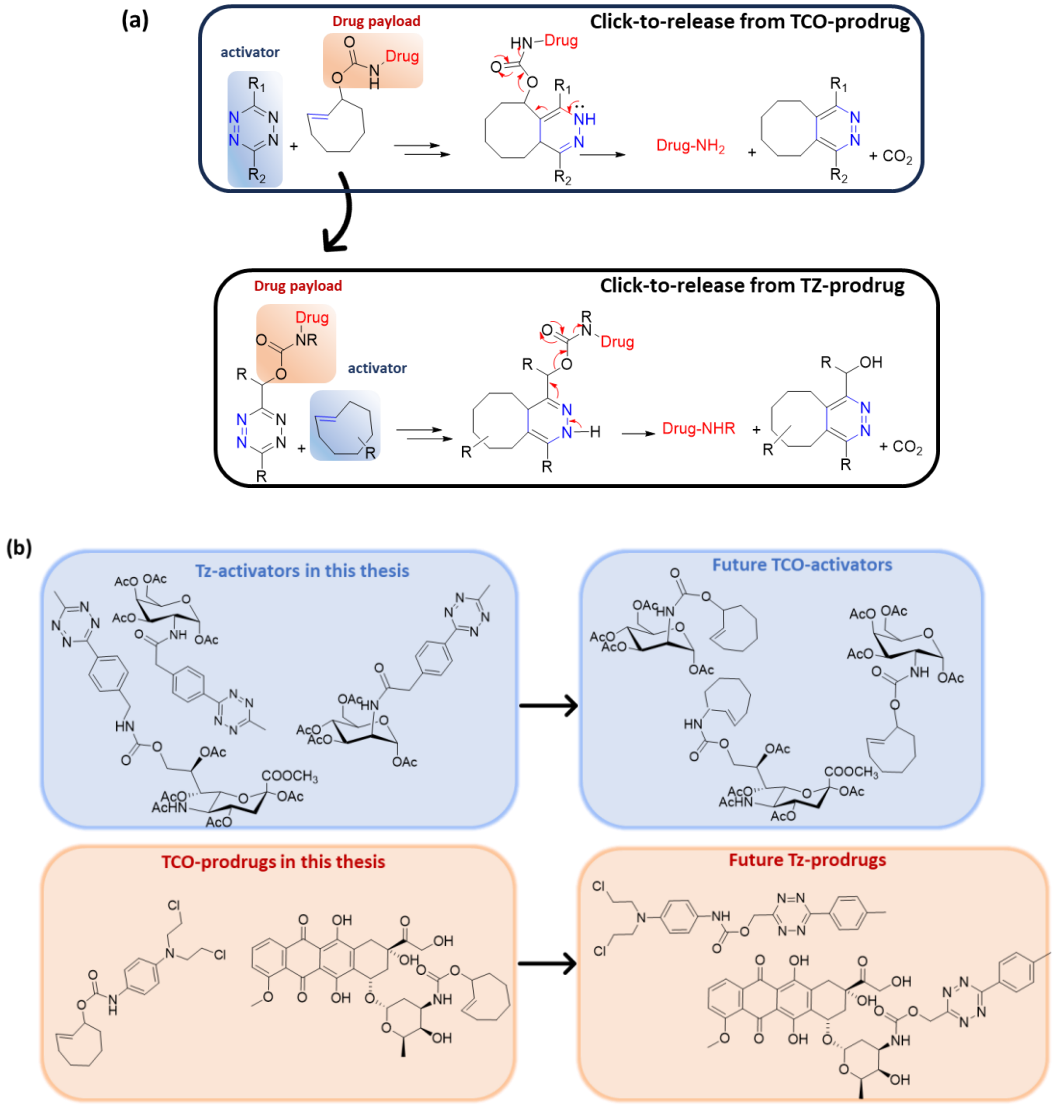


Figure 2. (a) Click-to-release reaction of TCO-prodrugs with Tz-activators and the modifications for the reaction for a click-to-release reaction between Tz-prodrugs and TCO-activators. (b) Chemical structures of the synthesised Tz-activators and TCO-prodrugs in this thesis and the proposed future Tz-prodrugs and TCO-activators.

6.4.2. Extension of the targeted prodrug activation strategy to include other bioorthogonal reactions

In section 6.4.1, TCO-modified monosaccharides are proposed to be developed for MGE and TCO expression on breast cancer cell surfaces' glycans. These TCO reporters could also be used to activate azide prodrugs by 1,3-dipolar cycloaddition reaction (further discussed in **Chapter 2, section 2.4.**). The only targeting mechanism that has been reported to be used with this reaction to increase its cancer-targeting, is active targeting. A TCO activator was conjugated to a cetuximab mAb to target EGFR-expressing melanoma cells and subsequently activate a 4-azidobenzyl carbamate doxorubicin prodrug.[73,74] In this thesis, MGE was demonstrated as a novel approach to target breast cancer, which propounds its further application for selective delivery of other bioorthogonal activators (e.g. TCO reporters) to breast cancer cells **(Fig. 3)**. The rate of the 1,3-cycloaddition between TCO and azide

is reported to be 10^{-2} $M^{-1}s^{-1}$ which is an order of magnitude faster than that of the Staudinger ligation (10^{-3} $M^{-1}s^{-1}$). This reaction rate was shown to be sufficiently rapid for the complete release of doxorubicin from the 4-azidobenzyl carbamate doxorubicin prodrug and full restoration of its cytotoxicity.[73] This suggests that utilizing the 1,3-azide-TCO cycloaddition reaction would be more advantageous than the Staudinger ligation for prodrug activation in terms of release kinetics and full restoration of parent drug activity.

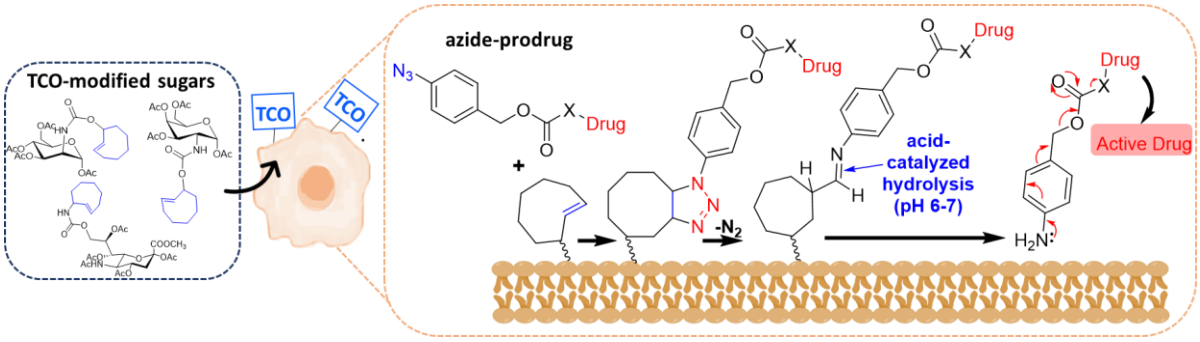


Figure 3. Proposed schematic illustration of MGE labelling of breast cancer cells' surfaces using TCO-modified sugars leading to TCO expression. This is then followed by targeted activation of azide-prodrugs *via* 1,3-dipolar cycloaddition.

6.4.3. Extension of the targeted prodrug activation strategy to other cancer types and to include other chemotherapies

Beside breast cancer, TACAs are reported to also be overexpressed in lung, pancreatic, and colon cancers,[37] which suggests the possibility of utilising the developed cancer-targeting prodrug activation strategies **(Chapters 3 and 4)** for targeting these cancer types **(table 8)**. Lung and colorectal cancers are the first and second leading causes of cancer-related deaths worldwide, respectively, also they are the second and third most prevalent cancer types, respectively according to the WHO.[75] Their high prevalence and mortality rates contribute to the clinical need for maximising the effectiveness of their treatments for example by increasing the selectivity of their currently used chemotherapies.

Table 8: Mortality rates of lung, pancreatic, and colon cancers, their current chemotherapy options, and their reported overexpressed TACAs as new therapeutic targets.

	Lung cancer	**Pancreatic cancer**	**Colon cancer**	**Ref**
Mortality rate (2020)	1.8 million deaths (18% of all cancer-related deaths)	> 50, 000 deaths	> 930, 000 deaths	[76–78]
Chemotherapy treatment options	Platinum-based *regimen (Carboplatin/cisplatin) with pemetrexed* or paclitaxel (in non-small cell lung cancer)	FOLFIRINOX (a combination of drugs): -FOL: folinic acid -F: 5-fluorouracil -IRIN: Irinotecan -OX: Oxaliplatin*	Combination of 2 or 3 of the following: -5-fluorouracil -Oxaliplatin* -Irinotecan -Trifluridine and tipiracil	[79–83]
Overexpressed TACAs	-Sialyl Lewisx "sLe^x" -Sialyl Lewisa "sLe^a" -Sialyl Tn "sTn" -Polysialic acid "PSA" -Fucosyl GM1	-Sialyl Lewisx "sLe^x" -Sialyl Lewisa "sLe^a" -Sialyl Tn "sTn" -Polysialic acid "PSA"	-Sialyl Lewisx "sLe^x" -Sialyl Lewisa "sLe^a" -Sialyl Tn "sTn"	[35–37]

*(further suggestions are provided herein for the highlighted compounds)

The approach could also be broadened by including a wider range of chemotherapeutic agents within the prodrugs (including the highlighted chemotherapies in **table 8**). Cytotoxic chemotherapeutics that lack cancer selectivity and comprise a chemical functional group that is essential for their cytotoxicity can be derivatised into a prodrug. In this thesis, doxorubicin and *N*-mustard were selected as they have a free amino group that was derivatised to afford the TCO- and triphenylphosphine-prodrugs. Other suitable chemotherapies such as pemetrexed, chlorambucil, and pyridostatin could be utilised, as it has been reported that masking their free carboxylic groups and amino group, respectively, decreases their cytotoxic effects **(Fig. 4)**.[84–87]. Platinum chemotherapies such as oxaliplatin (a platinum(II) complex) are reported to have less systemic cytotoxicity when used as platinum(IV) complex prodrugs which suggests that they could be converted into bioorthogonal prodrugs through attaching groups such as TCO and triphenylphosphine to axial positions of the platinum(IV) complex **(Fig. 4)**.[88]

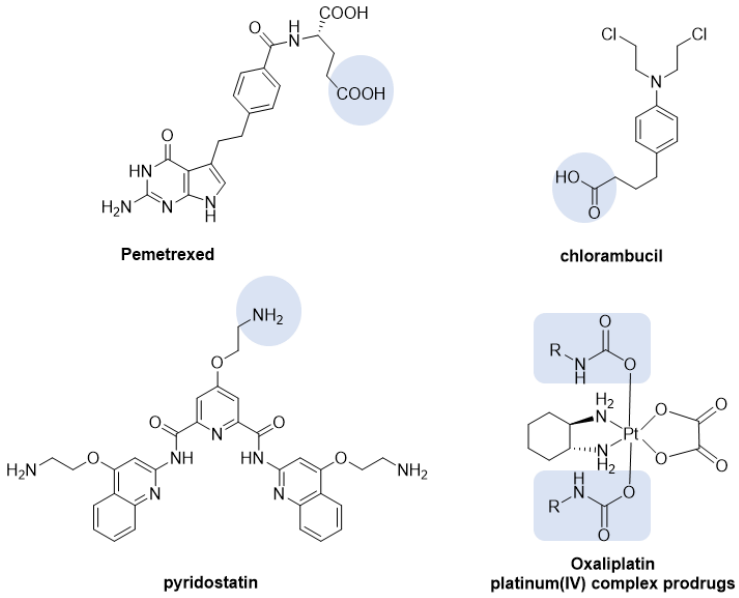


Figure 4. Chemical structures of chemotherapy candidates for incorporation within bioorthogonal prodrugs.

6.5. Concluding remarks

In conclusion, the work presented in this thesis validates the feasibility of using the bioorthogonal Staudinger ligation and tetrazine ligation reactions for prodrug activation in breast cancer. MGE is also introduced as a novel targeting approach to increase the selectivity of the prodrug activation strategy in breast cancer. In addition, the feasibility of using PEG-azide and PEG-Tz as bioorthogonal activators was also shown *in vitro*. Further work is still required to test the selectivity of breast cancer-targeting by MGE and the EPR effect *in vivo* in breast cancer-animal models. Future possible work is also suggested to improve many aspects for the prodrug activating approach including improving the serum stability of the compounds, utilisation of other bioorthogonal reactions, and extending the approach to other cancer types and chemotherapies.

Given the current research interest in bioorthogonal prodrugs, and the novelty of the work presented in this thesis, further research could bring significant impact within academic, industrial and clinical settings by optimising the reactivity, stability, and selectivity of the proposed prodrug strategies.

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