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Self-assembling antitumour prodrugs for localised drug delivery

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Declaration

The work presented in this thesis is submitted to the University of Nottingham in support of my application for the degree of Doctor of Philosophy. No part has been submitted for any other degree at The University of Nottingham or any other institution. The work presented herein has been carried out by myself, except for the cases defined below:

- The in vitro stability studies in rat and human plasma reported in Chapter 4 have been carried out by Lee Jong Bong of University of Nottingham.
- SEM images in Chapter 3 were acquired with Christine Grainger-Boulton of the University of Nottingham.
- TEM images in Chapter 3 and Chapter 4 were acquired with Dr. Mike Fay of the University of Nottingham.
- Temperature-dependent ^1H -NMR studies described in Chapter 4 were carried out with Dr. Huw Williams of the University of Nottingham.

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Chapter 1: Insights into low molecular mass gelators: a focus on drug delivery and tissue engineering applications. K. J. Skilling, F. Citossi, M. Ashford, T. D. Bradshaw, B. Kellam and M. Marlow, *Soft Matter*, 2014, 10, 237-256

Chapter 3: Antitumour benzothiazoles. Part 32: DNA adducts and double strand breaks correlate with activity; synthesis of 5F203 hydrogels for local delivery. E. L. Stone, F. Citossi, R. Singh, B. Kaur, M. Gaskell, P.B. Farmer, A. Monks, C. Hose, M.F.G. Stevens, C-O. Leong, M. Stocks, B. Kellam, M. Marlow and T.D. Bradshaw, *Bioorganic and Medicinal Chemistry*, 2015, 23, 6891-6899.

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Abstract

Localised cancer therapy is a developing strategy used to overcome the systemic toxicity associated with intravenous systemic chemotherapy, which still represents the primary route of administration for the majority of current anticancer agents. Low molecular weight gelators (LMWGs) have recently gained increasing popularity as drug delivery platforms for localised cancer therapy: they are small molecules, which self-assemble into a 3D network via non-covalent interactions. Due to their inherent biocompatibility, LMWGs represent a viable alternative to the extensively explored polymer based drug delivery systems. One such drug delivery approach, based on LMWGs, involves the synthesis of gelator-based prodrugs of chemotherapeutic agents; however, to date, there remains a limited number of anticancer prodrugs that have gelation properties.

Therefore, the present work aims to find new chemotherapeutic agents that display gelation properties by modification of the parent drug with known self-assembling groups. Two anticancer agents have been evaluated: the clinical antimetabolite methotrexate (MTX) and the experimental benzothiazole derivative 5F 203, both characterised by significant anticancer activity but systemic toxicity. Therefore, formulation of MTX and 5F 203 as LMWGs-based prodrugs for localised cancer therapy was considered a useful strategy to overcome the systemic toxicity of these antitumour drugs.

Different synthetic approaches were explored to formulate self-assembling MTX prodrugs. The most successful one involved the synthesis of MTX derivatives bearing alkyl chains and aromatic groups at the α or both α and γ carboxylic acid terminals of MTX. Unfortunately, preliminary gelation tests performed on MTX acyl derivatives via a solvent-switch method, revealed lack of self-assembling properties. Therefore, the MTX conjugates developed were not suitable for localised drug delivery applications.

Due to the absence of self-assembling properties of MTX conjugates, the potential gelation behaviour of novel derivatives of another anticancer candidate, the benzothiazole agent 5F 203, was considered, and two amide prodrugs series of 5F 203 were investigated. Amongst the compounds tested for gelation, 5F 203

succinic acid conjugate (**68a**) revealed formation of a hydrogel at physiological pH. Rheological measurements confirmed its LMWG nature, showing formation of a cross-linked gel network. In vitro growth inhibitory assays against breast (MCF-7) and ovarian (IGROV-1 and OVCAR-4) carcinoma cell lines showed overall activity of 5F 203 amide prodrugs in inhibiting cell proliferation. Release studies from the gel matrix of **68a** revealed a release of the derivative and the active drug over 3 days, thus confirming its potential application as a depot formulation for localised delivery of 5F 203.

In order to improve the rate of conversion of prodrugs into the parent amine 5F 203, compared to the previous amide series developed, acyloxyalkoxycarbonyl derivatives of 5F 203 were synthesised. Gelation tests displayed self-assembling behaviour for derivatives **76a-76c** and rheological studies confirmed the LMWG nature of the new entities. The acyloxyalkoxycarbonyl prodrugs revealed in vitro potencies similar to those displayed by 5F 203, when tested against MCF-7 and IGROV-1 cell lines. The isobutyl carbamate prodrug (**76a**) proved to be the most potent of the series, showing hydrolysis into the active drug 5F 203, when incubated in either PBS buffer, rat or human plasma; release from the gel matrix also showed release of 5F 203 in PBS within 72 h.

The outcomes from this work have therefore provided a basis for future optimisation and development of LMWG derivatives of 5F 203, as depot formulations for localised delivery of this anticancer agent.

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Abbreviations

1D	One-dimensional
3D	Three-dimensional
AFM	Atomic force microscopy
APA	4-(N-(2,4-diamino-6-pteridinylmethyl)-N-methylamino) benzoic acid
Boc	tert-butyloxycarbonyl
CDCl ₃	Deuterated chloroform
CD	Circular dichroism
CHCl ₃	Chloroform
CH ₂ Cl ₂	Dichloromethane
COSY	Correlation spectroscopy
D ₂ O	Deuterium oxide
DCC	Dicyclohexylcarbodiimide
DIPEA	N,N-diisopropylethylamine
DMAP	4-(dimethylamino) pyridine
DMF	N,N'-dimethylformamide
DMSO	Dimethylsulfoxide
DMSO-d ₆	Deuterated dimethylsulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ESI-MS	Electrospray Ionisation-Mass spectroscopy
Et ₂ O	Diethyl ether
EtOAc	Ethyl acetate
Fmoc	9-fluorenylmethoxycarbonyl
G'	Storage modulus
G''	Loss modulus
GdL	Glucono δ lactone
Glu	Glutamic acid
Gly	Glycine
HATU	2-(7-aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HOBt	1-hydroxybenzotriazole
HPLC	High Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
LMWG	Low molecular weight gelator
LVE	Linear Viscoelastic
Lys	Lysine
MeOH	Methanol
MgSO ₄	Magnesium sulfate
Mp	Melting point
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
NaOH	Sodium hydroxide
Na ₂ SO ₄	Sodium sulfate
NMM	N-methylmorpholine
NMP	N-methyl-2-pyrrolidone
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffered Saline
Phe	Phenylalanine
Ppm	Parts per million
r.t.	Room temperature
SEM	Scanning Electron Microscopy
t-Bu	Tert-butyl
TEM	Transmission Electron Microscopy
T _{gel}	Temperature of gelation
TIPS	Triisopropylsilyl ether
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
t _R	Retention time
Tyr	Tyrosine
UV-VIS	Ultraviolet-visible
Z (or CBZ)	Carboxybenzyl

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Chapter 1 : Introduction

This introductory chapter will review the current state of cancer and the existing localised cancer therapies, particularly focusing on low molecular weight gelators as innovative drug delivery systems. Then descriptions of the anticancer agents studied in the ensuing chapters, methotrexate and 5F 203, will be provided. Finally, the project aims and objectives will be detailed.

1.1. Cancer: the 21st century plague

Cancer is considered the leading cause of morbidity and mortality worldwide according to the latest reports from the International Agency for Research on Cancer.¹ In 2012, about 14.1 million of new cancer cases occurred globally and 8.2 million of people died because of cancer, with lung, breast, colorectal and stomach cancers accounting for 40% of all diagnosed cases. As risk factors, tobacco is considered the most important one, being the main cause of cancer in 2008.² Viral infections, consumption of alcohol, differences in weight and diet account for development of breast, gastric, liver and colorectal cancer. The latest data about cancer incidence by country show a rapid increase of cancer cases in the most developing countries, due to the “Westernisation” trend, because of their rapid societal and economic growth.¹ Projections to 2030 forecast an increase of cancer cases of 23.6 million every year. Also, according to one of the last reports published in the British Journal of Cancer in 2015, one person out of two, under the age of 65, will likely be diagnosed with cancer at some point in his life.³

1.1.1. Hallmarks of cancer

Cancer is the result of a multistep process in which normal cells evolve to a neoplastic state according to specific capabilities, known as hallmarks of cancer, which allow tumour growth and dissemination. Hanahan and Weinberg firstly described and classified the six hallmarks of cancer (Figure

1.1) and, more recently, described two further hallmarks additionally elucidating two enabling characteristics that play a key role in cancer development.⁴

At the base of cancer formation, i.e. tumorigenesis, two enabling characteristics are fundamental: presence of genomic instability, which leads to chromosomal rearrangements, and occurrence of an inflammatory state promoted by immune cells.

Tumour growth is then the result of six main characteristics: one of the most important is represented by sustaining proliferative signalling, which means that cells proliferate in an uncontrolled manner, possibly as a result of a major supply of growth factors derived from normal cells or direct production from the same cancer cells. Alterations or overexpression of oncogenic growth factor receptors are also considered to be a cause of proliferative signalling: for example, the human epidermal growth factor receptor-2 (HER2) determines activation of downstream pathways (i.e. mitogen-activated protein kinase, MAPK), eventually resulting in sustaining proliferation.⁵

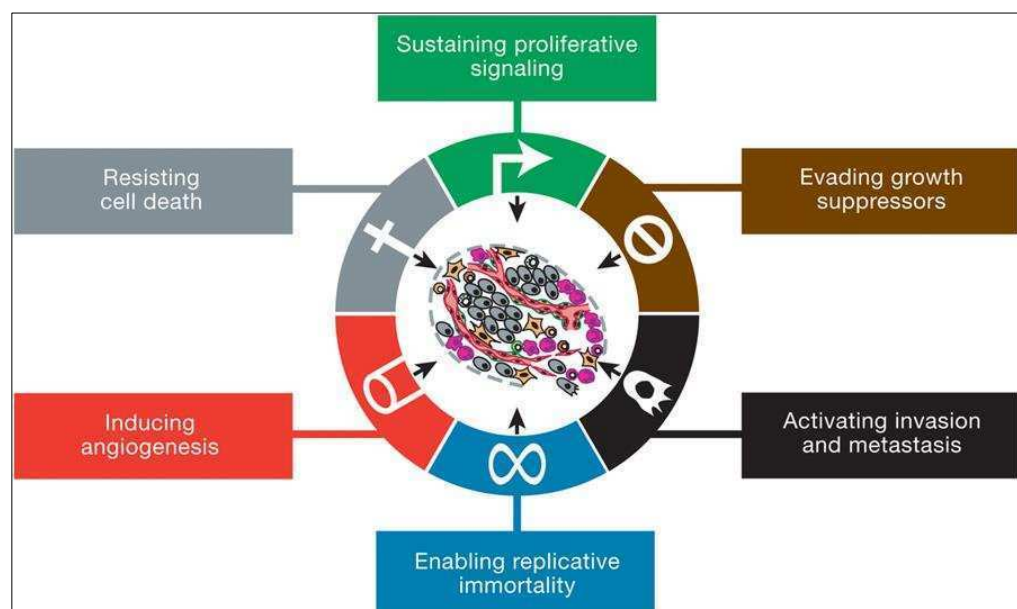


Figure 1.1: Hallmarks of cancer. Taken from Ref. 4.

Another factor involved in enhancement of cells proliferation is disruption of negative-feedback mechanisms that inhibit different types of proliferative signalling. For example, mutations of the Ras oncogene, involved in the regulation of intracellular signalling networks, lead to increased proliferation.⁴

Resisting programmed cell death is another hallmark capability of neoplastic cells, which imparts resistance to apoptosis due to suppression of pro-apoptotic trigger proteins, such as Bcl-2 and Mcl-1.⁶ A key role in tumour development is also played by the tumour protein p53, considered the guardian of the genome. This protein is the main one involved in tumour suppression, stress and DNA damage response by inducing apoptosis via upregulation of pro-apoptotic proteins (Noxa and Pluma BH3). Inactivation of p53, due to different factors, such as activation of p53-inactivating proteins of oncogenic viruses, leads to cell survival and tumour progression.⁷

From a macroscopic point of view, tumours are the result of an unlimited replicative capacity (immortality) of cancer cells, which are able to replicate for an infinite number of cell cycles compared to normal cells. This characteristic is mainly due to presence of telomeres, i.e. the last region of DNA that protects the ends of chromosomes from end-to end fusions. In immortalised cells, telomeres do not shorten and they do not prevent deterioration and fusion with close chromosomes, leading to alteration to the normal cell cycle. It has been calculated that ~90% of immortalised cells, including human cancer cells, express the enzyme telomerase (which restores telomere length) at functionally significant levels.⁴

Other two important hallmarks of cancer include the ability of tumours to induce angiogenesis and activate metastasis: these factors are related to each other, as the growth of blood vessel networks which supply nutrients to tumour cells contributes to development of the invasion-metastasis cascade.

In the recent years, identification of two new emerging hallmarks of cancer contributed to develop a better understanding of the tumour complexity. One of them is represented by reprogramming energy metabolism: indeed, cancer cells show the ability of producing glucose in aerobic conditions, favouring

the glycolysis metabolism, compared to normal cells, in which glucose is mainly produced in anaerobic conditions. This metabolic switch has been associated to activation of oncogenes (RAS) or inactivation of tumour suppressors (p53).

The role played by the immune system in resisting or suppressing tumour growth is another still-unresolved emerging hallmark capability. In fact, the immune system represents a barrier for the progression of tumours: studies from genetically engineered mice demonstrated that mice deficient of some components of the immune system are more likely to develop carcinogen-induced tumours compared to the immunocompetent controls.

All these elements integrated together explain the complex biology of cancer. Indeed, due to cancer's several and elaborated mechanisms of growth, a complete understanding of tumorigenesis is still required to allow a development of more efficient cancer therapies. One of the emerging specific cancer treatments is currently represented by localised cancer therapy.

1.1.2. Current localised cancer therapies for treatment of solid tumours

The most common malignant cancers are represented by solid tumours: they consist of solid masses of cancer cells and may occur in any solid part of the body. About 85% of the total solid cancers are represented by carcinomas, i.e. malignant tumours of epithelial cells; the other 15% are sarcomas, i.e. they affect the mesenchymal layer, such as bone, cartilage, muscle and fat.⁸

In terms of cancer therapy, nowadays many treatment options are available: surgery still remains the first choice for treatment of primary solid tumours; radiotherapy, anticancer drug cocktails and monoclonal antibodies are used as continuation treatments after surgical resection in attempt to prevent any recurrent cancer growth. Also, vaccination and screening programmes are emerging strategies used to control and prevent the initiation of cancer.⁹

In spite of the many treatment options mentioned above, cancer therapy is still a hard challenge. Many chemotherapeutic drugs present low solubility

problems associated with a low bioavailability, necessitating increased drug administration frequency, leading to systemic toxicity and drug resistance. Indeed, intravenous systemic chemotherapy is not specifically targeted to the tumour and rarely reaches high therapeutic levels within the tumour site, frequently accumulating in healthy organs and leading to systemic toxicity. One example is represented by the commonly used antineoplastic drug paclitaxel: upon intravenous (i.v.) injection, half of the dose is excreted within 24 hours and only 0.5% of the initial dose reaches the targeted tumour site (i.e. lungs).¹⁰

Drug resistance represents another important factor related to poor effectiveness of cancer treatments. Tumours can be intrinsically drug-resistant, or they can develop over time an acquired resistance against the anticancer agents used to treat them. Sometimes cross-resistance to drugs with a similar mechanism of action can also occur. It has been calculated that drug resistance is the main cause of treatment failure in 90% of cancer patients. The main mechanisms involved in development of drug resistance are related to the mechanism of action of antineoplastic drugs: they include drug inactivation, excessive drug efflux or alterations in drug target.¹¹

Due to these limiting factors, new strategies are being rapidly developed in cancer treatment: an example is represented by localised drug delivery either at the resection site or intra-tumourally, a targeted therapy which recently demonstrated to be an effective alternative to systemic delivery, with some examples of polymer-based controlled release formulations already on the market.¹⁰

Localised delivery of a drug at the solid tumour site is mainly used for prevention of cancer recurrence after surgical removal of a primary solid tumour, where undetected malignant cells are left at the resection site. The main advantages of localised chemotherapy over the traditional systemic treatments include:

- enhancing drug concentration at the tumour site due to the localised delivery, resulting in less waste of drug;
- promoting controlled and prolonged drug release in order to guarantee drug uptake by cancer cells in all phases of the cell cycle;

- diminishing drug administration frequency;
- decreasing systemic side effects.

Brachytherapy represents the first example of localised drug delivery used in clinical applications: it consists of implantation of radioactive seeds at the tumour site as treatment of inoperable primary tumours or as prevention after surgical resection. Brachytherapy showed efficacy specifically for treatment of prostatic cancer; it has also been used for lung and cervical cancers.^{12, 13} However, due its handling problems, limited availability and toxicity, brachytherapy use has not successfully progressed.¹⁰

Polymer-based formulations for localised drug delivery are emerging as promising targeted cancer therapies: wafers, thermogels, nanoparticles and millirods loaded with chemotherapeutic drugs represent only a few examples, and some of them are already in use for clinical applications (Figure 2.1).

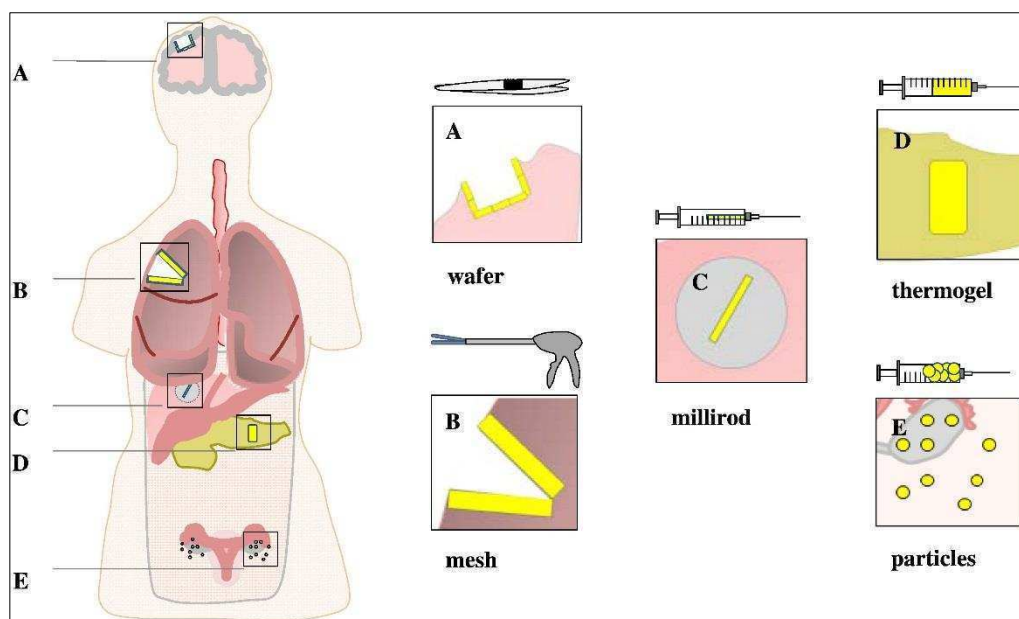


Figure 2.1: Examples of localised chemotherapy devices at different treatment sites and their relative mode of administration. Taken from Ref. 10.

The Gliadel Wafer[®] is a polyanhydride wafer containing the anticancer drug carmustine and it is currently used for treatment of glioma, a malignant brain cancer. It was introduced in the early 1980's and it represents the most successful example of localised cancer therapy.¹⁰ This drug delivery device

is mainly used as prevention of cancer recurrence after surgical excision: the wafers are deposited around the resection site of the primary tumour and allow the local drug delivery, circumventing the blood-brain-barrier issue, a main problem that limits chemotherapeutic agent bioavailability. The wafers resulted in increased survival times up to one year for patients with malignant glioma and were shown to be biodegradable and well-tolerated.¹⁴

Polymer-based gels represent another example of successful drug delivery platforms for intratumoural cancer therapy. Their gel nature allows an extended and controlled release of chemotherapeutic drugs from the gel matrix to the tumour site. Bhattarai et al. recently reviewed the use of injectable biodegradable chitosan-based hydrogels for the drug delivery of anticancer drugs and elucidated the potential clinical applications of this class of gels.¹⁵

Microspheres formed by paclitaxel-loaded polyphosphoester microparticles, developed with the name of Paclimer[®], have been used locally for prevention of recurrent ovarian cancer; however, onset of peritoneal abnormalities and inflammatory response were observed around the microsphere site, suggesting poor tolerability and biocompatibility of these microspheres.¹⁶

Besides the drug delivery systems reported above, which are already in the clinical arena, other formulations for localised delivery, e.g. nanoparticles containing paclitaxel and polymer millirods loaded with doxorubicin, have demonstrated efficacy in vivo, showing promise as targeted therapies.¹⁰

In spite of the numerous examples of successful localised cancer therapies, research is still ongoing to address the challenges required for the development of ideal cancer drug delivery platforms:

- intratumoural drug delivery is still ineffective in preventing distal metastasis growth because of localised application, therefore it can be used only as treatment of primary solid tumours in early stage cancer patients;
- more research is needed to determine safe, biocompatible and biodegradable polymers, to avoid onset of inflammatory responses in situ;

- high drug loading is required to ensure prolonged drug release following a single administration;
- potential interactions between polymers and therapeutic agents need to be avoided, in order to reduce the related side effects and increase drug's efficiency.

In order to fulfil the characteristics reported above, numerous drug delivery systems have been developed;^{17, 18} one of them is represented by the emergent low molecular weight gelators (LMWGs).

1.2. Self-assembling low molecular weight gelators for drug delivery applications

Over the last two decades, LMWGs have rapidly become an attractive group of soft biomaterials with exceptional tuneable properties that make them suitable for many applications.¹⁹

LMWGs are molecules with a molecular mass ≤ 3000 Da, which form gels in small amounts (typically <2 % wt) of water or organic solvents.¹⁹ The definition of a gel has changed and evolved over the last century. In the late 1970's, Flory gave a comprehensive definition of a gel stating that “a substance was a gel if it possessed a continuous microscopic structure with macroscopic dimensions that are permanent and solid-like in rheological behaviour despite being mostly liquid”.²⁰

LMWGs are termed hydrogels, if they are mainly aqueous in nature, even if occasionally a small amount of solvent is needed to solubilise the gelator molecule before addition of water; conversely, gels whose liquid phase is predominantly organic are called organogels.

1.2.1. Gelation by LMWGs

Gels are organised into a hierarchical structure, represented by primary, secondary and tertiary structures (Figure 3.1). The self-assembly of LMWGs into a three dimensional (3D) network is the result of a tiered process. As explained by Estroff and Hamilton, initial assembly into a primary structure is given by one dimensional (1D) order non-covalent interactions, such as hydrogen bonds, van der Waals and electrostatic interactions and π - π stacking.²¹

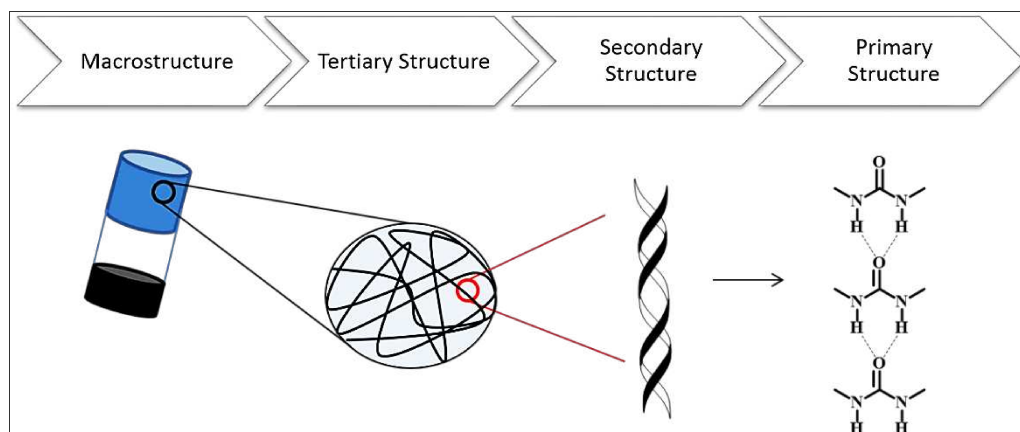


Figure 3.1: The primary, secondary, tertiary and macrostructure of a self-assembled LMWG. The primary structure is here exemplified by hydrogen bonds between individual urea molecules. Self-assembling of the gelator in a secondary structure, here represented by a helical structure, is given by non-covalent interactions. The tertiary structure signifies the overall network, i.e. a branching structure of elongated fibres. Taken from Ref. 19.

This first step is crucial for determining aggregation into 1D fibres, as the overall gelation process is the result of a delicate balance between the molecules' ability to aggregate or dissolve in solution. The secondary structure consists of the formation of self-assembled aggregates, such as fibres, tapes, ribbons, micelles, vesicles or lamellae, whose morphologies mainly depend on gelators' molecular structure. The tertiary structure is the result of interactions between aggregates, and determines whether they assemble into a gel or precipitate.^{19, 22}

In spite of the numerous studies about LMWGs, to date, conclusive mechanisms that explain the self-assembly process have not been reported. In most cases, gelation has been defined as a “frustrated” crystallisation, as both processes are characterised by an initial nucleation.^{23, 24} However, they differ in the process that leads to dimensional packing of the molecules: whilst in crystallisation molecules are organised into a well-defined 3D order, the gelator molecules need first to form 1D aggregates (i.e. fibres) to allow a subsequent less-ordered cross-linking into a 3D network.^{22, 25}

1.2.1.1. Factors determining the self-assembly

In relation to the molecular structure, the presence of some functional groups in the gelator molecule is fundamental for determining gelation, as they promote formation of non-covalent interactions necessary for self-assembly:

- Amides (RCONHR') and ureas (RNHCONHR') form complementary hydrogen bonding interactions due to the presence of hydrogen bond donors (NH) and acceptor groups (C=O).
- Long chain alkanes promote van der Waals forces and solvophobic interactions, as these functional groups are poorly soluble in the solvents used to form the gel and contribute to the gelation process by reducing the solubility of the gelator in the solvent.
- Nucleobases can form hydrogen bonds and π - π stacking interactions.
- Carbohydrates contain numerous O-H residues which generate hydrogen bonding.¹⁹

Chirality also plays an important role in gelation: indeed, a large proportion of existing gelators contain at least one stereogenic centre. This is due to the stabilising effect that chirality confers to the self-assembly process, at the nanoscale level. Smith widely described the effect of chirality in gelation, showing how the chiral information translates from the molecular level to the formation of self-assembled aggregates. For a further description of chirality effects on LMWGs, the reader is referred to valuable reviews.^{26, 27}

Although gelators' intermolecular interactions play a key role in gel formation, the solvent used is also a determinant for the self-assembling process, and this factor has often been underestimated. Recently, Zurcher and McNeil reported some tools to rationally design and identify gelator scaffolds and solvents for gel formation. They suggested that an initial screening of the gelator in different solvents should be carried out to evaluate the solubility of the gelator, this being the first requirement to promote dissolution of the molecule in the solvent.²⁸

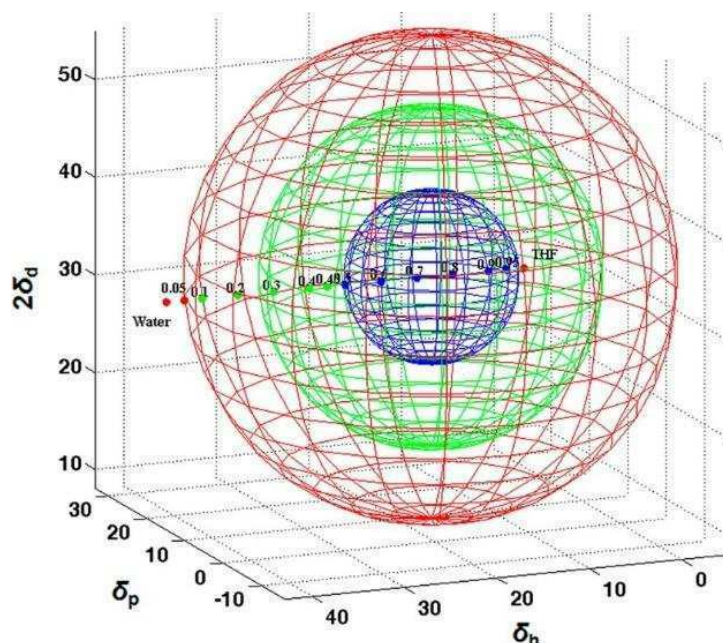


Figure 4.1: Example of plot of Hansen solubility data for a sugar-based gelator in THF/water, predicting formation of solutions (blue), gels (green) and insoluble precipitates (red). Taken from Ref. 30.

Studies on the solvent parameters have also recently been considered, such as dielectric constants, octanol/water partition coefficients and Hansen solubility parameters (HSP). In particular, Weiss et al. demonstrated the importance of HSP, i.e. a method to predict the solubility of a material into a solvent defined by the parameters δ_d (dispersive interactions), δ_p (polar interactions) and δ_h (hydrogen bonding interactions); they showed that the mathematic determination of the Hansen space (i.e. the three dimensional space defined by parameters δ_d , δ_p and δ_h , Figure 4.1) of a gelator molecule is an accurate approach for predicting different solvents that can be used for gelation.^{29, 30}

1.2.1.2. Stimuli-triggered gelation methods

Along with the rational design of LMWGs, several studies have been carried out to develop specific triggers for initiation of gel formation. They mainly consist of physical (heat, mechanical forces, UV-vis light), chemical (pH change with acid-base reagents) or enzyme-triggered methods. This section provides an overview of the most reported gelation techniques for LMWGs used for drug

delivery purposes; further methods have been thoroughly reviewed by Zhang et al.³¹

Temperature-switch method: this procedure is probably the most commonly used for gel formation and it has been applied to many classes of LMWGs, ranging from hydrogels to organogels. High temperatures favour increased solubility of the gelator, required prior to self-assembly; subsequent lowering of the temperature results in a reduction of solubility and formation of self-assembled aggregates, due to the presence of 1D non-covalent interactions. It has been observed that the rate at which gels are heated/cooled alters the rate of nucleation and, thus, of fibre formation. Amongst the numerous examples of temperature-responsive gels, a particular hydrogel (**1**) has been reported by Kiyonaka et al. (Figure 5a.1): they showed how this glycosylated amino acid-based gel had different phase transitions depending on the temperature changes (Figure 5b.1).³²

pH-switch method: this procedure can be specifically applied for compounds with acidic or basic moieties, which are poorly soluble at neutral pH, to obtain hydrogels. Solubility of the gelator is firstly achieved by increasing or lowering the pH, depending on the acidic or basic nature of the molecule, and the corresponding salts are then formed, i.e. the gelator molecule is ionised and has increased solubility. Gelation occurs afterwards on lowering or increasing the pH with an inorganic acid or base (i.e. hydrochloric acid or ammonia), and the gelator in its non ionised, less soluble form then self-assembles via non-covalent interactions.^{33, 34} Adams and his group also introduced an alternative procedure to achieve gelation of compounds bearing a carboxylic acid terminus via pH-switch, using the pH modifier glucono- δ -lactone (GdL). Further details on the mechanism of GdL are discussed in Chapter 3.³⁵

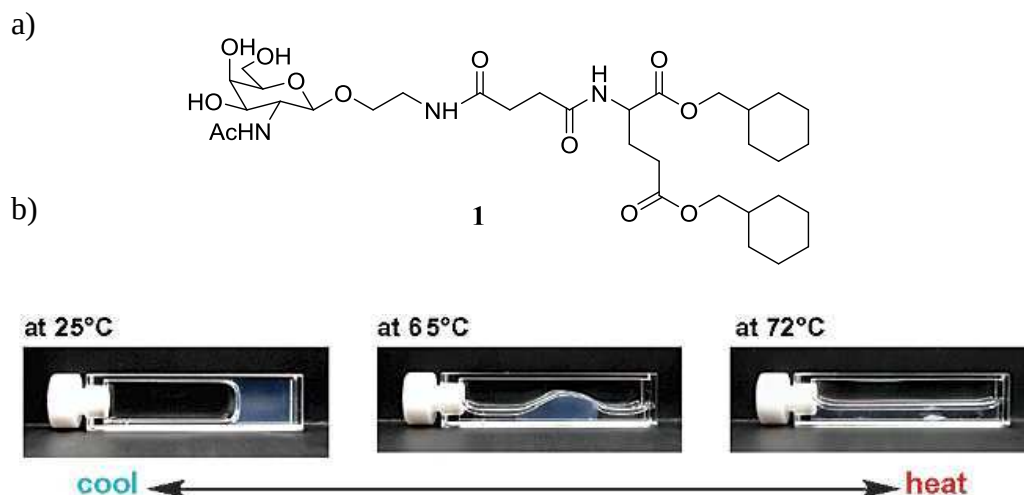


Figure 5.1: (a) Molecular structure of glycosylated amino acid-based hydrogel **1**. (b) Thermally induced phase transition of the hydrogel of **1**. At 25° C water molecules are immobilised in the gel matrix; they are partially expelled at 65 °C; at 72 °C, the compound completely precipitates into a white solid. Taken from Ref. 32.

Enzyme-triggered method: this mechanism consists of triggering the gelation of a self-assembling building block from a precursor molecule with an enzymatic reaction (i.e. hydrolysis). As most of the enzymes' substrates are amphiphilic in nature, mimicking the structure of small self-assembling molecules, such as short peptide sequences, is the simplest approach to this method. Guilbaud and co-workers reported the example of gelation of ionic peptides triggered by a protease. They demonstrated that incubation of the non-gelling precursor tetrapeptide FEFK with the enzyme thermolysin induced gelation of the initial solution. The species responsible for the self-assembly were found to be octapeptides, derived from a reverse-hydrolysis reaction. This method consists of using an enzymatic reaction to couple two precursors together, which form gelling ionic-complementary peptides.³⁶

In order to apply this gelation method for drug delivery purposes, one of the main requirements is to have precursors with biological activity.

Xu and his group successfully reported the first example of a supramolecular hydrogel based on the anticancer agent paclitaxel, commercially known as Taxol[®], with an enzyme-triggered self-assembly. As illustrated in Figure 6.1, they developed a non-gelling precursor based on Taxol[®] (2), covalently conjugated with the self-assembling motif Naphthalene-Phe-Phe-Lys-Tyr- and an enzyme-cleavable group (phosphate). Upon addition of an alkaline phosphatase enzyme, the group was cleaved, allowing the self-assembly of Taxol[®] derivative 3 into nanofibres, from which the antineoplastic agent was slowly released into aqueous medium. Moreover, the newly synthesised hydrogelator and the precursor conserved cytotoxic activity similar to Taxol[®], when tested against HeLa cells.³⁷ This work exemplifies the importance of this gelation method in biomedical applications.

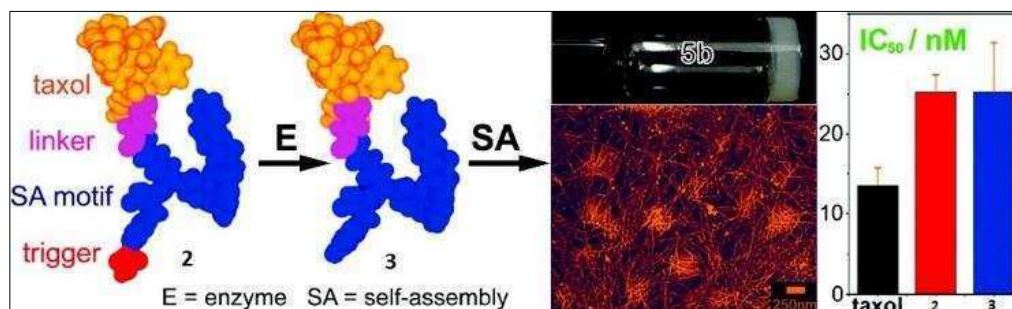


Figure 6.1: Schematic synthesis of taxol precursor (2) and the enzyme-triggered hydrogelator (3), which self-assembles into fibres. Both precursor and hydrogel retain the activity of taxol. Adapted from Ref. 37.

1.2.2. Classification of LMWGs

Since the 1990's several authors started reviewing and classifying LMWGs: in 1997 Terech and Weiss offered a comprehensive review of organogels³⁸ and a few years later Hamilton and Estroff summarised existing hydrogelator literature.²¹ In spite of several reviews published to date and the rational molecular approaches attempted to justify the gelator nature of some molecules,³⁹⁻⁴⁴ the main observation that emerged from these studies is that most of LMWGs have been discovered through serendipity rather than by design. Indeed, even if amphiphilicity can be considered the most common feature of gelator molecules, not all LMWGs are amphiphilic. Therefore, a large amount of research into LMWGs has been focused on the synthesis of new gelator

molecules via the modification and adaptation of existing structures, whereas the rest has been obtained serendipitously.³⁹

In the field of organogels, the first examples reported in the literature were the ALS compounds (**4**, Figure 7.1), i.e. compounds having an aromatic group (A) connected through a functionalised linker (L) to a steroidal derivative (S), usually cholesterol, which demonstrated the ability to gel in most organic solvents. Weiss and co-workers studied this group of organogelators, demonstrating the importance of the aromatic rings (A) in gelation, as they provided the π - π interactions necessary for self-assembly; the linker (L), possessing ester, carbamate, urea or amide functionalities, contributed to other non-covalent interactions, such as hydrogen bonds.⁴⁵

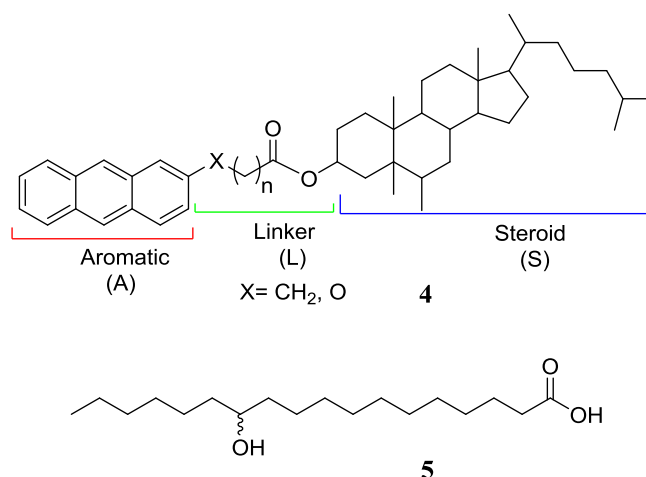


Figure 7.1: Generic structure of ALS (**4**) and 12-hydroxystearic acid (**5**).

Fatty acid derivatives have also been reported to be good organogelators, with 12-hydroxystearic acid (**5**, Figure 7.1) being the most representative example.⁴⁶ Their ability to gel organic solvents depends on the carbon chain length, as longer moieties provide more hydrophobic interactions.⁴⁷

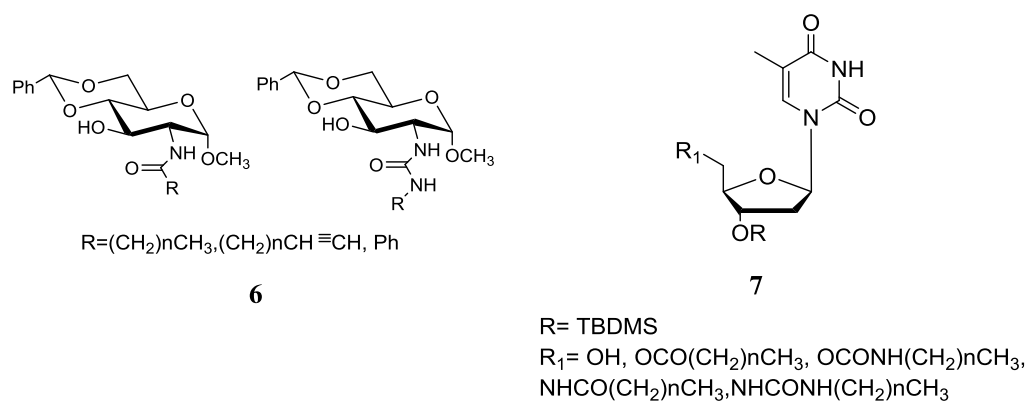


Figure 8.1: Molecular structures of glucosamine derivative (6) and thymidine-based organogels (7).

Among the biomolecules, carbohydrate, amino acid and nucleoside derivatives proved to be both efficient organogelators and hydrogelators.

As organogelators, amide and urea derivatives of glucosamine (6),⁴⁸ L-leucine-based cationic surfactants,⁴⁹ dipeptide-based compounds⁵⁰ and thymidine-based derivatives (7)⁵¹ are some examples (Figure 8.1).

Among the hydrogelators, amino acids and dipeptides represent the most studied group of gelators: a detailed description of these two classes of LMWGs and their properties will be provided in Chapters 2 and 3.

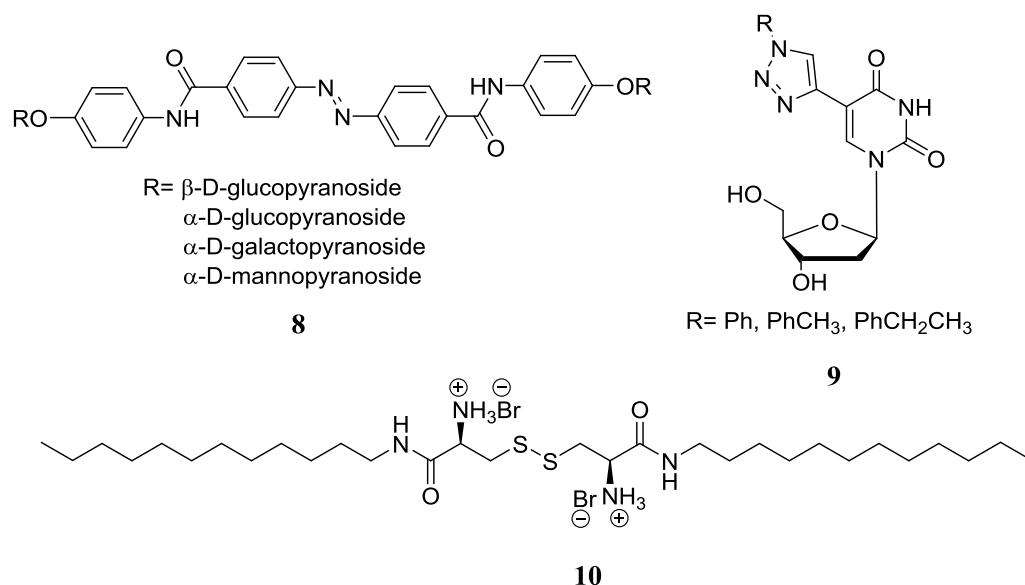


Figure 9.1: Examples of structures of azobenzene-glucopyranosyl derivatives (8), 2'-deoxyuridine hydrogelators (9), and gemini surfactants (10).

Carbohydrates account for another family of hydrogelators: Shinkai and his group pioneered the field of sugar-based hydrogels reporting azobenzene-glucopyranosyl derivatives (**8**).⁵² Among nucleosides, thymidine derivatives,⁵³ 2'-deoxyuridine hydrogelators (**9**)⁵⁴ and diacyluridinophosphocholine derivatives with different acyl chains formed stable gels in aqueous solutions.⁵⁵ Lastly, also gemini surfactants (**10**), i.e. amphiphilic molecules possessing, in sequence, a long hydrocarbon chain, an ionic group, a rigid spacer, a second ionic group, and another hydrocarbon tail, demonstrated to be efficient hydrogelators at low concentrations (Figure 9.1).⁵⁶

1.2.3. Characterisation of LMWGs

Due to the hierarchical structure and complex nature of gels, a full characterisation of LMWGs, from macroscopic to nanoscale level, is still considered a challenge. Therefore, many different approaches can be applied to provide a comprehensive understanding of the self-assembly process of gelators, such as mechanical, microscopy and spectroscopy techniques.

Gels used in drug delivery are required to be mechanically and chemically stable, allowing release of therapeutic agents from the gel matrix. Moreover, in order to promote a targeted drug delivery, a gel's stiffness and network structure are fundamental to ensure its ability to remain localised in a body cavity or tissue.⁵⁷

The first approach is the macroscopic observation of the samples with the so-called “table-top” rheology techniques: they represent preliminary tests used to monitor the gel-sol transition and measure the temperature at which this phenomenon occurs upon heating (T_{gel}). The “tube inversion” test is the simplest method that determines gel formation: if, after gelation, the vial containing the gel is inverted and no flow occurs upon inversion, it can be assumed that the sample has a certain degree of elasticity. Furthermore, once the gel is formed, it is also possible to measure the T_{gel} by immersing the vial into a water bath and heating the sample, to determine the temperature at which the gel is not self-supporting anymore. The “dropping ball” method is an alternative to the previous technique: a small ball is placed on top of a gel and its descent through the gel is observed. Determination of T_{gel} is carried out at different gelator

concentrations by varying the temperature. Both these methodologies are ideal preliminary screens for gelation, because they provide an overall idea of gel formation by initial macroscopic observation. However, their qualitative nature makes them not always reliable and can provide misleading results.

Rheological studies offer a deeper insight into the mechanical properties of gelators, allowing a quantitative characterisation of the tertiary structure of the gel network and distinguishing between weak and strong gels.

When measuring the response of materials to stress-strain, oscillation and stress relaxation, a number of different parameters are defined. G' is called the storage modulus and measures the deformation energy stored by a sample during a shear process; it represents the elastic behaviour of a sample. G'' is called the loss modulus and it is a measure of the deformation energy used by the sample; it represents the viscous behaviour of a sample. Measurements of these parameters against frequency, strain, time or temperature allow determination of the gel's response to different stresses, and investigation of the interactions responsible for aggregation (Figure 10.1). For solid-like gels, G' exceeds G'' by at least an order of magnitude and the presence of a linear viscoelastic (LVE) region is evident, i.e. a region in which both moduli are independent on stress applied. T_{gel} , rate of assembly and the viscoelastic properties are the main variables studied, with strain sweeps and frequency sweeps being the most common methods used.⁵⁸⁻⁶⁰ Moreover, different geometries of plates can be used, such as parallel plates, cone and plates, cup and vane.⁶¹

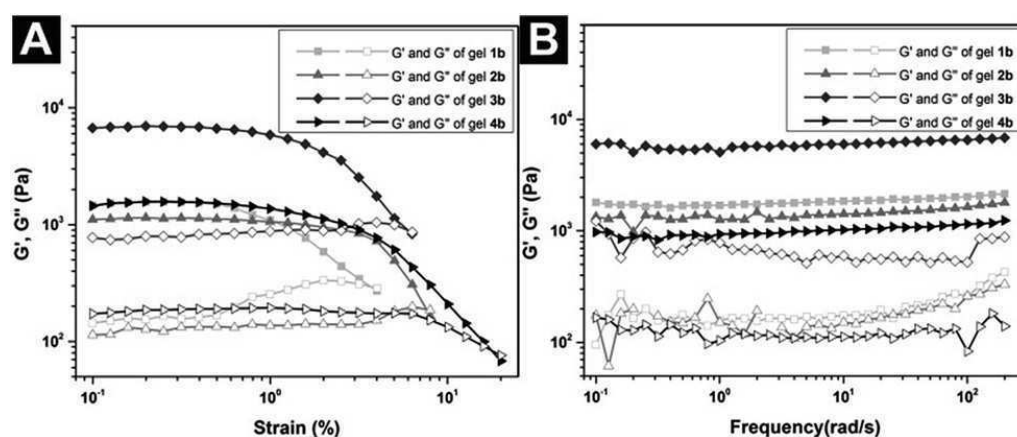


Figure 10.1: Strain sweep (A) and frequency sweep (B) of an enzyme-triggered hydrogelator formed by nucleobases, amino acids and saccharide. Taken from Ref. 59.

Along with the description of mechanical properties of gels offered by rheology, microscopy techniques provide characterisation of the inner morphology of the 3D network of gels, giving important information on the shape of gels' aggregates at a nanometer scale, such as fibres, tapes, ribbons, ranging from 0.01 to 1000 nm. Optical microscopy can provide information about gel structure, but due to its low spatial resolution is not commonly used.⁶² Nowadays, electron microscopy (EM) techniques, such as Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) represent the most frequently used techniques to image the nanostructures of gels at a high resolution (0.2 nm). In both SEM and TEM, the gel sample needs to be first dehydrated (either under ambient conditions or under vacuum), forming a xerogel, which is the collapsed form of the gel (Figure 11.1). In TEM imaging a heavy-metal staining often needs to be applied to increase the image contrast.⁶³

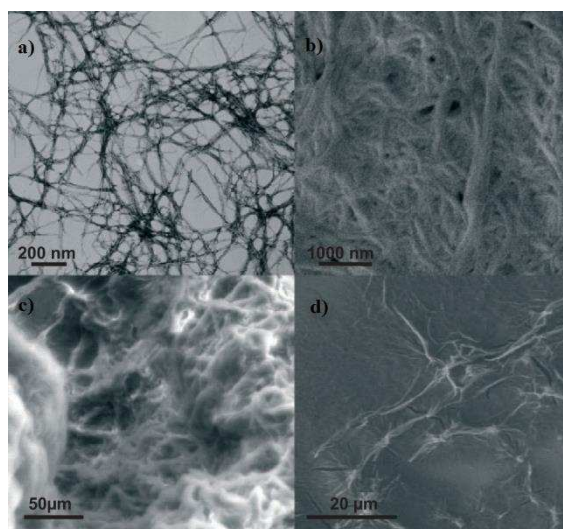


Figure 11.1: (a) TEM image of the fibrous scaffold of self-assembled Fmoc-diphenylalanine hydrogel. (b) Cold-field emission-gun HR scanning electron microscopy (HR-SEM) image of the scaffold fibrils. (c and d) Environmental SEM (E-SEM) images of the 3D, natural, spacious structure of the hydrogel. Adapted from Ref. 63.

Use of cryogenic techniques, such as CryoSEM/TEM, is increasing, as they allow imaging of gels in their hydrated state, without risk of disrupting the self-assembled network.⁶⁴

Atomic force microscopy (AFM) is a scanning probe technique that gives information on the nanoscale morphology of a gel in addition to fibre width and height, without need of dehydration.³⁸

In the last 15 years, X-ray techniques, such as small-angle X-ray scattering (SAXS) and the similar small-angle neutron scattering (SANS) have emerged as innovative characterisation techniques for resolving supramolecular gel structures, as they provide useful information about organisation of fibrillar networks into a 3D order on a mesoscopic scale (1–1000 nm).⁶⁵

Spectroscopic techniques are used to give a deep understanding of the intermolecular organisation of gelators (i.e. primary structure) that cannot be provided by any other technique. Solution state and, more recently, solid state nuclear magnetic resonance (NMR) have been used to study the spatial distribution of gelator species in the gel network and to distinguish between immobilised and solvated molecules in assemblies gels. Indeed, molecules entrapped in the solid-like portion of a gel are characterised by reduced relaxation times (T_2) due their slow diffusion. On the contrary, free monomers in assembled gels have longer T_2 . Therefore, NMR signals of the molecules incorporated within the gel network are broader compared to the free molecules that don't belong to the solid-like phase, due to their reduced molecular motion.³⁵ These effects can be monitored by varying the temperature to promote the gel-sol transition (Figure 12.1).

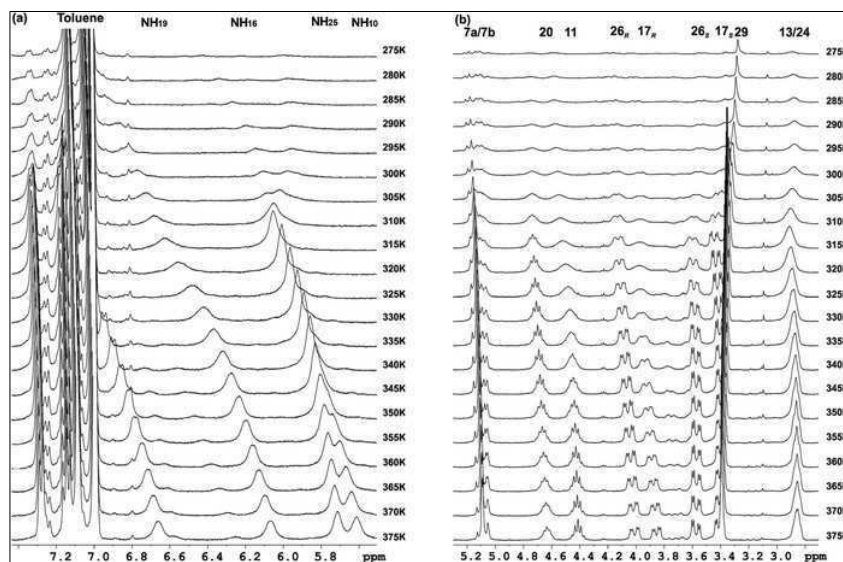


Figure 12.1: Gelation process of a cyclobutane-based peptide monitored by variable-temperature ^1H NMR spectroscopy: (a) NH region from 5.6 ppm to 7.3 ppm. (b) Aliphatic region from 2.8 ppm to 5.2 ppm. Taken from Ref. 68.

Moreover, by integration of the NMR signals, after addition of an internal standard, it is possible to quantify what is immobilised in the solid-like state of the gel and what is in the solvated form.⁶⁶ Simple NMR measurements have also been used to calculate the gel-sol transition temperature (T_{gel}) and the critical gelation concentration values associated with T_{gel} .^{67, 68}

Infrared spectroscopy (IR) is often used to determine the hydrogen bond interactions between gelator molecules. When hydrogen bonds form, a change in bond strength occurs, resulting in shifts of wave number. Also, variable-temperature IR experiments can provide useful information about weak interactions with temperature changes. A drawback of this technique is represented by the liquid part of the gel, which may obscure important parts of the spectrum.^{19, 66, 69}

Fluorescence spectroscopy is used particularly with gelators containing fluorophores, to study the weak interactions that contribute to gelation, such as π - π stacking. Indeed, fluorophores have been often reported to form supramolecular gels due to their hydrophobic surfaces; when they are in the self-assembled state they are characterised by high emission intensities and formation of excimers, whilst in solution they are represented by monomers (Figure 13.1).^{70, 71} Moreover, increased fluorescence intensities can be attributed to formation of aggregate structures that are not present in the solution state.⁷²

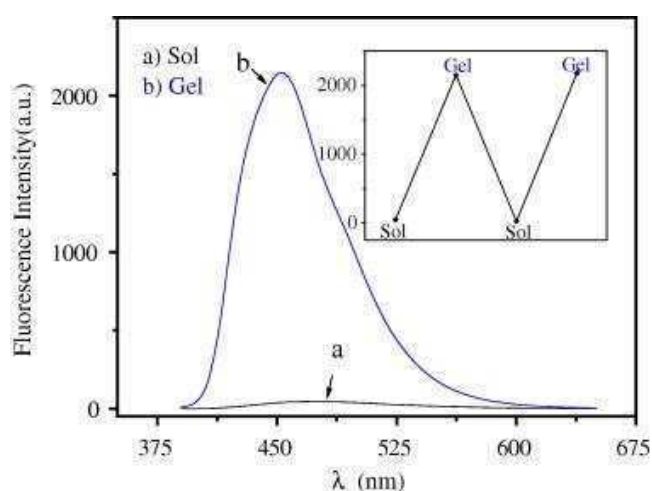


Figure 13.1: The fluorescence spectra of silole (silacyclopentadiene)-based gelators in n-hexane before and after gel formation; the reversible variation of the emission intensity at 453 nm accompanying the solution–gel phase transition is shown. Taken from Ref. 70.

Similarly to fluorescence, ultraviolet-visible spectroscopy (UV-Vis) is applied for characterisation of gelators containing aromatic moieties, for example, to detect changes in hydrophobic interactions within gel formation.⁶⁵

Use of circular dichroism (CD) is associated with analyses of gelators having chiral centres. In particular, CD measures the absorption of left and right circularly polarised light emitted by a chiral gelator as a function of wavelength, giving positive or negative signals. This technique is particularly useful for studying formation of nanoscale chiral aggregates leading to hierarchical self-assembly.^{19, 65}

1.2.4. The innovative role of LMWGs in localised drug delivery

In recent years, LMWGs have emerged as a new potential strategy in localised drug delivery compared to the current polymer-based platforms, with the aim to improve the therapeutic effect especially of drugs that have limited efficacy due to poor solubility, low bioavailability and short plasma half-life.¹⁹ Due to the absence of other excipients in the gel matrix, LMWGs have the advantages of being biodegradable and non-toxic. Therefore, they can be promising new carriers for therapeutic agents.

There are currently three different drug delivery approaches based on LMWGs:

- a therapeutic agent is physically entrapped in the gel matrix and the drug is released from the gel after passive diffusion and gel degradation (Figure 14a.1) ;
- the active drug is covalently conjugated to a functional group in order to create an amphiphilic prodrug which self-assembles under certain conditions forming a gel that can then be degraded to release the active agent in a controlled manner (Figure 14b.1);
- in a similar manner to the previous method, in addition to the functional group, an enzyme-labile moiety is covalently conjugated to the therapeutic compound and the self-assembly occurs only upon enzymatic cleavage (Figure 14c.1).

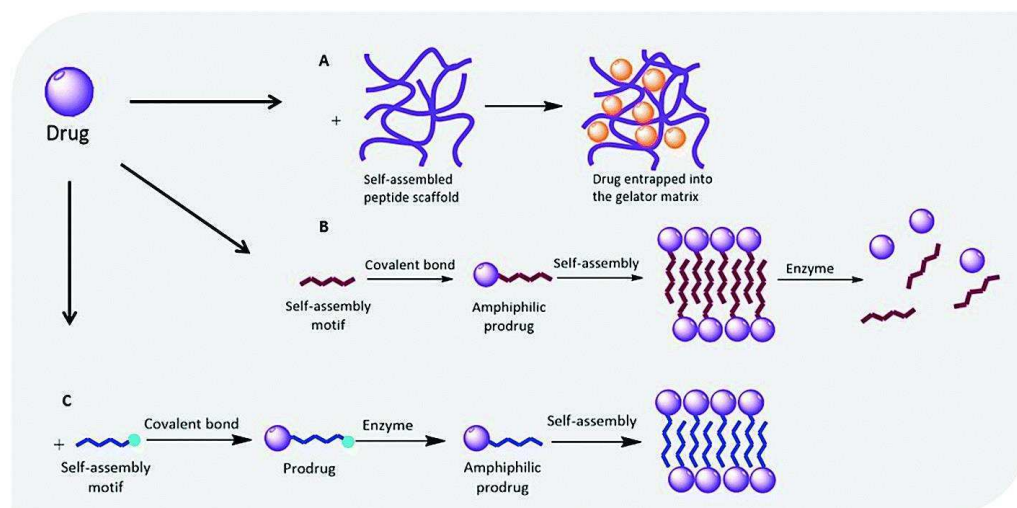


Figure 14.1: The three different approaches of LMWGs for targeted drug delivery. Taken from Ref. 19.

1.2.4.1. LMWGs as drug delivery scaffolds

The first drug delivery approach based on LMWGs consists of using a gel network as scaffold for the passive release of therapeutic drugs.

The most frequently used LMWGs scaffolds are represented by peptide hydrogels, due to their well-known gelation properties and high biocompatibility and biodegradability. Zhang and his group pioneered the field of peptide hydrogels for drug delivery: they reported a self-assembling long chain peptide formed by 16 amino acids (RADA16), which was used as a scaffold for release of different dyes, such as phenol red and bromophenol blue, demonstrating that the rate of passive diffusion of the molecules correlated with the dyes' structure and the peptide concentration in the gel.⁷³ The same group also studied the controlled release properties of enzymes such as lysozyme and trypsin from the self-assembling peptide Ac-(RADA)₄-CONH₂, showing different release based on protein molecular weight and density of peptide fibres. In order to improve the drugs' controlled release, they also suggested synthesising tailor-made peptide hydrogels for each compound to be delivered from the gel matrix, depending on its size and physicochemical properties.⁷⁴ Adams et al. studied the release of model drugs from Fmoc protected amino acid hydrogels, such as Fmoc-phenylalanine and Fmoc-tyrosine, and calculated the rate of drug release, demonstrating that the drugs' diffusion followed Fick's law.⁷⁵

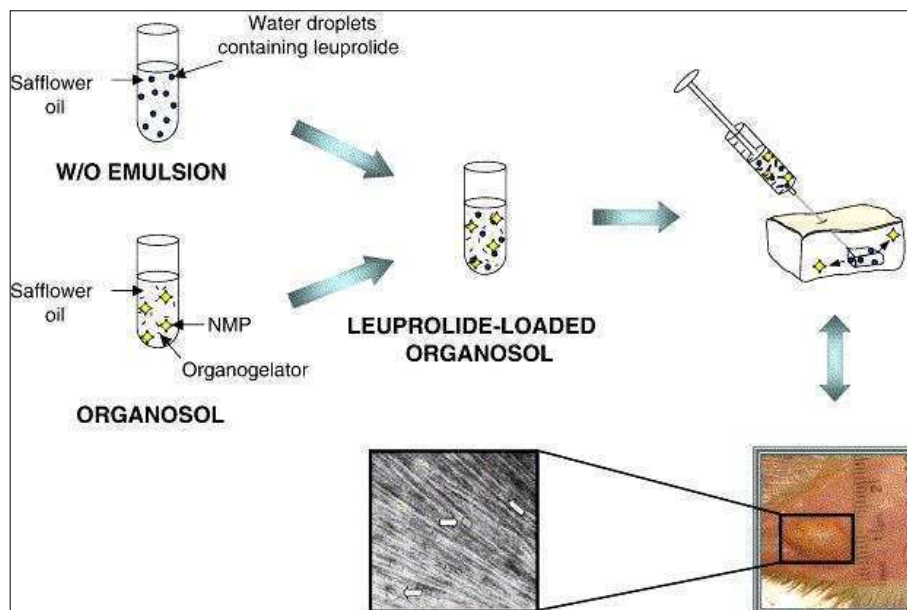


Figure 15.1: Schematic representation of preparation and administration of in situ-forming implants. The formulations were prepared by mixing the L-alanine organosol with an emulsion of leuprolide. Gelation was firstly inhibited by addition of NMP; then, upon subcutaneous injection of the system, NMP diffused out, allowing gelation of the implant. The bottom panel represents photomicrograph of the gel (left) with entrapped water droplets and a subcutaneous implant 24 h after injection (right). Taken from Ref. 78.

Gels have also been used as drug delivery devices for antitumour agents, as they increase in situ drug concentration and efficacy and minimise the side effects associated with systemic administration. Stupp and co-workers used self-assembling peptide fibres as carriers for anticancer drugs. They encapsulated the hydrophobic chemotherapeutic agent camptothecin into a self-assembling compound consisting of a hydrophobic palmitic tail linked to an Ala₄-Gly₃-Glu₃ sequence. Studies demonstrated enhancement of the drug's solubility of 50-fold and the ability of the amphiphilic fibres to inhibit tumour growth in vitro and in vivo.⁷⁶

Due to their reduced biocompatibility and risk of local inflammation, applications of organogel scaffolds in drug delivery are limited. However, the lack of hydrophilic excipients make organogels ideal carriers for poorly-water soluble drugs. Leroux et al. developed organogels formed by N-lauroyl L-alanine derivatives and soybean oil or medium chain triglycerides, showing in vitro sustained release of fluorescein isothiocyanate-dextran over a 20 day period. After subcutaneous injection in rats, the organogel formed in situ, revealing the potential of this LMWG for drug delivery applications.⁷⁷ Indeed, the same group

used an N-stearoyl L-alanine organogel in safflower oil for site-specific delivery: they formulated leuprolide, a hormone for treatment of prostate tumour and endometriosis, with this organogel and administered it in situ in rats, revealing release of the anticancer drug over a period of 14 to 25 days (Figure 15.1).⁷⁸ Furthermore, biocompatibility studies on L-alanine organogels in safflower oil showed minimal inflammatory reactions to these drug carriers, proving the safety of these biomaterials for drug delivery.⁷⁹

1.2.4.2. Self-assembling LMWGs prodrugs

The second and third strategies of LMWGs for drug delivery are quite similar, as they both involve the synthesis of amphiphilic self-assembling prodrugs derived by covalent conjugation of the active drug with functional groups. After the self-assembly, these systems release the therapeutic agent via hydrolysis or enzymatic degradation. However, the third approach slightly differs from the second one because, together with a functional group, an enzyme-labile moiety is introduced in the molecular structure, and this makes the amphiphilic compound an enzyme-triggered substrate for the self-assembly.

Both strategies, which have been applied for the development of LMWGs self-assembling prodrugs, possess significant advantages compared to the approach described in the previous section. In order to overcome some limitations of hydrogel-based scaffolds, such as low drug encapsulation and burst drug release, these two new approaches have been introduced with the aim of increasing drug loading, since each part of the gel contains drug molecules. Moreover, no stabilising agents are used, as the gel consists of the prodrug itself, therefore no excipient-drug interactions can arise.⁸⁰

The choice of the gelating group to be covalently conjugated to the drug is pivotal: amino acids, di- or tri-peptides, fatty acids and surfactants represent functional moieties that have been reported to gel and can be used in the synthesis self-assembled prodrugs.¹⁹

Different classes of self-assembling prodrugs have been developed and investigated, ranging from antibacterial to anti-inflammatory and chemotherapeutic agents.

Xu and co-workers reported the first example of a self-assembling prodrug hydrogel: they modified the antibiotic vancomycin by introducing a pyrene group, responsible for π - π stacking (Figure 16.1), at the C-terminal of the vancomycin backbone via an amide bond.⁸²

Kim et al. developed low molecular weight hydrogels based on the anti-inflammatory agent ibuprofen, by addition of different dipeptides to the free carboxylic acid group of the active compound. The Gly-Gly derivative was the most successful in displaying self-assembling properties in water; the group also carried out drug release studies, by incubating the gel matrix with a carboxypeptidase. Results showed release of the active drug ibuprofen from the hydrogel matrix, as a result of enzymatic degradation of the prodrug.⁸³ Organogelators based on the non-steroidal anti-inflammatory drug (NSAID) ketoprofen have also been reported: they were obtained by derivatisation of the drug with long hydrocarbon chain of fatty acids via a diacylhydrazine linker and they were found to self-assemble in carbon tetrachloride.⁸⁴

Vemula is also another pioneer of self-assembling prodrugs: his group synthesised amphiphilic and bola-amphiphilic derivatives of paracetamol that formed hydrogels at low concentrations (0.4-1.5 % w/v). They demonstrated that the enzymatic-triggered method can be applied to these hydrogels: incubation of the gel with lipase induced gel-sol transition in less than 48 hours, meaning that paracetamol was efficiently released from the gelator prodrug.^{85, 86}

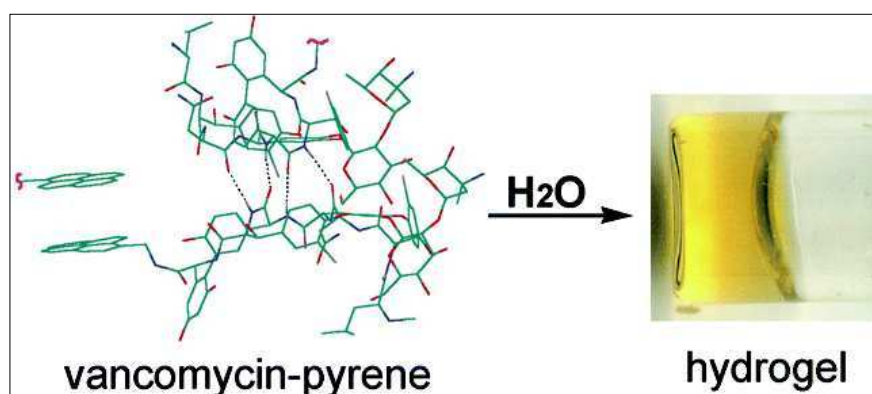


Figure 16.1: Illustration of π - π stacking and multiple hydrogen bonding (dotted lines) in the vancomycin-pyrene hydrogel (left) and optical image of the gel (right). Taken from Ref. 82.

In addition to the examples cited above, the introduction of self-assembling LMWGs prodrugs in drug delivery is revealed to be innovative for the development of local delivery of anticancer drugs, particularly for those patients with solid tumours. In fact, existing chemotherapeutic approaches are failing to successfully treat many intractable solid tumours, due to aggressiveness of disease, increased tumour size and drug resistance. Moreover, formulation of an antitumour drug as a self-assembling LMWG prodrug can improve drug physicochemical properties, such as solubility and bioavailability. As a consequence, LMWGs can be used as drug delivery platforms for intra-tumoural delivery: these systems can be employed to locally release antineoplastic drugs, increasing their in situ concentration and reducing the side effects associated with systemic administration.⁸⁷

So far, most of the examples in the literature of chemotherapeutic LMWGs are the ones based on the anticancer drugs Taxol[®] and camptothecin.

Xu and his group were the first to report the self-assembly of a paclitaxel derivative via an enzyme-triggered method (section 1.2.1.2), that exemplifies the third strategy of LMWGs in drug delivery.³⁷

With a similar approach, Yang et al. used the same antineoplastic drug to develop a folic acid-paclitaxel hydrogel: folic acid was chosen because of its known propensity to form stable tetramers; this moiety was then linked to Taxol[®] via a phosphorylated and succinated tripeptide Gly-Tyr-Lys, designed to act as a substrate for the enzyme phosphatase and increase water solubility. Upon enzymatic dephosphorylation after addition of the phosphatase, the Taxol[®] derivative exhibited self-assembling properties in water, forming a hydrogel with nanosphere morphology. The self-assembling derivative also displayed the ability to conserve a similar anticancer activity to that of Taxol[®].⁸⁸ These results proved the successful use of folic acid as a versatile functional group in the synthesis of hydrogelators of hydrophobic drugs.^{89, 90}

The same group also reported another example of hydrogel (**11**) based on Taxol[®], formed by the active drug linked via an ester bond to succinic acid connected to oxidised glutathione (Figure 17.1). Gelation occurred in phosphate buffered saline (PBS), after hydrolysis of the ester bond between Taxol[®] and succinic acid.

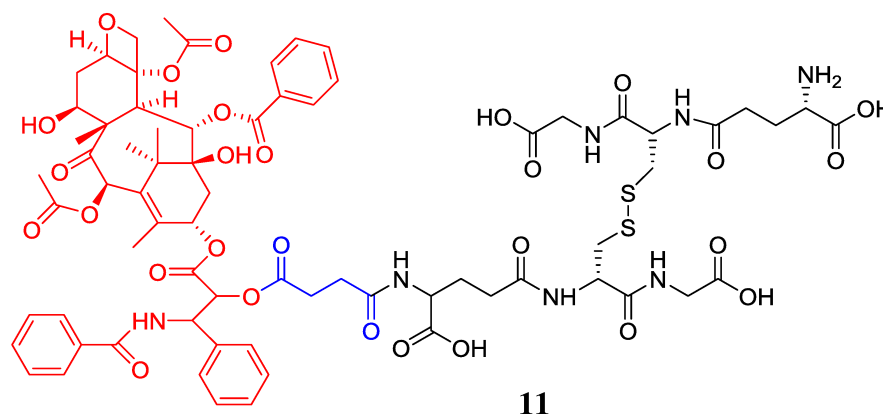


Figure 17.1: Chemical structure of precursor of hydrogelator **Taxol-succinic acid-GSSG (11)**. Taken from Ref. 91.

The innovative nature of this work, compared to previous Taxol[®] gelators, was revealed in in vivo studies. Results showed that when the hydrogelator was locally injected, it was more efficient in hindering tumour growth and preventing breast cancer metastasis than the traditional Taxol[®] intravenous injection (Figure 18.1).⁹¹

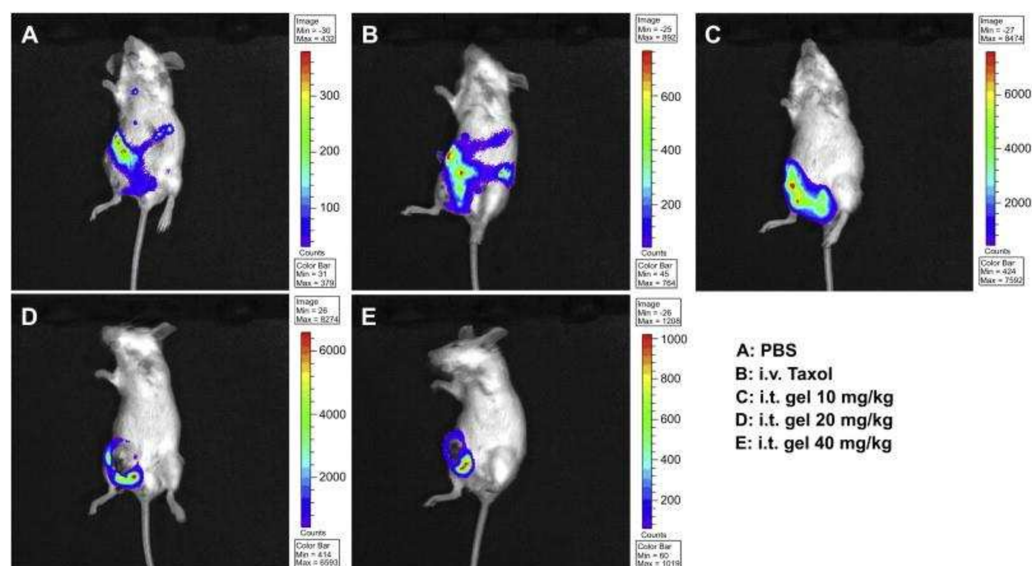


Figure 18.1: Bioluminescence imaging on luciferase breast-tumour bearing mice after 12 days of treatment. Taken from Ref. 91.

Recently, the same group also reported the development of a supramolecular hydrogel of curcumin, a compound known for its anticancer properties. They

synthesised a curcumin-based precursor, covalently linked to the peptide sequence Phe-Phe-Glu-ss-Glu-Arg-Gly-Asp.

Upon incubation with glutathione, disulphide bond reduction occurred, with subsequent self-assembly of the precursor into nanofibres, which exhibited all the properties typical of LMWGs. However, when tested *in vivo* in xenografted mice bearing breast tumours, the gel didn't display potency in inhibiting cancer growth, thus suggesting applications of this hydrogel only for topical cancer treatment.⁹²

Cui and his group developed amphiphilic derivatives of the antineoplastic agent camptothecin (CPT): they synthesised CTP conjugates connected by a reducible disulfylbutyrate (buSS) linker to a Tau-peptide derivative (Figure 19.1). The conjugates self-assembled in water, forming nanofibres or nanotubes, depending on the number of CPT attached in the molecule (one, two or four CPT per CPT-conjugate). Two CPT conjugates containing a non-reducible linker were also developed, as control molecules. The group performed release tests in presence and absence of the reducing agent glutathione, in order to demonstrate the key role played by the reducible linker in releasing the active drug. CPT derivatives bearing the reducible linker showed faster release of CPT in the presence of glutathione than in its absence. The compound with the non-reducible linker, susceptible to ester hydrolysis only, did not show any difference in release between the two conditions tested. Moreover, they demonstrated that the self-assembled nanostructures can act as reservoirs to guarantee continuous release of CPT in the presence of glutathione.⁹³

All these studies prove the practical high potential of chemotherapeutic LMWGs in the treatment of solid tumours and suggest that more investigations need to be carried out for expanding and developing this emerging field.

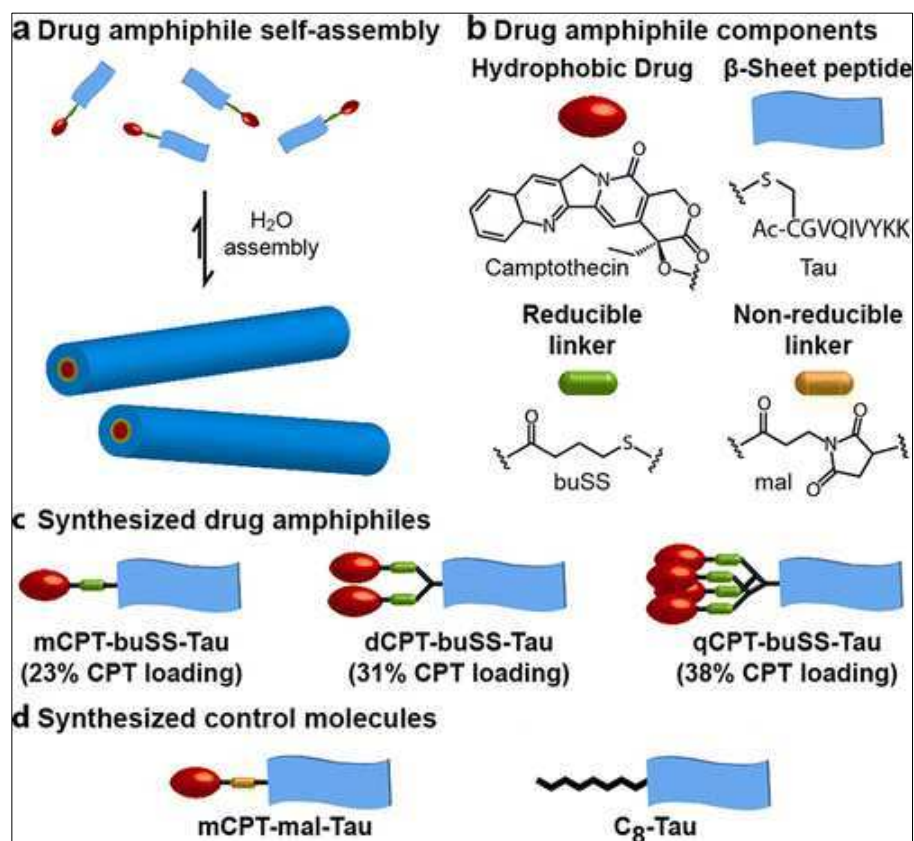
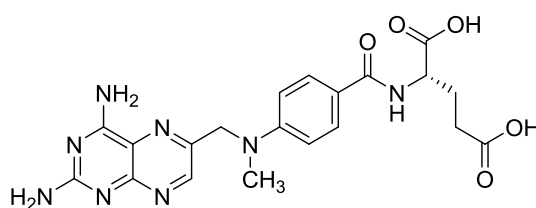


Figure 19.1: Schematic representation of CTP amphiphilic conjugates: (a) Self-assembled drug amphiphiles (DA) nanostructures. (b) Three key component parts of the drug amphiphiles: the drug CPT, the Tau- β -sheet-forming peptide, and the buSS biodegradable linker. (c) The synthesised CPT DAs with quantitative drug loadings of 23%, 31%, and 38%. (d) The two synthesised control molecules. Taken from Ref. 93.

1.3. A traditional chemotherapeutic drug: the antimetabolite methotrexate

Methotrexate (MTX) **12** (Figure 20.1), also known as amethopterin, is an antifolate, a folic acid antagonist. Developed in the early 1950s, it is still one of the leading drugs used for several medical applications, ranging from treatment of many types of cancer (i.e leukaemia, lymphoma, breast and bladder cancer, osteogenic sarcoma)⁹⁴ to autoimmune diseases (rheumatoid arthritis, psoriasis, Crohn's disease)^{95, 96} and ectopic pregnancies.⁹⁵

The first attempts to synthesise folic acid analogues started after studies that demonstrated a remission of disease in patients affected by leukaemia, as a consequence of induced folic acid deficiency. This discovery led to the development of antagonists of folic acid: the first one that proved its efficacy in treatment of acute lymphocytic leukaemia in children was aminopterin. After this, the mechanism of action of aminopterin was clarified revealing that it was able to inhibit the enzyme dihydrofolate reductase (DHFR, i.e. an enzyme involved in the conversion of folic acid and dihydrofolic acid into tetrahydrofolic acid). Isolation and full characterisation of this enzyme led to the quest to seek more selective and potent antifolates. As a result, in 1956 methotrexate was first introduced in the treatment of choriocarcinoma and it showed greater efficacy compared to aminopterin, thus supplanting its use in the clinic.⁹⁵



12

Figure 20.1: Molecular structure of MTX (**12**)

MTX, named as (S)-2-[[4-[[[(2,4-diaminopteridin-6-yl)methyl]methylamino]benzoyl] amino]pentanedioic acid, differs from folic acid in the presence of a 4-amino group on the pterin ring in place of a hydroxyl group and a methyl group at the N¹⁰ position.

MTX can be administered orally or parenterally: in oral absorption, it shows an average oral bioavailability of 36%.⁹⁷ After intravenous injection, it rapidly distributes and concentrates mostly in the liver, bile, kidneys and skin and it binds to serum albumin (~50%). The metabolism of MTX is mainly due to bacterial intestinal flora during its enterohepatic circulation. Its major metabolite is 4-(N-(2,4-diamino-6-pteridinylmethyl)-N-methylamino) benzoic acid (APA), formed by the cleavage of the amide bond operated by carboxypeptidases.⁹⁸ After treatment at high doses, the 7-hydroxymethotrexate metabolite is also formed, derived from the action of oxidases in human liver.

1.3.1. Methotrexate mechanism of action, resistance and toxicity

MTX's cytotoxicity derives from its intracellular mechanism of action. MTX, being an analogue of folates, is metabolised in the same pathway: it is a competitive antagonist of folates, acting as a substrate of the DHFR enzyme involved in biosynthesis of factors, such as the nucleoside thymidine and purines, essential for DNA and RNA synthesis.

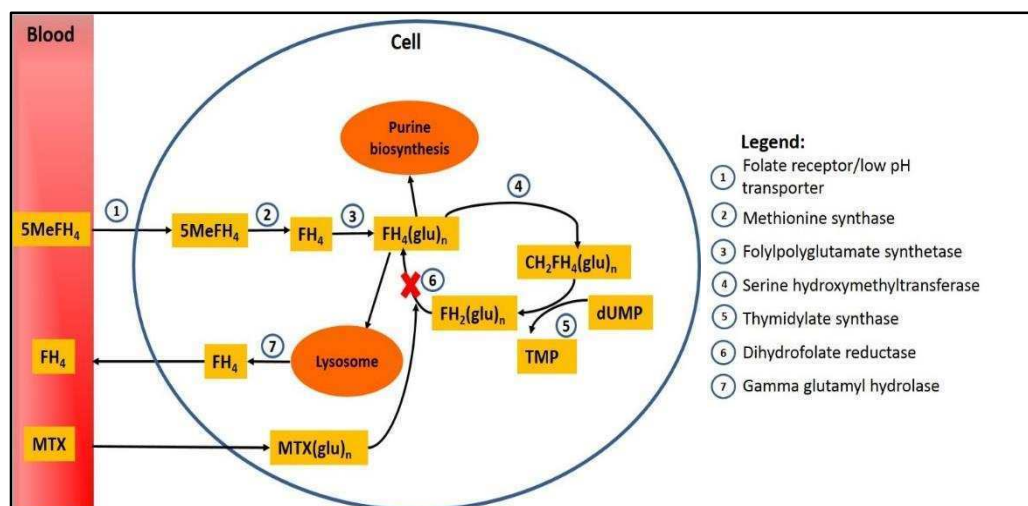


Figure 21.1: Folate metabolism and MTX mechanism of action.

Consequently, it acts during G1 and S phases of the cell cycle, having a greater effect on tissues with a high cellular turnover, such as many tumour cells.⁹⁹

Normal folate metabolism (Figure 21.1) starts with 5-methyltetrahydrofolate (5MeFH₄), the major folate present in human serum, which is carried into cells and converted into tetrahydrofolate (FH₄) by methionine synthase, which also synthesises methionine from homocysteine. FH₄ is then polyglutamylated into FH₄(glu)_n, and methylated as 5, 10-methylenetetrahydrofolate (CH₂FH₄(glu)_n), which is a substrate of thymidylate synthase for the synthesis of thymidine monophosphate. The resulting FH₂(glu)_n is then converted by the enzyme DHFR into FH₄(glu)_n. CH₂FH₄ is also transformed into 10-formyl tetrahydrofolate, an important cofactor for purine biosynthesis. Eventually, polyglutamylated FH₄ is transported into lysosomes and transformed into FH₄ monoglutamate by gamma glutamyl hydrolase and then released outside the cells via an energy dependent transporter.⁹⁵

Being an analogue of folates, MTX enters cells via the same mechanisms of transport: it can bind to reduced folate receptor, which has a higher affinity for MTX than for folates;¹⁰⁰ it can be transported by a pH and temperature-dependent carrier with high-affinity to reduced folates, particularly across intestinal epithelia.¹⁰¹ Once across the cellular membrane, MTX is polyglutamylated by the enzyme folylpolyglutamyl synthetase which adds up to five glutamate groups to the γ -carboxyl glutamate of native MTX.

MTX polyglutamates represent an important step of MTX mechanism of action: firstly because they bind to DHFR with an equal or even greater affinity than the unmetabolised drug; secondly because they can be considered as an intracellular MTX depot, which exit the cells more slowly compared to native MTX. Indeed, the greater the polyglutamylation, the higher the retention time within cells. For these reasons, MTX can be considered a prodrug since it needs to be polyglutamylated in order to be activated.^{101, 102}

After MTX introduction in the clinic, drug resistance was shown to occur in some patients, thus resulting in failure of cancer treatment. Later studies demonstrated that resistance to MTX was related to its mechanism of action, particularly to inhibition of DHFR.⁹⁵ Indeed, studies on experimental systems found that MTX resistance is characterised by increased DHFR

activity due to gene amplification and a reduction of binding affinity of DHFR to the drug. Moreover, decreased MTX transport into cells, due to a reduced folate carrier expression, has been associated with MTX resistance. Other less common mechanisms include decreased MTX polyglutamylation, resulting in diminished cell retention, and an increase in folate pools, which competes with MTX for DHFR binding.⁹⁵

MTX chronic use can also lead to severe systemic side effects: gastrointestinal mucositis, hepatitis, immunosuppression, alopecia, renal dysfunction, skin disorders are described as main toxicities associated with its use.⁹⁹

1.3.2. Methotrexate prodrugs

As discussed above, even though MTX is still one of the most commonly used drugs for many diseases, its therapeutic efficacy is hindered by some critical aspects: the main problem is represented by drug resistance, which often occurs when MTX is initially effective; secondly, it has a short plasma half-life, which requires high doses of administration producing high cell toxicity in normal cells.^{103, 104} For these reasons, after MTX's discovery, many attempts have been carried out in order to overcome these problems and one of the main strategies involved the synthesis of MTX prodrugs.

Prodrugs are chemically modified parent drugs characterised by pharmacological inactivity but with better properties in terms of solubility, absorption and bioavailability. The conversion from the prodrug into the parent drug occurs by hydrolysis. An enzymatic reaction (i.e. enzymatic hydrolysis) could also promote the release of the active compound in one or two enzymatic steps. Prodrugs are synthesised by covalently linking the parent drug with different functional groups, depending on the drug characteristics to be improved. The most commonly used linkers in prodrug synthesis are ester bonds, but drugs can be also functionalised with other groups forming amides, carbamates or oximes, even though their use is limited by a higher enzymatic stability in vivo compared to esters.⁸¹

Rosowsky, a pioneer during the late 1970s in MTX prodrug synthesis, prepared a series of MTX analogues with higher lipophilicity compared to the native compound, in order to improve MTX uptake into cells via membrane passive diffusion rather than active transport. His group synthesised mono- and diester derivatives of MTX (**13**, Figure 22.1) functionalised at the α or γ glutamic acid carboxylic terminus (monoesters) or at both positions (diesters), demonstrating their potential anticancer activity in vitro.¹⁰⁵⁻¹⁰⁸

Later on, Huennekens et al. derivatised the MTX α -carboxyl group of the glutamic acid moiety with an amide bond, reporting the synthesis of α -phenylalanine (**14**, Figure 22.1) and α -peptides prodrugs of MTX. In vitro incubation of these prodrugs with carboxypeptidase A led to conversion of MTX α peptides into native MTX, demonstrating in this way the key role of enzymatic hydrolysis in parent drug activation.^{103, 109, 110}

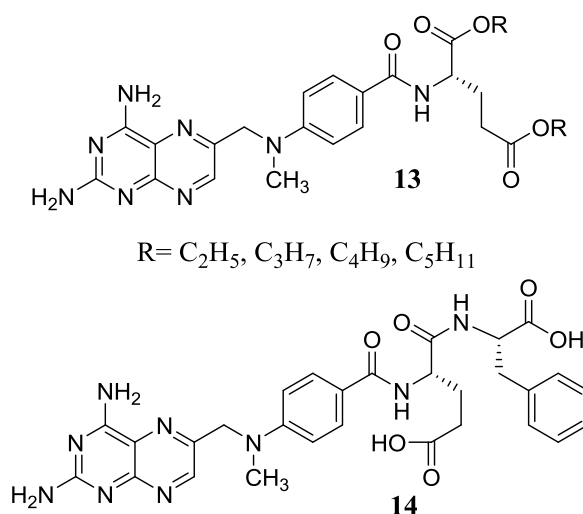


Figure 22.1: Molecular structures of MTX prodrugs **13** and **14**.

In order to overcome MTX transport resistance, lipophilic MTX conjugates have been synthesised to promote the passive internalisation of the drug into malignant cells. Pignatello and his group prepared MTX derivatives formed by an amide linker between α and γ glutamic acid carboxylic terminal and short-chain alkylamino acids (**15**, Figure 23.1), these prodrugs maintained the in vitro cytotoxicity of MTX.¹¹¹

1.4. A new antitumour candidate: the benzothiazole 5F 203

In medicinal chemistry, compounds based on the heterocyclic benzothiazole represent an important class of molecules with several biological properties, such as antimicrobial, antitubercular, antimalarial, antiviral and antidiabetic activities.¹¹² The first reports of the cytotoxic effects of benzothiazoles were described in the 1950s;¹¹³ however, the first comprehensive in vitro and in vivo studies about benzothiazoles were developed in the late 1990's, when Stevens' and Bradshaw's group investigated the antitumour properties of benzothiazole-based compounds against different cancer cell lines.¹¹⁴ In particular, the group pioneered the development of benzothiazole compounds, that led to the identification of the clinical trial candidate Phortress.¹¹⁵

In a research programme aimed to study the activity of polyhydroxylated-2-phenylbenzothiazoles against human cancer cell lines,¹¹⁶ the intermediate compound 2-(4-aminophenyl)benzothiazole (CJM 126, **17**, Figure 24.1) displayed growth inhibitory activities against MCF-7 and MDA-468 human-derived breast cancer cell lines at nanomolar concentrations ($GI_{50} < 1 \mu M$, being GI_{50} the concentration of a test compound required to inhibit cells' growth by 50%). However, a biphasic dose-response relationship, i.e. the occurrence of a second proliferative phase at high concentrations (Figure 25.1), emerged when the compound was tested at concentrations between $1 \mu M$ and $30 \mu M$, with formation of proliferating colonies. At concentrations $> 30 \mu M$ the compound displayed again cytotoxic activity. Structure activity relationship (SAR) studies on CJM 126 and its analogues led to development of compound DF 203 (**18**, Figure 24.1), finding that introduction of a methyl group at the 3' position of the benzothiazole ring led to an extended cytotoxic activity. In vivo studies of DF 203 in mice bearing breast carcinoma demonstrated consistency of the compound in inhibiting tumour growth in all xenograft models.¹¹⁷

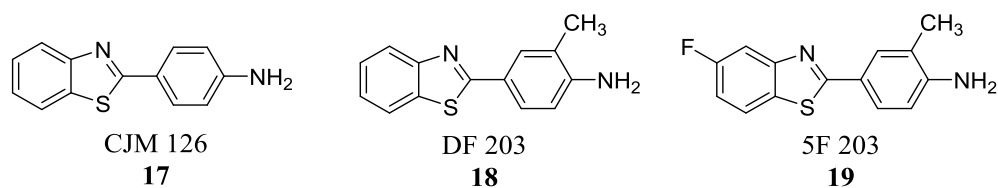


Figure 24.1: Molecular structures of CJM 126 (**17**), DF 203 (**18**) and 5F 203 (**19**).

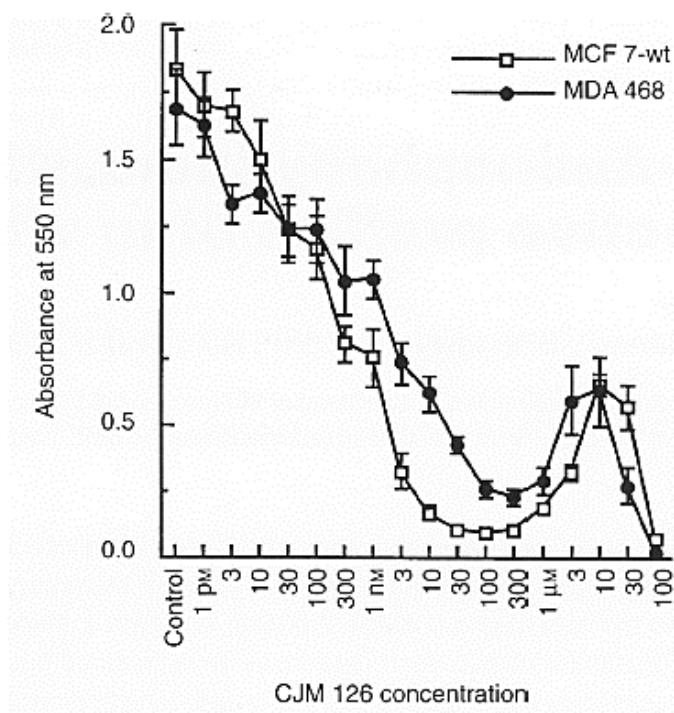


Figure 25.1: Example of biphasic dose-response of CJM 126 against human breast carcinoma cell lines MCF-7 and MDA 468. Taken from Ref. 117.

More in vitro studies of DF 203 against human derived cancer cell lines, revealed selective cytotoxicity against particular breast (MCF-7, MDA-MB-468, T-47D, SK-BR-3), ovarian (IGROV-1, OVCAR-3, OVCAR-5) and renal (TK-10) cell lines, whilst some other breast (MDA-MB-435), ovarian (OVCAR-8), colon (KM-12) and central nervous system (CNS) (SNB-19) cells showed resistance against this compound ($GI_{50} > 100 \mu M$). DF 203 displayed also in vivo activity in arresting tumour growth against HCT-116 human colon xenograft tumours.¹¹⁷ However, in vitro, DF 203 still showed a biphasic dose-response. Moreover, at high concentrations, production of the inactive metabolite 6OH 203 was observed, meaning that oxidation (6-hydroxylase activity) was the main metabolic pathway of this family of 2-(4-aminophenyl)benzothiazoles (later determined to be mediated by the enzyme cytochrome P450 1A1, Figure 26.1). Consequently, the biphasic dose-response relationship of DF 203 could have been explained by the lack of cytotoxicity of the metabolite 6OH 203 ($GI_{50} > 10 \mu M$): metabolite's production at high concentrations led to diminished growth inhibitory activity of DF 203.¹¹⁸

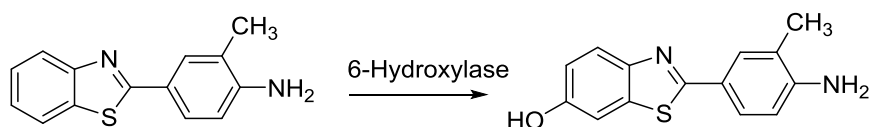


Figure 26.1: Structures of DF 203 and its main metabolite 6OH 203.

In order to overcome these limitations, fluorination was adopted as a strategy to prevent undesired hydroxylation. Therefore, a study aimed to develop mono and di-fluorinated derivatives of DF 203 was carried out.¹¹⁹ Although all the synthesised compounds retained a high potency ($GI_{50} < 1$ nM) in breast, colon and renal cancer cell lines, the 4- and 6-fluorobenzothiazoles still elicited the biphasic dose-response relationship. However, the 5- and 7-fluoro isomers displayed a conventional dose-response, and negligible production of 6OH 203 metabolite was detected in the presence of sensitive MCF-7 breast carcinoma cell lines. When tested in the National Cancer Institute (NCI) panel, the 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203, **19**, Figure 24.1) showed stark selectivity and enhanced potency against human-derived cancer cell lines. In vivo, 5F 203 demonstrated to be highly selective against MCF-7 breast cancer xenograft tumours, whilst it was not active in resistant MDA-MB-435 models.¹²⁰

1.4.1. 5F 203 mechanism of action

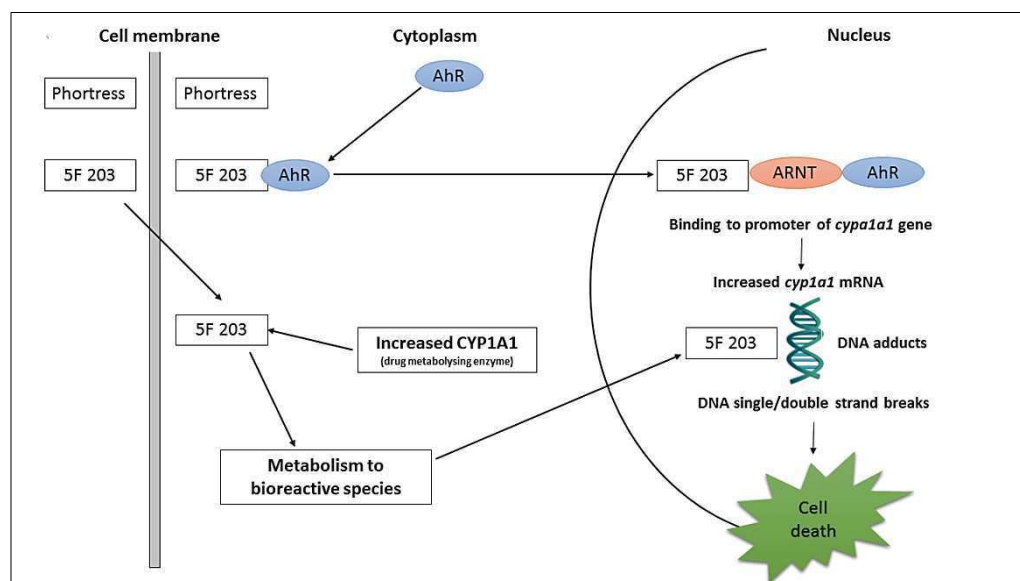


Figure 27.1: Mechanism of action of 5F 203 and its main prodrug Phortress.

Due to the unique cytotoxicity pattern of the 2-(4-aminophenyl)benzothiazoles compounds in the NCI panel, studies to explain their innovative mechanism of action were carried out.

Both DF 203 and 5F 203 were shown to induce expression and activation of cytochrome P450 1A1 (CYP1A1) within sensitive tumour cells.^{121, 122} After binding to the cytosolic arylhydrocarbon receptor (AhR), the activated complex of 5F 203 and the receptor translocated to the nucleus to induce transcription of gene *cyp1a1* and consequent expression of CYP1A1. Incubation of DF 203 with recombinant CYP enzymes revealed a 6-OH metabolite formed in significant quantities following incubation with CYP 1A1. Cells were exposed to DF 203 or 5F 203 and lysates prepared, showing a CYP 1A1 dose-and time-dependent induction. These experiments corroborated the role of CYP 1A1 in 5F 203's mechanism of action. Subsequently, after induction of CYP1A1 and in the presence of NADPH, DF 203 was converted into a more electrophilic reactive species, which subsequently bound to DNA, leading to formation of DNA single strand breaks (SSB) and double strand breaks (DSB). These DNA lesions induced DNA damage, cell cycle arrest and apoptosis (Figure 27.1).

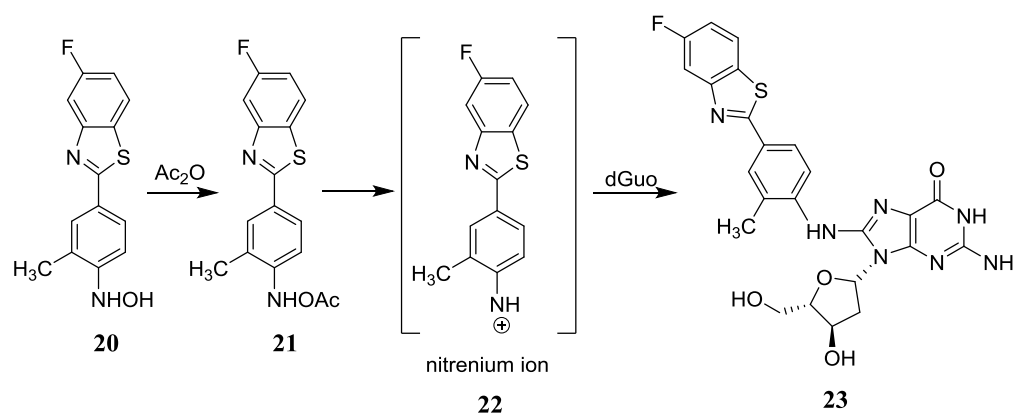


Figure 28.1: Formation dGuo adducts between the hydroxylamine product of 5F 203 (obtained by oxidation of 5F 203 with CYPs 1A1 and 2W1) and deoxyguanosine. Adapted from Ref. 125.

Formation of DNA adducts was corroborated both in vitro and in vivo studies, with detection of adducts in sensitive cells only (MCF-7 and IGROV-1).^{115, 123, 124} Wang and Guengerich also proved that nitrenium intermediates represented the reactive species responsible for formation of DNA adducts: by incubating 5F 203 with recombinant CYPs 1A1 or 2W1, they demonstrated generation of a hydroxylamine product (**20**). Reaction of this compound with deoxyguanosine (dGuo) led to generation of dGuo adducts via formation of nitrenium species (Figure 28.1).¹²⁵

1.4.2. 5F 203 prodrugs and related toxicity

In the development programme of antitumour benzothiazoles, the next stage was to find an adequate route of drug administration able to guarantee a high drug bioavailability and, possibly, to avoid first pass hepatic metabolism. Therefore, a parenteral formulation was chosen; however, 5F 203 lipophilicity and poor aqueous solubility were limiting factors for the development of the formulation.

To overcome these physicochemical problems, the first attempt involved the synthesis of sulfamate salts of 5F 203; however, degradation into the active free amine occurred at acidic conditions ($\text{pH}=4$) and at high temperatures ($50\text{ }^{\circ}\text{C}$).¹²⁶ Therefore, a new strategy was introduced and it consisted in the synthesis of amino acid prodrugs of 5F 203, by covalently conjugating the active drug with previously Boc-protected amino acids via an amide bond.

The L-Lysyl- (**24**) and L-alanyl-amide (**25**) prodrugs of 5F 203 (Figure 29.1) were synthesised and demonstrated appreciable water solubility (at pH=5, >53.2 mg/mL, at pH=7.4, 0.39 mg/mL) and good stability, showing no degradation over a 45 days period when kept at 25 °C at pH=4.5.¹²⁷ In vitro evaluation of both prodrugs firstly consisted of assessing prodrug uptake and conversion into the parent 5F 203: results demonstrated that, when incubated with sensitive cancer cells (i.e. MCF-7), both compounds were rapidly depleted from media and converted into the active drug after 24 hours. Also, growth inhibitory assays showed that the activity was retained, with GI₅₀ values in the nanomolar range. The lysylamide prodrug Phortress displayed better pharmacokinetic profiles compared to the alanylamide counterpart.

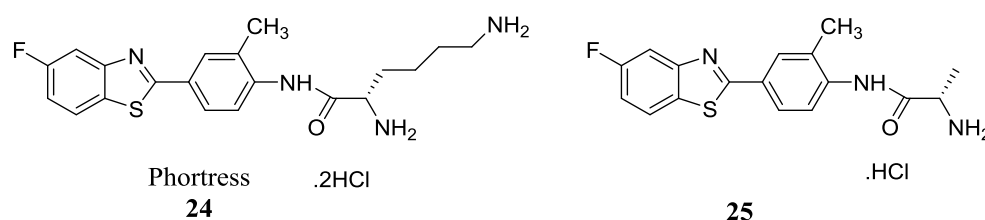


Figure 29.1: Chemical structures of 5F 203 lysylamide prodrug prodrugs (**24**, Phortress) and alanylamide prodrug (**25**).

Furthermore, mechanistic studies confirmed the previous theory, according to which 2-(4-aminophenyl)benzothiazoles derivatives induce activation of CYP1A1 enzyme in sensitive cells and, at the same time, they are also substrates of this enzyme, thus proving the ‘smart’ mechanism of action of this class of drugs.¹²⁸ In preclinical in vivo studies Phortress demonstrated superior pharmacokinetic properties in mice and dogs, with rapid conversion of the prodrug into the parent 5F 203. Plasma concentrations of 5F 203 between 2 and 4 μ M were maintained for ~6 hours. In terms of efficacy against tumour growth, the effective in vitro activity was retained in vivo, with Phortress suppressing the growth of MCF-7 and IGROV-1 carcinomas in xenograft models. Negligible gastric toxicity and lethargy were observed only in two dogs. In view of these positive preclinical studies, the lysylamide prodrug was selected as candidate to undergo Phase I clinical evaluation.¹²⁰ In clinical trials, Phortress was administered via i.v. injection to 50 patients with mesothelioma, renal and

colorectal carcinoma, in a dose range of 3-50 mg/m² for consecutive 21 day cycles. 28% of patients reached a stable stage of disease. However, increasing pulmonary and hepatic toxicities and overall lack of efficacy led to study termination.¹²⁹

1.5. Aims and objectives of the PhD

The overall aim of this PhD project is the development of novel self-assembling gelator prodrugs for localised drug delivery at solid tumour resection sites, such as breast and ovarian cancers. The main approach will consist of synthesis of amphiphilic compounds, afforded through the covalent conjugation of biomolecules, such as peptides and amino acids, to the main drug via a hydrolysable linker (ester, amide or carbamate bonds). In particular, two different anticancer agents will be considered, i.e. the antimetabolite methotrexate and the benzothiazole derivative 5F 203, to investigate the effect that molecular structure has on the self-assembly and to study which different strategies can be adopted to promote gelation. The reasons why these two different anticancer agents have been chosen are numerous: they are both effective antineoplastic agents whose efficacy is however hindered by resistance (in the case of methotrexate) and toxicity; moreover, their completely different chemical structures make them interesting compounds to study which synthetic approaches are required to create self-assembling prodrugs. Further objectives are to characterise the newly synthesised biomaterials to confirm their gelator nature in terms of electron microscopy, spectroscopy techniques and mechanical properties by rheological studies. In vitro assays will also be carried out to evaluate the growth inhibitory properties of the new prodrugs against human-derived cancer cell lines. Release studies at physiological conditions will be also performed in order to assess the ability of the gelators to release the drugs from the gel matrix over time and to demonstrate their use as depot formulations for localised cancer therapy.

- In **Chapter 2**, different synthetic approaches are explored to formulate self-assembling methotrexate prodrugs, via solid or liquid phase, using dipeptides as functional groups. Moreover, with the aim to promote gelation, an alternative strategy will be undertaken. Complete characterisation of the synthesised compounds will be carried out in order to confirm the structure and the purity of the products.

- In **Chapter 3**, the self-assembling behaviour of two series of 5F 203 amide prodrugs is investigated. Self-assembling properties via solvent-switch and pH-triggered methods will be investigated. Characterisation of the gelators will be carried out to assess the LMWG nature of the synthesised materials together with in vitro studies.
- In **Chapter 4**, the gelation behaviour of a series of 5F 203 carbamate prodrugs is explored. These conjugates aim to self-assemble via a solvent-switch technique. Characterisation of the synthesised materials will be carried out along with in vitro growth inhibitory studies.

Finally, in **Chapter 5** the findings described in Chapter 2, 3 and 4 will be summarised and discussed in the light of further optimisation and development.

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Chapter 2: Methotrexate conjugates – peptide and acyl modified antifolate derivatives

The following chapter will firstly give an overview of LMWGs based on folic acid, dipeptides and long alkyl chain derivatives. Then, it will describe the synthesis and the self-assembling studies carried out on MTX peptide and acyl derivatives.

2.1 Introduction

Acquired resistance to MTX, its short plasma half-life and systemic toxicity represent the main limiting factors that led to a quest for innovative targeted therapies to deliver this drug.¹ Several polymer-based gel formulations of MTX have been reported, with the attempt of promoting a sustained delivery of MTX from the polymeric gel matrix.

Injectable thermosensitive hydrogels based on polyethylene glycol (PEG) triblock copolymers were reported by Miao and his group as an intra-articular drug delivery system for MTX. In particular, they developed amphiphilic thermosensitive polymers that were able to self-assemble into micelles at 25 °C. In vitro release studies of different concentrations of MTX revealed a sustained profile of the drug from the polymeric gel matrix, with the best formulation allowing a cumulative release of 74% within 15 days. After intra-articular injection in vivo, MTX-loaded hydrogels were able to decrease the clearance rate of MTX from the joint cavity and to allow a controlled localised delivery, suggesting their potential as targeted therapy for arthritis diseases.²

Azadi et al. recently reported another example of polymeric gels loaded of MTX, in which they formulated MTX into chitosan hydrogel nanoparticles. In vitro release studies showed a sustained release of MTX (65-70%) from the nanogels over 48 hours, with a release depending on both diffusion of MTX from the nanoparticles and disintegration of the polymeric gel network. In vivo studies performed via intravenous administration of MTX-

loaded nanogels displayed remarkably higher brain concentrations of the drug (10-fold) compared to the controls that received free MTX, due to gel formulation's ability to prevent MTX renal excretion.³

The examples described above demonstrate the potential of polymeric gel-based formulations of MTX as alternatives to traditional systemic delivery systems of this anticancer drug. However, to date no examples of LMWGs based on MTX derivatives have been reported in the literature.

2.1.1 Self-assembly of folic acid and derivatives into LMWGs

MTX is an antifolate compound having a similar structure to folic acid, differing from the presence of a 4-amino group on the pterin ring in place of a hydroxyl group and a methyl group at the N¹⁰ position (Figure 1.2).

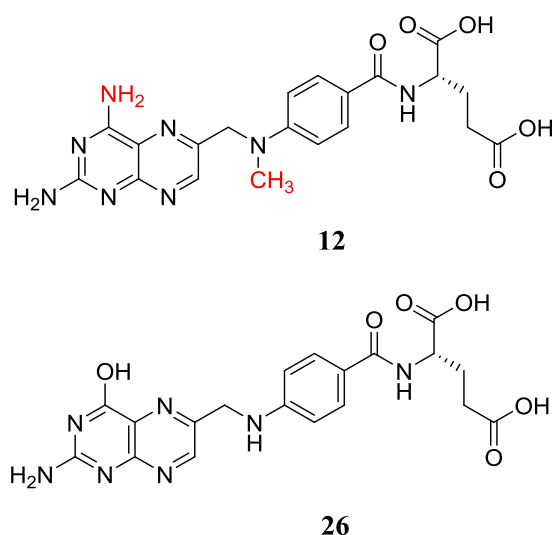


Figure 1.2: Molecular structures of MTX (**12**) and folic acid (**26**) and their structural chemical differences (highlighted in red).

The first reports on folic acid's self-assembly into supramolecular aggregates were extensively described by Kato and his group, who reported the formation of liquid crystalline cubic structures of folic acid derivatives⁴ and subsequently developed self-assembled columnar structures of folic acid conjugates.⁵

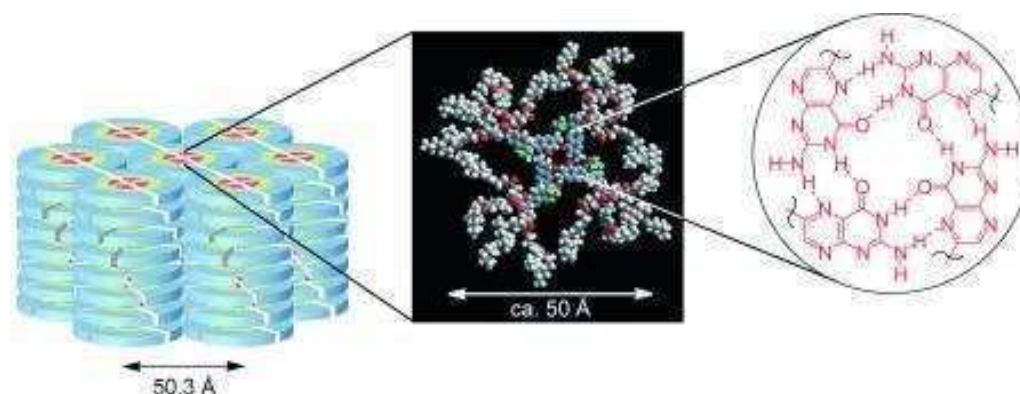


Figure 2.2: Schematic representation of self-assembled folic acid derivatives into hexagonal columnar phases. Taken from Ref. 5.

In both studies, self-assembly occurred via hydrogen bonds between the pterin rings of the molecule, with formation of folic acid tetramers.

In the last study, the group described the importance of chirality in the formation of supramolecular structures. They synthesised two enantiomers and a diastereomer of a folic acid derivative formed by conjugation of the molecule to lipophilic alkyl chains. Self-assembly of the L-enantiomer into chiral columnar aggregates occurred in the presence of sodium triflate (Figure 2.2), whilst circular dichroism studies on the diastereomer demonstrated that it was not able to self-assemble into chiral supramolecular structures. In non-polar solvents (i.e. chloroform and dodecane), all the compounds spontaneously self-assembled, driven by weak interactions such as hydrogen bonds, ionic interactions and π - π stacking.

Xing et al. recently described the gelator properties of folic acid in mixtures of DMSO and water, showing formation of different self-assembled structures, ranging from vesicles to nanofibres, depending on folic acid concentrations (Figure 3.2). In particular, studies revealed the pivotal role of L-glutamic acid in the formation of helical one dimensional order fibres. In addition, the π - π stacking and the hydrogen bonds among the pterin rings promoted the self-assembly. Moreover, the compound revealed self-healing properties, thus suggesting its use for ink or painting applications. These studies finally demonstrated the multi-responsiveness of folic acid gelator to different triggers, such as heating, acid-base or light changes, thus proving its potential as a smart material scaffold for different applications.⁶

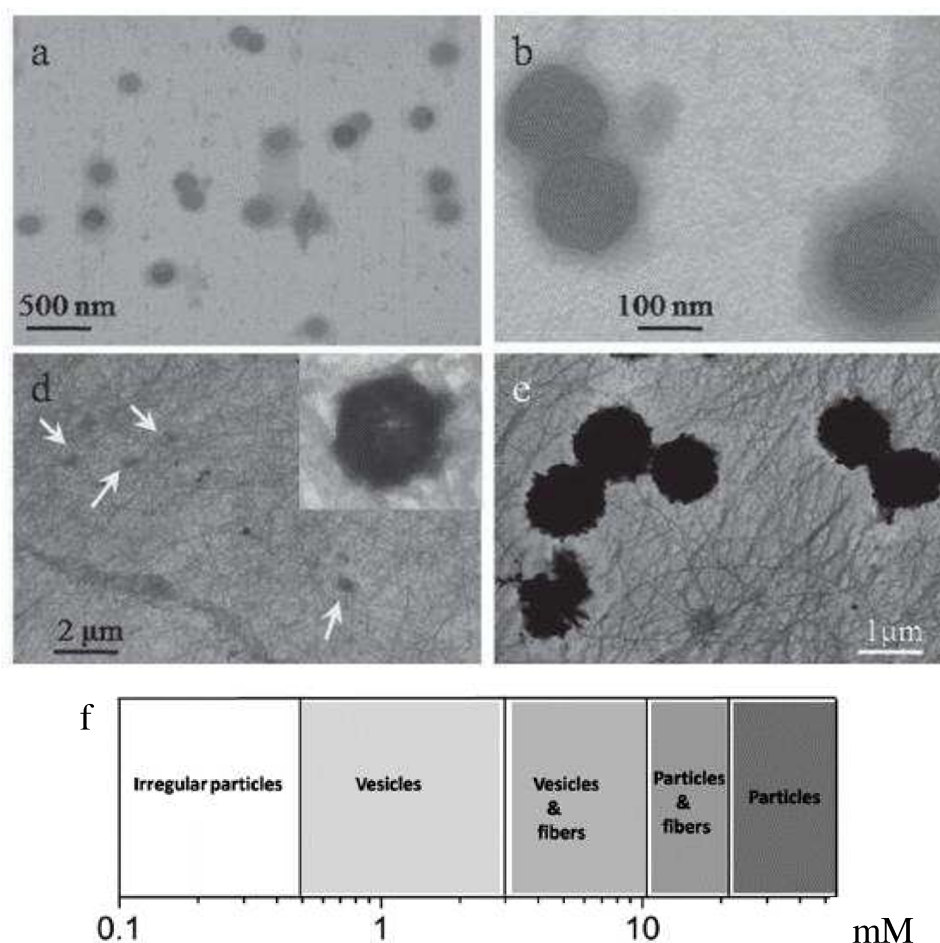


Figure 3.2: TEM images of samples with different concentrations of folic acid: a) and b) 1 mM, d) 10 mM, e) 20 mM, f) phase diagram of the topologies obtained related to concentration of folic acid using the sample with 60% DMSO. Adapted from Ref. 6.

Cui and co-workers focused on the self-assembly of folic acid in mixtures of water and methanol. They demonstrated the organogelation properties of folic acid at concentrations of 1% w/v, with formation of different self-assembled aggregates (i.e. nanofibres and lozenge-shaped platelets) depending on the ratios of water and methanol used. Circular dichroism studies also revealed induced chirality in all the supramolecular structures formed. On the contrary, when MTX was tested for self-assembling properties in mixtures of water and methanol, no formation of self-assembled structures occurred in all the conditions studied, thus meaning that replacement of the hydroxyl group of folic acid with the amine moiety of MTX does make a difference in formation of hydrogen bonds required for the self-assembly. These results confirmed the importance of hydrogen bonds in the molecular arrangement of folic acid into tetramers, as a first step towards the formation of self-assembled structures.⁷

As already described in the previous chapter (section 1.2.4.2), folic acid was also used by Yang et al. in several studies, as a self-assembling motif to design folic acid-paclitaxel hydrogels for drug delivery purposes.^{8, 9}

All the studies reported above demonstrate the propensity of folic acid to form self-assembling aggregates and the lack of gelation properties of the analogue MTX due to subtle changes in the molecular structure.

2.1.2 LMWGs based on dipeptide derivatives

Amino acids (Chapter 3, section 3.1.2) and peptide derivatives represent an important class of LMWGs which have been used for many drug delivery and tissue engineering applications, due to their biocompatibility, easy synthetic pathways and good mechanical strength.^{10, 11}

The first dipeptide derivative to be reported as a hydrogelator was 9-fluorenylmethoxycarbonyl-phenylalanine-phenylalanine (Fmoc-Phe-Phe or Fmoc-FF, **27**), with a Fmoc group at the N-terminus, which self-aggregated in water at room temperature forming transparent gels (Figure 4.2).^{12, 13} Subsequently, studies on Fmoc-FF were reported enabling deeper comprehension of the self-assembling mechanism of the dipeptide derivative, suggesting that gelator molecules were arranged according to a π - π interlocked β -sheet architecture (Figure 4.2).¹⁴ Adams and his group thoroughly investigated the factors that affect the mechanical properties of Fmoc-FF in order to obtain suitable gels for biological applications: they demonstrated that pH and gelation methods are the main parameters affecting the rheological characteristics of this gel.¹⁵

After the discovery of Fmoc-FF as a gelator, several changes in the molecular structure of the dipeptide were carried out developing libraries of LMWGs based on different amino acids and protecting groups, to investigate the effects of structural changes on the self-assembled compounds.

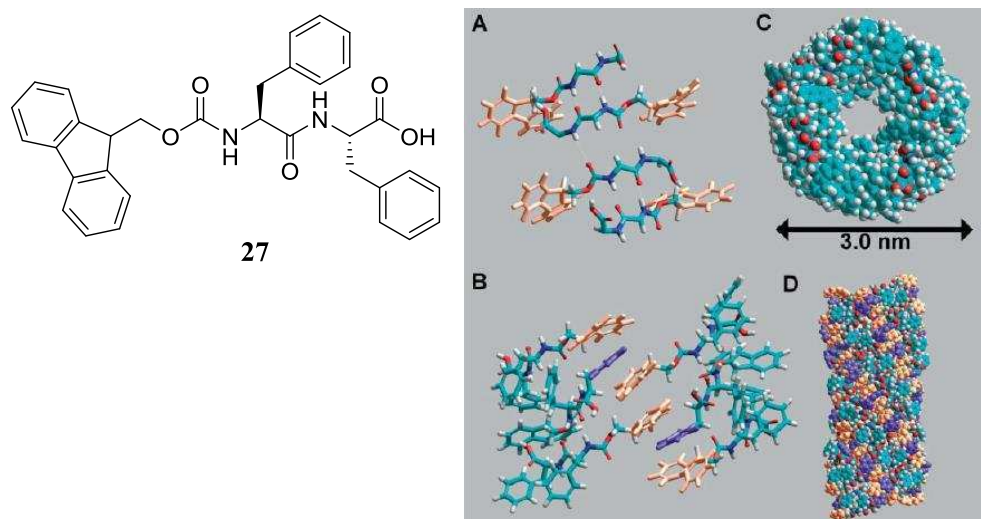


Figure 4.2: (left) Molecular structure of Fmoc-Phe-Phe **27**; (right) A) model structure of Fmoc-FF peptides arranged in an anti-parallel β -sheet pattern, B) interlocking of Fmoc groups from alternate β -sheets to create π -stacked pairs; C) cylindrical structure (top view) that derives from interaction between the fluorenyl groups; D) cylindrical structure (side view). Adapted from Ref. 14.

For example, substitution of the amino acid phenylalanine with glycine in the Fmoc-FF sequence led to the self-assembly of all the different combinations of peptides tested, even with diverse gelation methods, meaning that the main driving force for the self-assembly of Fmoc-protected dipeptides is a combination of hydrophobic forces and π - π stacking interactions of the fluorenyl groups.¹⁶

Gazit et al. studied the biophysical properties of some different Fmoc-peptide hydrogels, such as Fmoc-Phe-Gly, Fmoc-2-naphtalene and Fmoc-Arg-Gly-Asp-Phe, finding that the main contribution to the self-assembly process is given by interactions between the aromatic moieties.¹⁷

Xu and his group developed supramolecular hydrogels based on β -amino acid derivatives, i.e. amino acids with the amino group linked to the β -carbon instead of the α -carbon as in the traditional biological amino acids. Design of these modified peptides was carried out to prevent proteolysis, a common drawback of peptide-based therapeutics. They found that the naphthalene- β -alanine and naphthalene- β -diphenylalanine derivatives (**28**, Figure 5.2) were able to form clear gels at physiological conditions, and fluorescence spectroscopy studies confirmed the role of π - π stacking of the aromatic moieties in the self-assembly process. Therefore, due to their prolonged bioavailability these hydrogels can be

used for biological applications.¹⁸ The same group reported stronger hydrogels compared to the previous ones, by preparing a library of naphthalene-dipeptide hydrogelators (**29**, Figure 5.2), which formed superhelical nanofibres, at low gelator concentrations (0.07 wt%). Biocompatibility studies revealed that these LMWGs can be used as building blocks for new gelators and for biomedical applications.¹⁹

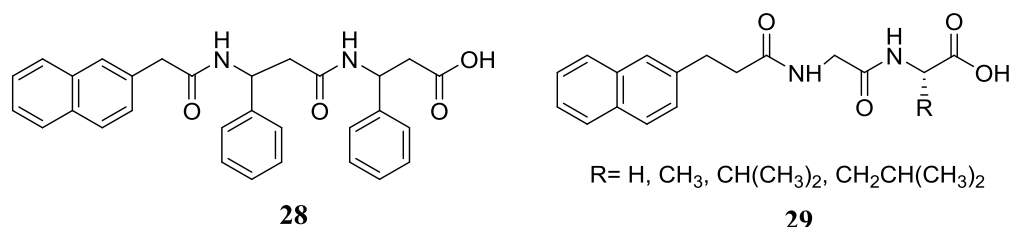


Figure 5.2: Molecular structure of naphthalene-β-diphenylalanine (**28**) and naphthalene-dipeptide hydrogelators (**29**).

2.1.3 LMWGs with long alkyl chains

In the stage of molecular design of potential LMWGs, the strategy of introducing an alkyl chain functionality in the molecule has been widely adopted by several groups, due to the hydrophobic and van der Waals interactions that the chains confer to the self-assembly process (Figure 6.2).²⁰

In his attempt to provide a rational design of supramolecular gelators, Dastidar found that alkyl chains represent a common feature of LMWGs and identified this group as one of the most important in the synthesis of potential gelling agents.²¹

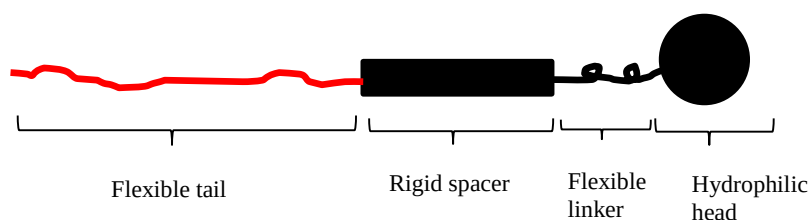
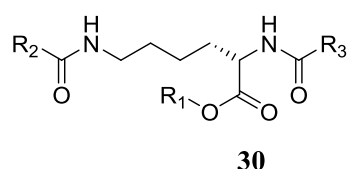


Figure 6.2: Schematic representation of an ideal amphiphilic gelator: the flexible tail (in red) represents one of the four components. Adapted from Ref. 20.

The role of the functionalised linker in ALS compounds has been previously described and examples of LMWGs based on fatty acid derivatives have been provided (section 1.2.2).

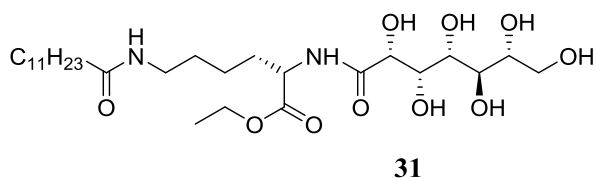
In a recent paper, Weiss and Zhang thoroughly reviewed the role of long chain, naturally-occurring fatty acids, particularly focusing on structural modifications that have been carried out to obtain suitable gelators of a wide range of solvents. They described LMWGs based on n-alkanes, mono- and di-hydroxyalkanoic acids, unsaturated fatty acids and alkali metal salts, finding that subtle changes in the molecular structure of gelators can lead to important differences in the self-assembly process.²²

Hanabusa and co-workers pioneered the field of LMWGs based on L-lysine-derivatives bearing long alkyl chains. As organogelators, they developed L-lysine conjugates linked to alkyl chains of different lengths via different bonds: they synthesised urea-urea, amide-urea and amide-amide compounds (**30**, Figure 7.2), in order to evaluate the contribution that both hydrogen bonding and van der Waals interactions provided to gelation.



Urea-urea type

- i. $R_1 = \text{CH}_3$, $R_2 = R_3 = \text{NHC}_6\text{H}_{13}$
- ii. $R_1 = \text{CH}_3$, $R_2 = R_3 = \text{NHC}_8\text{H}_{17}$
- iii. $R_1 = \text{CH}_3$, $R_2 = R_3 = \text{NHC}_{12}\text{H}_{25}$
- iv. $R_1 = \text{CH}_3$, $R_2 = R_3 = \text{NHC}_{18}\text{H}_{37}$



Amide-urea type

- v. $R_1 = \text{C}_2\text{H}_5$, $R_2 = \text{C}_{11}\text{H}_{23}$, $R_3 = \text{NHC}_6\text{H}_{13}$
- vi. $R_1 = \text{C}_2\text{H}_5$, $R_2 = \text{C}_{11}\text{H}_{23}$, $R_3 = \text{NHC}_{12}\text{H}_{25}$

Amide-Amide type

- vii. $R_1 = \text{C}_2\text{H}_5$, $R_2 = \text{C}_{11}\text{H}_{23}$, $R_3 = \text{NHC}_{12}\text{H}_{25}$

Figure 7.2: Molecular structures of L-lysine-based organogelators (**30**) and the super-hydrogelator **31**. Taken from Ref. 23.

Results demonstrated that the best gelators were the ones with an appropriate balance between these two non-covalent forces, possessing a urea-urea bond and long alkyl chains. Amongst the hydrogelators, they reported positively, negatively and non-ionic gelators based on L-lysine derivatives. In this last class they identified a “super-hydrogelator” **31** (Figure 7.2), as defined by the authors,

that consisted of a L-lysine core connected to a long alkyl chain on one terminus and to aldonamides on the other. Due to its ability to gel water, saline, acid and inorganic salts at concentrations of 0.05% w/v, it acquired the name of ‘super-hydrogelator’.²³

All these studies proved the propensity of long alkyl chain-based compounds to self-assemble and the key role that they play in the molecular design of LMWGs.

2.2 Materials and Methods

2.2.1 General procedures

2.2.1.1 Materials

Chemicals were purchased from commercial suppliers and were used without further purification. All solvents used were either analytical reagent or HPLC grade and were supplied by Fisher Scientific. Dry CH_2Cl_2 , DMF and DMSO were purchased by Acros Organics. Deuterated solvents were purchased from Cambridge Isotope Laboratories Inc. and Sigma Aldrich.

2.2.1.2 General methods

Unless otherwise stated, reactions were performed at room temperature (r.t) and were monitored by thin layer chromatography (TLC) on commercially available precoated aluminium-backed plates (Merck Kieselgel 60 F₂₅₄). Visualisation was carried out by examination under UV light (254 nm) or by staining with potassium permanganate (KMnO_4) solution. Organic solvents were evaporated in vacuo at temperatures between 35 °C and 45 °C (water bath). DMSO was removed by Genevac HT-4X/HCl centrifugal evaporator. Purification by silica gel flash column chromatography was performed using silica gel Sigma Aldrich (technical grade, 40-63 μm particle size).

2.2.1.3 Instrumentation

^1H NMR spectra were recorded on a Bruker 400 Ultrashield at 400 MHz at 25 °C. ^{13}C NMR spectra were recorded on a Bruker 400 Ultrashield at 100 MHz at 25 °C. Chemical shifts (δ) are recorded in parts per million (ppm). Solvents used for NMR analyses (reference peaks listed) were DMSO- d_6 ($(\text{CHD}_2)_2\text{SO}$ at δ_{H} 2.50 ppm, $(\text{CD}_3)_2\text{SO}$ at 39.52 ppm) and CDCl_3 (CHCl_3 at 7.26 ppm, CDCl_3 at 77.16 ppm). The significant multiplicities are described by singlet (s), doublet (d), triplet (t), quartet (q), septet (sept), broad singlet (bs) and multiplet (m).

Coupling constants (J) are recorded in Hz. Spectra were assigned using apposite COSY sequences and analysed with Bruker TopSpin 3.0.

High-resolution mass spectra (HRMS)-time of flight, electrospray (TOF-ES) were recorded using a Waters 2795 spectrometer.

Melting points were measured on a Gallenkamp Melting Point Apparatus.

Lyophilisation from water was carried out on an Edwards Modulyo FD freeze drier.

Reverse phase high performance liquid chromatography (RP-HPLC) was performed using a Waters 2767 sample manager, Waters 2525 binary gradient module, and visualised at 254 nm with a Waters 2487 dual wavelength absorbance detector. Spectra were analysed using MassLynx.

Analytical RP-HPLC was performed using a Phenomenex Luna 5 μm C8 (2) 100 Å column (150 mm x 4.60 mm) at a flow rate of 1 mL/min. The retention time (t_R) of products is reported using an analytical RP-HPLC method of 0-24 min gradient of 5% to 95% solvent B in solvent A, 24-26 min held at 95% solvent B in solvent A, 26-27 min 95% to 5% solvent B in solvent A, 27-30 min held at 5% solvent B in solvent A (solvent A= 0.05% TFA in H_2O , solvent B= 0.05% TFA in 9:1 v:v $\text{CH}_3\text{CN}:\text{H}_2\text{O}$).

Preparative HPLC was performed using a Phenomenex Luna 10 μm C8 (2) 100 Å column (150 mm x 30.00 mm) at a flow rate of 20 mL/min. and UV detection at 254 nm. Three different preparative RP-HPLC methods were used.

Method A: 0-24 min gradient of 40% to 80% solvent B in solvent A, 24-25 min gradient of 80% to 95% solvent B in solvent A, 25-29 min held at 95% solvent B in solvent A, 31-34 min 95% to 40% solvent B in solvent A.

Method B: 0-24 min gradient of 5% to 95% solvent B in solvent A, 24-27 min held at 95% solvent B in solvent A, 27-29 min 95% to 5% solvent B in solvent A, 29-33 min held at 5% solvent B in solvent A.

Method C: 0-22 min gradient of 10% to 85% solvent B in solvent A, 22-24 min held at 85% solvent B in solvent A, 24-26 min 85% to 10% solvent B in solvent A, 26-30 min held at 10% solvent B in solvent A.

For all methods, solvent A= 0.05 % TFA in H_2O and solvent B= 0.05 % TFA in 9:1 v:v $\text{CH}_3\text{CN}:\text{H}_2\text{O}$.

Liquid chromatography-mass spectrometry (LC-MS) was performed on a Shimadzu UFLCXR system coupled to an Applied Biosystems API2000, and visualised at 220 nm (channel 2) and 254 nm (channel 1). LC-MS was performed using a Phenomenex Gemini-NX C18 110 Å, column (50 mm x 2 mm x 3µm) at a flow rate 0.5 mL/min. t_R of products is reported using a LC-MS gradient method: pre-equilibration run for one min at 5% B, 1-2 min 5% to 98% solvent B in solvent A, 2-4 min held at 98% solvent B in solvent A, 4-4.5 min 98 to 10% solvent B in solvent A, 4.5-5 min held at 10% solvent B in solvent A (solvent A= 0.1% formic acid in H₂O, solvent B= 0.1% formic acid in CH₃CN).

2.2.1.4 Determination of resin substitution based on ‘Fmoc-substitution’ test

In order to determine the substitution level of resin, an accurately weighed sample of dry Fmoc-substituted resin (5-10 mg) was covered with freshly prepared piperidine 20% v/v in DMF (3 mL). The resin mixture was then agitated for 2-3 min and the resin was left for 2 h with the deprotecting solution. 300 µL of supernatant were withdrawn from the resin mixture, diluted to 3 mL of 20% piperidine solution and transferred to a UV cuvette and the UV absorbance read at 290 nm. The loading of the resin was then calculated from the following equation:

$$\text{Loading (mmol/g)} = \text{Abs}_{\text{sample}} / \text{mg of dried resin} \cdot 1.65$$

Equation 1.2: Calculation of resin loading

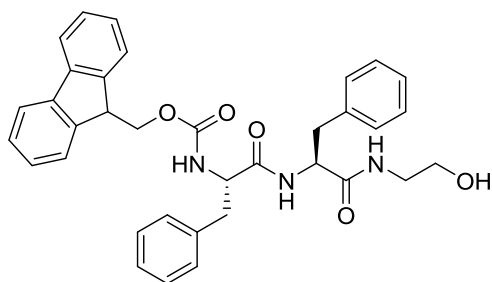
2.2.1.5 Gelation tests

Gelation tests were performed according to a solvent-switch method. The compounds were weighed using an OHAUS semi micro-analytical balance into 1.5 mL sample vials. DMSO was added to create a stock solution at 10% w/v. The solution was then sonicated in a Sonicor Ultra-sonic bath for one min and successively heated at 60 °C to help full dissolution of the compound. An aliquot (25 µL) of the stock solution was transferred in another 1.5 mL vial and the

remaining DMSO/ethanol and pre-heated (60 °C) ultra-purified water were added. Samples were then allowed to cool down to r.t. Vial inversion tests were subsequently carried out as preliminary screens for gelation. All samples prepared had a final concentration of 0.5% w/v.

2.2.2 Experimental data

(9H-Fluoren-9-yl)methyl((S)-1-(((S)-1-((2-hydroxyethyl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (32)



Compound **32** was synthesised on a glycinol 2-chlorotriyl resin (0.45 mmol/g of manufacturer substitution). The polymeric support (0.50 g, 0.23 mmol) was placed in a reaction glass vessel and allowed to swell overnight

with DMF (4 mL). Functionalisation with the first amino acid residue Fmoc-Phe-OH (0.26 g, 0.68 mmol) was performed using HBTU (0.26 g, 0.68 mmol), HOBT (0.09 g, 0.68 mmol) and NMM (0.10 mL, 0.90 mmol,) dissolved in the minimum amount of DMF. The reaction mixture was added to the resin and reacted for 1.5 h, with occasional swirling. The resin was successively flow washed with copious amounts of DMF and an estimation of the level of first residue attachment was carried out (section 2.2.1.4). A further double coupling was performed yielding a resin loading of 0.39 mmol/g. In order to prevent side reactions on the remaining active sites of the resin, it was capped with acetic anhydride (0.10 mL, 1.13 mmol) and DIPEA (0.20 mL, 1.13 mmol) in DMF for 45 min and the procedure was repeated twice. The resin was washed with DMF to remove acetic anhydride and DIPEA residues. Fmoc-deprotection of the first amino acid was carried out by treating the resin with a solution of piperidine 20% v/v in DMF (3 mL) 3 x 15 min. The resin was then washed with DMF and the second residue Fmoc-Phe-OH (0.17 g, 0.45 mmol) was coupled to the resin using HBTU (0.17 g, 0.45 mmol), HOBT (0.06 g, 0.45 mmol) and NMM (0.07 mL, 0.60 mmol,) and the mixture was stirred for 1.5 h. A double coupling was performed. The resin was then washed with DMF, CH₂Cl₂ and dried under a

stream of nitrogen for 3 h. Cleavage was performed by treating the resin with TFA: H₂O (95:5 v/v, 5 mL) for 1 h under stirring. The cleavage mixture was filtered off and washed with TFA: H₂O (95:5 v/v). The filtrate was evaporated under a stream of nitrogen, precipitated with cold diethyl ether, and the crude product was collected after filtration with cold diethyl ether, to afford a white solid (0.06 g, 0.10 mmol, 65% yield).

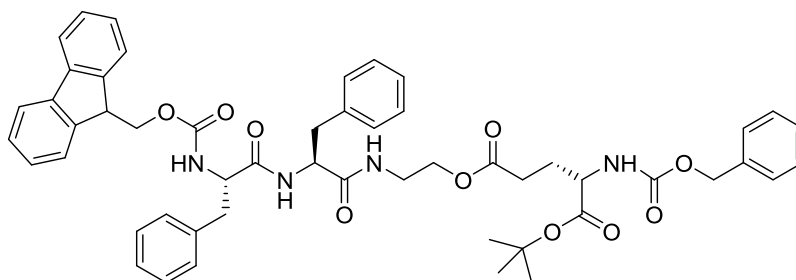
¹H NMR (DMSO-*d*₆): δ 8.08 (d, *J*=8.9 Hz, 1H, NH-CH), 7.96 (t, *J*=5.80 Hz, 1H, NH-CH₂), 7.87 (d, *J*=7.6 Hz, 2H, H-4, H-5, fluorenyl ring), 7.64-7.58 (m, 2H, H-1, H-8, fluorenyl ring), 7.55 (d, *J*= 8.7 Hz, 1H, NH-CH), 7.43-7.36 (m, 2H, H-3, H-6, fluorenyl ring), 7.33-7.26 (m, 2H, H-2, H-7, fluorenyl ring), 7.25-7.19 (m, 8H, H-2', H-3', H-5', H-6', H-2'', H-3'', H-5'', H-6'' Phe rings), 7.14-7.19 (m, 2H, H-4', H-4'', Phe rings), 4.55-4.47 (m, 1H, CH-CH_AH_B), 4.34-4.29 (m, 1H, CH-CH_AH_B), 4.18 (m, 1H, CH-CH₂), 4.14-4.07 (m, 2H, CH₂-O), 3.98 (bs, 1H, OH), 3.37-3.30 (m, 2H, CH₂-OH), 3.15-3.05 (m, 2H, NH-CH₂), 2.99 (dd, *J*=5.5, 14.2 Hz, 1H, CH_AH_B), 2.91 (dd, *J*=4.2, 14.2 Hz, 1H, CH_AH_B, Phe), 2.83 (dd, *J*=8.2, 13.1 Hz, 1H, CH_AH_B), 2.71 (dd, *J*=10.5, 13.4 Hz, 1H, CH_AH_B).

¹³C NMR (DMSO-*d*₆): δ 171.60, 171.18, 156.12, 144.22, 144.17, 141.11, 138.53, 138.01, 137.88, 129.73, 129.66, 128.08, 127.53, 126.71, 126.66, 125.80, 125.70, 120.53, 66.12, 60.11, 56.59, 54.36, 46.99, 41.88, 38.45, 37.86.

***m/z*:** HRMS (TOF ES⁺) C₃₅H₃₆N₃O₅⁺ [MH]⁺ calculated 578.2650; found 578.2683.

HPLC *t*_R: 8.05 min (single peak).

5-(2-((S)-2-((S)-2-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-phenylpropanamido)-3-phenylpropanamido)ethyl) 1-(tert-butyl) ((benzyloxy)carbonyl)-L-glutamate (33)



EDC (0.01 g,
0.05 mmol)
and HOBT
(0.01 g, 0.05
mmol) were

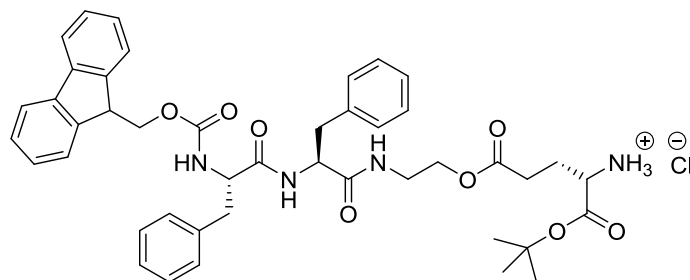
added to a solution of Z-Glu-OtBu (0.01 g, 0.04 mmol) in dry CH₂Cl₂ (3 mL) and the solution was stirred for 1 h at 0 °C. **32** (0.02 g, 0.040 mmol) solubilised in dry DMF (2 mL) was added dropwise, followed by addition of DMAP (0.003 g, 0.003 mmol). The reaction was then stirred at r.t. for 3 days. Cold diethyl ether was added to the reaction mixture, the precipitate was filtered off and the resulting solution was concentrated under reduced pressure. The crude material was purified with silica gel flash chromatography (gradient elution 2-10% MeOH in CH₂Cl₂) to afford a white powder (0.014 g, 0.02 mmol, 46% yield).

¹H NMR (DMSO-d₆): δ 8.15-8.08 (m, 1H, NH-CH), 7.96 (s, 1H, NH-CH), 7.88 (d, J=7.5 Hz, H-4, H-5, fluorenyl ring), 7.68-7.59 (m, 3H, NH-CH₂, H-1, H-8, fluorenyl ring), 7.54 (d, J=8.5 Hz, 1H, NH-CH), 7.44-7.12 (m, 19H, H-2', H-3', H-4', H-5', H-6', H-2'', H-3'', H-4'', H-5'', H-6'' Phe rings, H-2, H-3, H-6, H-7, fluorenyl ring, benzyl ring), 5.03 (dd, J=12.3, 20.9 Hz, 2H, CH₂ benzyl), 4.53-4.47 (m, 1H, NH-CH, Phe), 4.28-4.21 (m, 1H, NH-CH, Phe), 4.15-4.06 (m, 1H, CH-CH₂), 3.98-3.91 (m, 3H, NH-CH (Glu), CH₂-O), 2.96-2.93 (m, 1H, CH_AH_B, Phe), 2.93-2.89 (m, 3H, CH_AH_B(Phe), CH₂-NH), 2.88-2.82 (m, 1H, CH_AH_B, Phe), 2.75-2.71 (m, 3H, CH_AH_B(Phe), CH₂-O), 2.44-2.30 (m, 2H, CH₂-CH_AH_B, Glu), 2.03-1.90 (m, 1H, CH_AH_B, Glu), 1.84-1.74 (m, 1H, CH_AH_B, Glu), 1.38 (s, 9H, t-C₄H₉).

¹³C NMR (DMSO-d₆): δ 207.41, 172.60, 171.68, 171.55, 171.42, 162.76, 156.57, 156.10, 144.13, 141.11, 138.51, 137.87, 137.43, 129.78, 128.78, 128.50, 128.31, 128.22, 128.08, 127.53, 126.70, 125.79, 125.67, 120.57, 81.17, 79.45, 79.17, 66.11, 65.96, 62.94, 56.54, 54.38, 46.90, 37.89, 36.26, 30.36, 28.03, 26.39.

m/z: HRMS (TOF ES⁺) C₅₂H₅₇N₄O₁₀⁺ [MH]⁺ calculated 897.4070; found 897.4094.

(5S,8S,17S)-5,8-Dibenzyl-1-(9H-fluoren-9-yl)-20,20-dimethyl-3,6,9,14,18-pentaoxo-2,13,19-trioxa-4,7,10-triazahenicosan-17-aminium chloride (34)

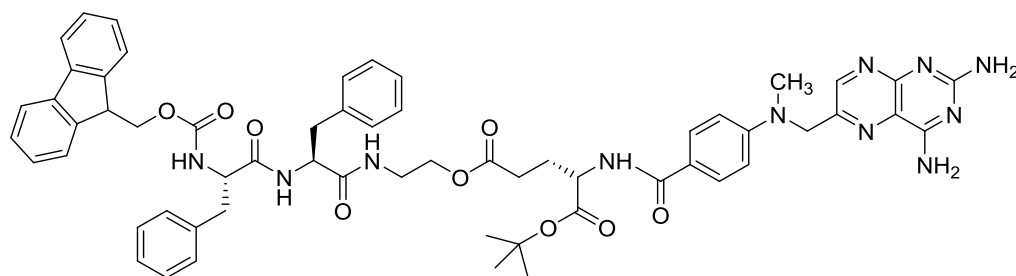


33 (0.02 g, 0.02 mmol) was dissolved in MeOH (4 mL) and HCl 0.1. M was added (0.15 mL, 0.02 mmol). The

reaction mixture was hydrogenated with 10% Pd/charcoal (0.003 g, 0.003 mmol Pd) for 2 h. The catalyst was filtered off and the filtrate was collected and concentrated under reduced pressure. The crude oily product (0.005 g) was utilised for the following coupling without further purification.

m/z: HRMS (TOF ES⁺) C₄₄H₅₁N₄O₈⁺ [MH]⁺ calculated 763.3701; found: 763.1821.

5-(2-((S)-2-((S)-2-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-phenylpropanamido)-3-phenylpropanamido)ethyl)-1-(tert-butyl)-4-(((2,4-diaminopteridin-6-yl)methyl)(methyl) amino)benzoyl)-L-glutamate (35)



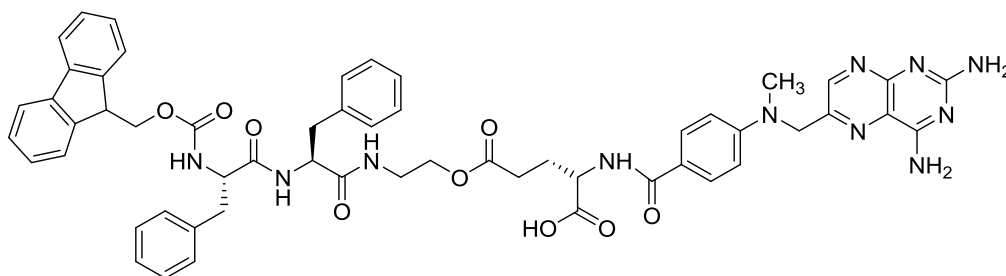
DIPEA (0.003 mL, 0.01 mmol) and HATU (0.003 g, 0.008 mmol) were added to a solution of APA (0.002 mg, 0.006 mmol) in 0.400 mL of dry DMSO. After 30 min of stirring, **34** (0.005 g, 0.006 mmol), solubilised in 0.400 mL of DMSO, was added to the reaction mixture and the solution was stirred for 3 h. DMSO was evaporated under reduced pressure to afford an

oily yellow crude mixture. The crude product was subjected to tert-butyl deprotection without further purification.

m/z: HRMS (TOF ES⁺) C₅₉H₆₄N₁₁O₉⁺ [MH]⁺ calculated 1070.4883; found 1070.4700.

LC-MS t_R: 2.64 min.

4-Amino-6-(((4-(((5S,8S,17S)-5,8-dibenzyl-17-carboxy-1-(9H-fluoren-9-yl)-3,6,9,14-tetraoxo-2,13-dioxo-4,7,10-triazaheptadecan-17-yl)carbamoyl)phenyl)(methylamino)methyl)pteridin-2-aminium 2,2,2-trifluoroacetate (36)

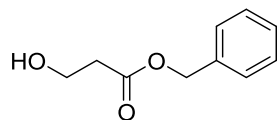


35 was dissolved in 3 mL of TFA 50% in CH₂Cl₂ and the reaction was stirred for 1 h. TFA was removed under a stream of nitrogen yielding a crude yellow semi-solid compound.

m/z: HRMS (TOF ES⁺) C₅₅H₅₆N₁₁O₉⁺ [MH]⁺ calculated 1014.4257; found 1014.3478.

LC-MS t_R: 2.49 min.

Benzyl 3-hydroxypropanoate (37)



A mixture of NaH 60% in mineral oil (0.007 g, 0.28 mmol) in benzyl alcohol (3.4 mL, 33.30 mmol) was stirred at -10 °C under nitrogen for 10 min. β-propiolactone (0.34 mL, 5.55 mmol) was added dropwise to the mixture and the reaction mixture was stirred at -10 °C for 30 min. The reaction was quenched with HCl (12 N), extracted 3 times with CHCl₃ (10 mL) and washed with H₂O. The CHCl₃ solution was dried over MgSO₄, filtered and the filtrate was concentrated under vacuum and

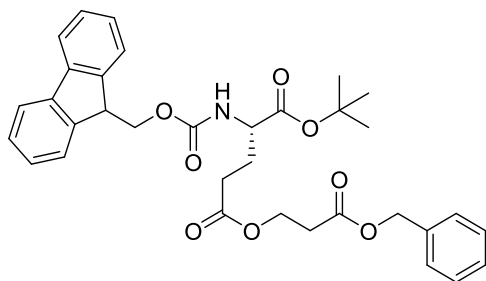
purified by silica gel flash chromatography (25% EtOAc in petroleum ether 40-60 °C) to afford **37** as a colourless oil (0.49 g, 2.72 mmol, 49% yield).

¹H NMR (CDCl₃): δ 7.38 (m, 5H, benzyl), 5.18 (s, 2H, CH₂-benzyl), 3.89 (t, J= 5.75 Hz, 2H, CH₂-OH), 2.79 (bs, 1H, OH), 2.64 (t, J= 5.70, 2H, CH₂-CO).

¹³C NMR (CDCl₃): δ 172.63, 135.80, 128.67, 128.51, 128.39, 128.32, 127.04, 66.50, 58.15, 37.20.

m/z: HRMS (TOF ES⁻) C₁₀H₁₂O₃ [M-H] calculated 179.0714; found 179.1039.

5-(3-(Benzyloxy)-3-oxopropyl)1-(tert-butyl)(((9H-fluoren-9-yl)methoxy)carbonyl)-L-glutamate (38)



DCC (0.33 g, 1.59 mmol) and HOBT (0.21 g, 1.59 mmol) were added to a solution of Fmoc-NH-Glu-OtBu (0.33 g, 0.78 mmol) in dry CH₂Cl₂ (12 mL) and the solution was stirred under

nitrogen. After 1 h, **37** (0.23 g, 1.29 mmol) solubilised in dry CH₂Cl₂ (3 mL) was added dropwise, followed by addition of DMAP (0.008 g, 0.063 mmol). The reaction was stirred overnight. Diethyl ether was added and the precipitate was filtered; the solution was concentrated under vacuum. The crude material was purified by silica gel flash chromatography (gradient elution 15-25% EtOAc in petroleum ether 40-60 °C) to yield **38** as a pale yellow oil (0.37 g, 0.63 mmol, 80% yield).

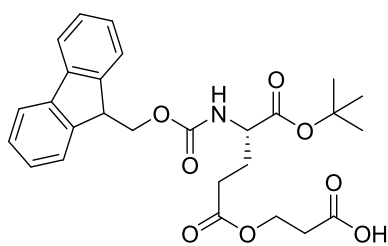
¹H NMR (DMSO-d₆): δ 7.90 (d, J= 8.3 Hz, 2H, H-4, H-5, fluorenyl ring), 7.75-7.66 (m, 3H, NH, H-1, H-8, fluorenyl ring), 7.45-7.30 (m, 9H, benzyl ring, H-2, H-3, H-6, H-7, fluorenyl ring), 5.17 (s, 2H, CH₂-benzyl), 4.37-4.19 (m, 5H, CH-CH₂, O-CH₂), 3.97-3.90 (m, 1H, NH-CH), 2.73 (t, J= 6.2 Hz, 2H, CH₂-COO), 2.38-2.29 (m, 2H, CH₂-CH_AH_B), 2.01-1.88 (m, 1H, CH_AH_B), 1.85-1.74 (m, 1H, CH_AH_B), 1.39 (s, 9H, t-C₄H₉).

¹³C NMR (DMSO-d₆): δ 172.47, 171.55, 170.79, 156.55, 144.28, 144.22, 141.21, 136.73, 136.47, 128.81, 128.49, 128.36, 128.27, 128.11, 127.47,

125.69, 120.62, 81.13, 66.15, 65.78, 60.17, 57.43, 54.03, 49.13, 47.14, 38.15, 33.72, 30.33, 28.06, 26.53.

m/z: HRMS (TOF ES⁺) C₃₄H₃₈NO₈⁺ [MH]⁺ calculated 588.2592; found 588.2565.

(S)-3-((4-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-5-(tert-butoxy)-5-oxopentanoyl)oxy)propanoic acid (39)



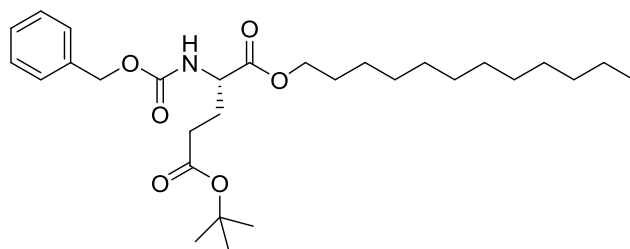
38 (0.20 g, 0.35 mmol) was dissolved in MeOH (7 mL) and hydrogenated with 10% Pd/charcoal (0.06 g, 0.06 mmol Pd) for 2 h. The catalyst was filtered off and the filtrate was collected and concentrated under reduced pressure. The residue was dissolved in EtOAc and washed with an acidic solution of citric acid 0.5 M to remove the Fmoc-protected side products. The organic phase was dried over MgSO₄, filtered and the filtrate was concentrated under reduced pressure and purified by preparative HPLC with method A to yield **39** as a colourless oil (0.06 g, 0.12 mmol, 35% yield).

¹H NMR (DMSO-d₆): δ 7.90 (d, J= 7.5 Hz, 2H, H-4, H-5, fluorenyl ring), 7.72 (d, J=7.6 Hz, 2H, H-1, H-8, fluorenyl ring), 7.68 (d, J= 7.9 Hz, 1H, NH), 7.42 (t, J= 7.4 Hz, 2H, H-3, H-6, fluorenyl ring), 7.33 (t, J= 7.4 Hz, 2H, H-2, H-7, fluorenyl ring), 4.32-4.27 (m, 2H, CH-CH₂), 4.25-4.17 (m, 3H, CH-CH₂, O-CH₂), 3.96-3.88 (m, 1H, NH-CH), 2.57 (t, J= 6.3, 2H, CH₂-COO), 2.39-2.30 (m, 2H, CH₂-CH_AH_B), 1.98-1.89 (m, 1H, CH_AH_B), 1.84-1.77 (m, 1H, CH_AH_B), 1.39 (s, 9H, t-C₄H₉).

¹³C NMR (DMSO-d₆): δ 172.53, 172.40, 171.55, 156.54, 144.28, 142.87, 141.21, 140.74, 130.13, 128.11, 127.54, 126.39, 125.74, 120.60, 90.03, 81.18, 66.04, 64.88, 60.49, 53.99, 49.04, 47.17, 33.72, 30.36, 28.12, 26.43.

m/z: HRMS (TOF ES⁺) C₂₇H₃₂NO₈⁺ [MH]⁺ calculated 498.2128; found 498.2156.

HPLC t_R: 8.88 min.

5-(tert-butyl) 1-dodecyl ((benzyloxy)carbonyl)-L-glutamate (40)

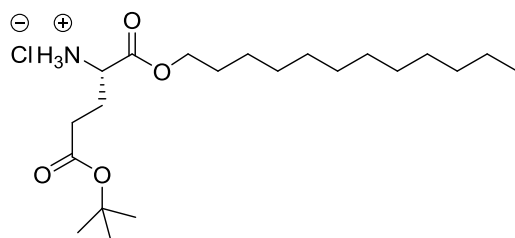
To a stirred solution of Z-Glu-OtBu (1.50 g, 4.45 mmol) and dodecan-1-ol (2.48 g, 13.35 mmol) in dry CH_2Cl_2 , DMAP (0.05 g,

0.45 mmol) and DCC (1.10 g, 5.34 mmol) were added and the reaction mixture was stirred for 1 h at 0 °C, then overnight at room temperature. After 23 h, the crude material was purified by silica gel flash chromatography (gradient elution 15-30% EtOAc in petroleum ether 40-60 °C) to yield **40** as a translucent oil (1.57 g, 3.11 mmol, 70% yield).

^1H NMR (DMSO- d_6): δ 7.72 (d, J = 7.9 Hz, 1H, NH), 7.39-7.29 (m, 5H, benzyl), 5.04 (s, 2H, CH_2 -benzyl), 2.35-2.21 (m, 2H, CH_2 - $\text{CH}_\text{A}\text{H}_\text{B}$), 4.11-3.97 (m, 3H, NH- CH , O- CH_2 - CH_2), 2.01-1.89 (m, 1H, $\text{CH}_\text{A}\text{H}_\text{B}$), 1.84-1.73 (m, 1H, $\text{CH}_\text{A}\text{H}_\text{B}$), 1.60-1.51 (m, 2H, O- CH_2 - CH_2), 1.38 (s, 9H, t- C_4H_9), 1.24 (bs, 18H, CH_2), 0.85 (t, 3H, J = 6.7 Hz, CH_3 - CH_2).

^{13}C NMR (DMSO- d_6): δ 172.44, 171.81, 156.60, 137.42, 128.75, 128.24, 128.12, 80.16, 65.93, 64.90, 53.54, 31.79, 31.51, 29.54, 29.49, 29.45, 29.21, 29.11, 28.55, 28.14, 26.58, 25.75, 22.57, 14.36.

m/z: HRMS (TOF ES^+) $\text{C}_{29}\text{H}_{48}\text{NO}_6^+$ $[\text{M}+\text{H}]^+$ calculated 506.3476; found 506.3515.

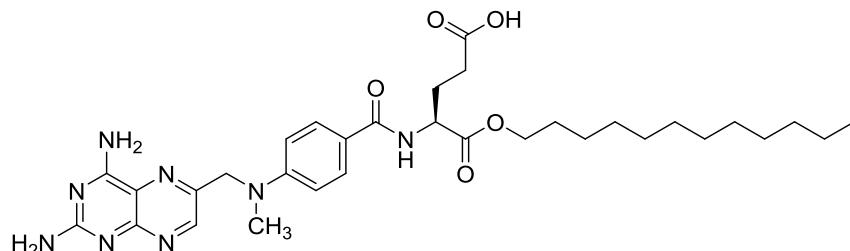
(S)-5-(tert-Butoxy)-1-(dodecyloxy)-1,5-dioxopentan-2-aminium chloride (41)

40 (0.26 g, 0.52 mmol) was dissolved in MeOH (121 mL) and HCl 0.1 M was added (4.66 mL, 0.47 mmol). The reaction mixture was hydrogenated with 10% Pd/charcoal

(0.03 g) for 2 h. The catalyst was filtered off and the filtrate was collected and concentrated under reduced pressure. The crude oily product (0.19 g) was utilised immediately for the following coupling without further purification.

m/z: HRMS (TOF ES⁺) C₂₁H₄₂NO₄⁺ [MH]⁺ calculated 372.3109; found 372.3171.

(S)-4-(4-(((2,4-Diaminopteridin-6-yl)methyl)(methyl)amino)benzamido)-5-(dodecyloxy)-5-oxopentanoic acid (42)



To a stirred solution of APA (0.03 g,

0.09 mmol) in 2 mL of dry DMSO, DIPEA (0.06 mL, 0.35 mmol), PYBOP (0.14 g, 0.26 mmol) and HOBt (0.04 g, 0.26 mmol) were added. After 30 min of stirring at 40 °C, **41** (0.05 g, 0.14 mmol), solubilised in 1 mL of dry DMSO, was added to the reaction mixture and the solution was stirred for 20 h at 40 °C. Purified water was then added (4mL) and a yellow solid precipitated out. The suspension was centrifuged to produce a yellow solid, which was dried under reduced pressure. The crude residue was suspended in 1.5 mL of dry CH₂Cl₂ and then TFA (1.5 mL) was added and the reaction was stirred for 2 h. TFA was removed under a stream of nitrogen yielding a crude yellow powder, which was purified by preparative HPLC with method B to give a yellow semi-solid product (0.02 g, 0.03 mmol, 31% yield).

¹H NMR (DMSO-d₆): δ 12.14 (bs, 1H, COOH), 8.65 (s, 1H, H-7), 8.32 (d, 1H, J= 8.1 Hz, NH-CH), 7.73 (d, J= 9.0 Hz, 2H, H-2', H-6'), 7.50 (bs, 2H, NH₂), 6.82 (d, J= 8.9 Hz, 2H, H-3', H-5'), 4.83 (s, 2H, CH₂-N-CH₃), 4.37 (m, 1H, CH-CH_AH_B), 4.02 (m, 2H, O-CH₂-CH₂), 3.23 (s, 3H, N-CH₃), 2.33 (t, 3H, J= 7.8 Hz, CH₂-CH_AH_B), 2.03 ((m, 1H, CH_AH_B), 1.94 (m, 1H, CH_AH_B), 1.53(m, 2H, CH₂-CH₂O), 1.31-1.11(bs, 18H, CH₂), 0.84 (t, 3H, J= 6.6 Hz, CH₃-CH₂).

¹³C NMR (DMSO-d₆): 13.93, 22.07, 25.24, 25.78, 28.07, 28.57, 28.69, 28.89, 28.96, 29.00, 30.21, 31.26, 52.02, 54.80, 64.23, 111.03, 121.04, 128.95, 148.71, 148.89, 150.03, 150.81, 157.95, 158.19, 158.44, 158.69, 159.07, 162.67, 166.38, 172.23, 173.78.

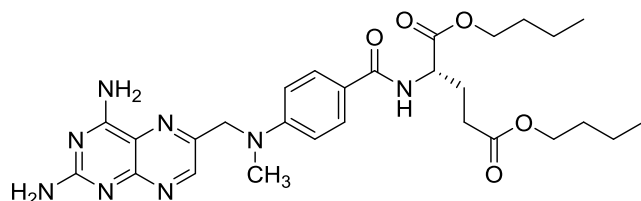
m/z: HRMS (TOF ES⁺) C₃₂H₄₇N₈O₅⁺ [MH]⁺ calculated 623.3664; found 623.3628.

HPLC t_R = 19.40 min.

General procedure for the synthesis of MTX diesters analogues (43):

MTX (1 eq) was dissolved in dry DMF at 0 °C and then DIPEA (5 eq), HOBt (3 eq.), EDC (3 eq.) were added. Alkyl or aromatic alcohol (20 eq, excess) and DMAP (0.5 eq) were then added to the reaction mixture. The reaction was stirred for 45-50 h at r.t., then it was diluted with water (5 mL) and extracted 3 times with EtOAc. Organic fractions were collected and dried over MgSO₄ and evaporated to give yellow products. Crude compounds were purified by preparative HPLC with method B, except for compound **43d** which was purified using method C.

Dibutyl 4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzoyl)-L-glutamate (**43a**)



Synthesis of **43a** was performed according to the general procedure, using MTX (0.08 g, 0.17 mmol),

DMF (3 mL), DIPEA (0.14 mL, 0.83 mmol), HOBt (0.07 g, 0.50 mmol), EDC (0.08 g, 0.50 mmol), butan-1-ol (0.30 mL, 3.30 mmol) and DMAP (0.01 g, 0.08 mmol). A yellow solid was afforded (0.05 g, 0.09 mmol, 48% yield).

¹H NMR (DMSO-d₆): δ 8.66 (s, 1H, H-7), 8.33 (d, 1H, J = 7.6 Hz, NH-CH), 7.72 (d, 2H, J = 8.2 Hz, H-2', H-6'), 6.82 (d, J = 8.7 Hz, 2H, H-3', H-5'), 4.84 (s, 2H, CH₂-N-CH₃), 4.39-4.33 (m, 1H, CH-CH_AH_B), 4.08-3.96 (m, 4H, O-CH₂-CH₂), 3.24 (s, 3H, N-CH₃), 2.40 (t, 2H, J = 7.9 Hz, CH₂-CH_AH_B), 2.11-2.01 (m, 1H, CH_AH_B), 2.01-1.89 (m, 1H, CH_AH_B), 1.56-1.47 (m, 4H, CH₂-CH₂O), 1.34-1.24 (m, 4H, CH₂-CH₃), 0.85 (t, 6H, J = 7.5 Hz, CH₃-CH₂).

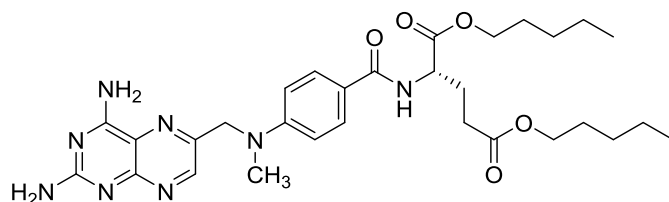
^{13}C NMR (DMSO- d_6): 172.75, 172.66, 166.85, 163.16, 158.33, 158.00, 151.26, 149.32, 129.46, 121.45, 111.53, 64.51, 64.08, 55.31, 52.34, 40.85, 30.67, 30.61, 26.24, 19.06, 18.98, 14.02.

m/z: HRMS (TOF ES⁺) $\text{C}_{28}\text{H}_{39}\text{N}_8\text{O}_5^+$ [MH]⁺ calculated 567.3038; found 567.3046.

Mp: 145-147 °C.

HPLC t_R = 16.32 min.

Dipentyl (4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzoyl)-L-glutamate (43b)



Synthesis of **43b** was performed according to the general procedure, using MTX (0.10 g, 0.22 mmol), DMF (3.5 mL),

DIPEA (0.19 mL, 1.10 mmol), HOBt (0.09 g, 0.66 mmol), EDC (0.10 g, 0.66 mmol), pentan-1-ol (0.48 mL, 4.40 mmol) and DMAP (0.01 g, 0.11 mmol). A yellow solid was afforded (0.08 g, 0.13 mmol, 61% yield).

^1H NMR (DMSO- d_6): 8.57 (s, 1H, H-7), 8.31 (d, 1H, J = 8.0 Hz, NH-CH), 7.72 (d, 2H, J = 8.8 Hz, H-2', H-6'), 6.82 (d, J = 9.0 Hz, 2H, H-3', H-5'), 4.79 (s, 2H, $\text{CH}_2\text{-N-CH}_3$), 4.40-4.32 (m, 1H, $\text{CH-CH}_A\text{H}_B$), 4.09-3.94 (m, 4H, $\text{O-CH}_2\text{-CH}_2$), 3.22 (s, 3H, N-CH₃), 2.40 (t, 2H, J = 7.5 Hz, $\text{CH}_2\text{-CH}_A\text{H}_B$), 2.10-2.01 (m, 1H, CH_AH_B), 2.01-1.91 (m, 1H, CH_AH_B), 1.60-1.47 (m, 4H, $\text{CH}_2\text{-CH}_2\text{O}$), 1.32-1.18 (m, 8H, $\text{CH}_2\text{-CH}_2\text{-CH}_3$), 0.89-0.75 (m, 6H, $\text{CH}_3\text{-CH}_2$).

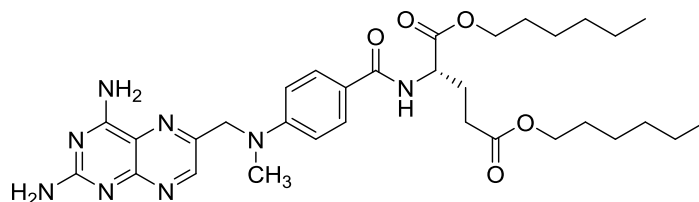
^{13}C NMR (DMSO- d_6): 190.67, 172.33, 172.22, 166.49, 162.72, 150.94, 149.09, 148.99, 129.01, 122.70, 121.58, 120.89, 111.05, 69.80, 64.34, 63.95, 54.88, 51.94, 40.43, 30.24, 27.81, 27.56, 27.50, 25.81, 21.79, 21.74, 13.86.

m/z: HRMS (TOF ES⁺) $\text{C}_{30}\text{H}_{43}\text{N}_8\text{O}_5^+$ [MH]⁺ calculated 595.3351; found 595.3339.

Mp: 156-158 °C.

HPLC t_R = 17.92 min.

Dihexyl 4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzoyl)-L-glutamate (43c)



Synthesis of **43c** was performed according to the general procedure, using MTX

(0.10 g, 0.22 mmol), DMF (3.5 mL), DIPEA (0.19 mL, 1.10 mmol), HOBT (0.09 g, 0.66 mmol), EDC (0.10 g, 0.66 mmol), hexan-1-ol (0.55 mL, 4.40 mmol) and DMAP (0.010 g, 0.11 mmol). A yellow solid was afforded (0.08 g, 0.13 mmol, 57% yield).

¹H NMR (DMSO-*d*₆): 8.72 (s, 1H, H-7), 8.34 (d, 1H, *J* = 7.3 Hz, NH-CH), 7.74 (d, 2H, *J* = 8.6 Hz, H-2', H-6'), 6.83 (d, *J* = 8.8 Hz, 2H, H-3', H-5'), 4.88 (s, 2H, CH₂-N-CH₃), 4.45-4.34 (m, 1H, CH-CH_AH_B), 4.09-3.95 (m, 4H, O-CH₂-CH₂), 3.25 (s, 3H, N-CH₃), 2.41 (t, 2H, *J* = 7.3 Hz, CH₂-CH_AH_B), 2.13-2.03 (m, 1H, CH_AH_B), 2.03-1.92 (m, 1H, CH_AH_B), 1.60-1.47 (m, 4H, CH₂-CH₂O), 1.32-1.17 (m, 12H, CH₂-CH₂-CH₂-CH₃), 0.88-0.78 (m, 6H, CH₃-CH₂).

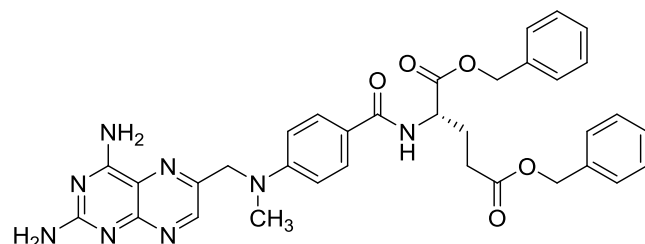
¹³C NMR (DMSO-*d*₆): 172.23, 172.11, 166.29, 162.67, 156.08, 151.06, 150.76, 148.78, 145.83, 128.98, 122.11, 121.16, 111.06, 104.28, 102.97, 64.33, 63.92, 54.82, 51.93, 30.82, 30.76, 30.21, 28.02, 25.78, 24.98, 21.93, 13.80, 13.75.

m/z: HRMS (TOF ES⁺) C₃₂H₄₇N₈O₅⁺ [MH]⁺ calculated 623.3664; found: 623.3712.

Mp: 169-171 °C.

HPLC t_R = 19.34 min.

Dibenzyl 4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzoyl)-L-glutamate (43d)



Synthesis of **43d** was performed according to the general procedure, using MTX (0.15 g, 0.33 mmol),

DMF (6 mL), DIPEA (0.29 mL, 1.65 mmol), HOBt (0.13 g, 0.99 mmol), EDC (0.15 g, 0.99 mmol), benzyl alcohol (0.68 mL, 6.60 mmol) and DMAP (0.02 g, 0.17 mmol). A yellow solid was afforded (0.10 g, 0.16 mmol, 49% yield).

¹H NMR (DMSO-*d*₆): 8.65 (s, 1H, H-7), 8.43 (d, 1H, *J* = 7.5 Hz, NH-CH), 7.76 (d, 2H, *J* = 8.7 Hz, H-2', H-6'), 7.38-7.25 (m, 10H, H-2''-H-6''), 6.84 (d, *J* = 9.3 Hz, 2H, H-3', H-5'), 5.13 (s, 2H, O-CH₂-benzyl), 5.07 (s, 2H, O-CH₂-benzyl), 4.84 (s, 2H, CH₂-N-CH₃), 4.55-4.46 (m, 1H, CH-CH_AH_B), 3.24 (s, 3H, N-CH₃), 2.54-2.47 (m, 2H, CH₂-CH_AH_B), 2.21-2.10 (m, 1H, CH_AH_B), 2.10-1.99 (m, 1H, CH_AH_B).

¹³C NMR (DMSO-*d*₆): 172.15, 171.98, 166.60, 162.74, 159.74, 150.88, 149.00, 148.30, 136.06, 129.02, 128.40, 128.05, 127.89, 127.65, 121.74, 120.95, 111.07, 65.83, 65.47, 51.94, 48.59, 30.14, 25.72.

***m/z*:** HRMS (TOF ES⁺) C₃₄H₃₅N₈O₅⁺ [MH]⁺ calculated 635.2725; found 635.2745.

Mp: 141-143 °C.

HPLC *t*_R = 16.10 min.

2.3 Results and Discussion

Due to folic acid's self-assembling properties and its similarity in structure to MTX, attempts in synthesising potential LMWGs based on MTX derivatives are here reported, using the strategy of covalently conjugating MTX to known self-assembling motifs, such as Fmoc-Phe-Phe or long alkyl chains.

2.3.1 Design and synthesis of MTX peptide conjugates

There have been many reports in the literature of MTX prodrugs synthesised in order to overcome the phenomenon of drug resistance and toxicity: mono and diesters, peptide-conjugates and alkylamino acids derivatives of MTX were synthesised to increase MTX's passive uptake into cells.²⁴⁻²⁹ Either selective or non-selective functionalisations of the α - or γ - carboxylates of glutamic acid have been reported, even though it has been demonstrated that the α - function plays a key role for the stability of MTX-DHFR binding.³⁰ On the contrary, the γ - carboxylic acid terminus showed only weak interactions with the target enzyme, therefore chemical changes can be performed in this region without modifying the affinity of the drug for DHFR.^{28, 31, 32} Furthermore, most of the synthetic strategies involved the use of 4-(N-(2,4-diamino-6-pteridinylmethyl)-N-methylamino) benzoic acid (**44**, APA) (Figure 8.2) as the building block in both liquid and solid phase syntheses.²⁸

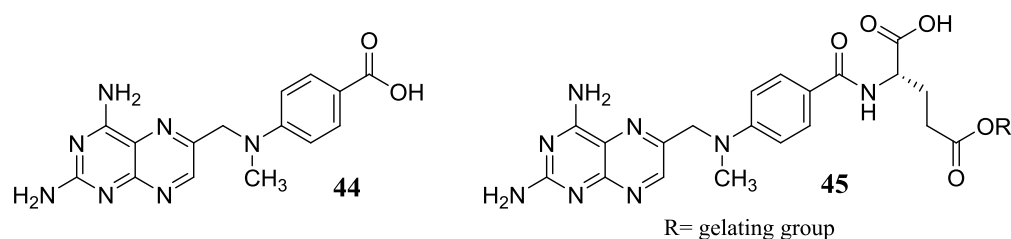
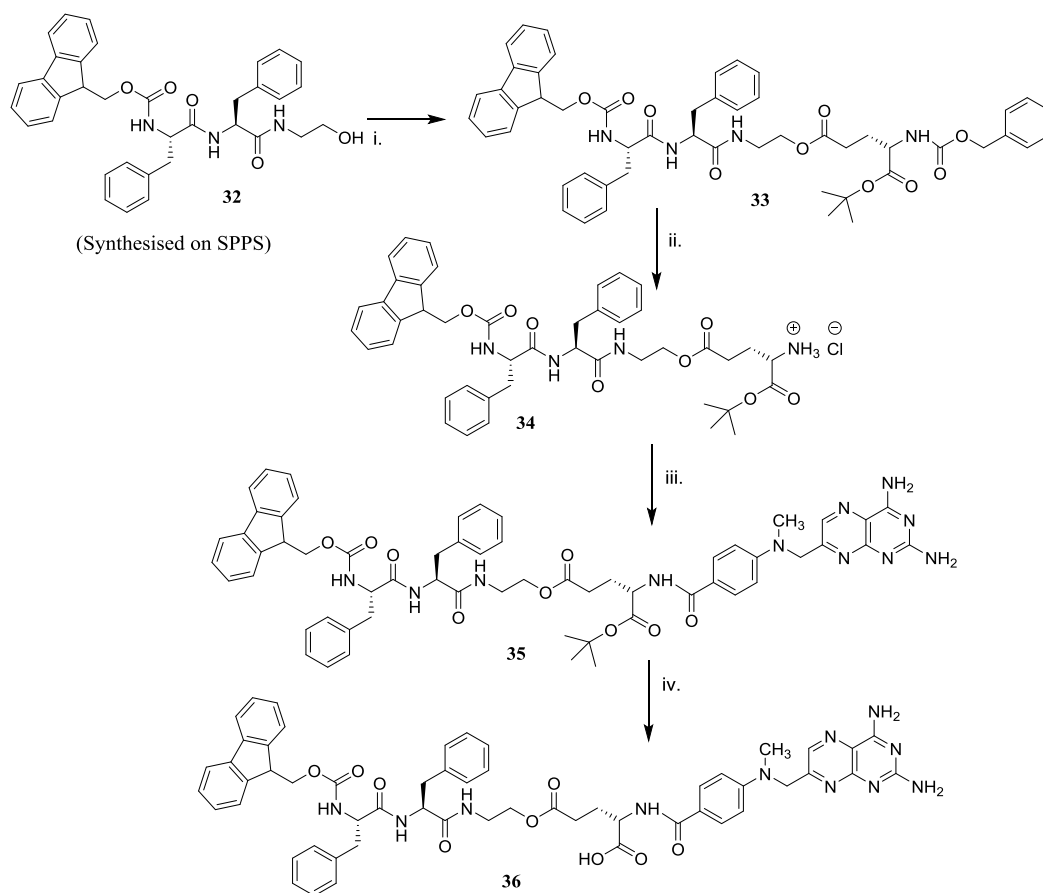


Figure 8.2: Molecular structures of building block APA (**44**); MTX esterified with “R” at the γ -carboxylic acid position (**45**).

Based on these premises, it was decided to synthesise γ - functionalised MTX conjugates using APA as starting material. MTX was derivatised via an ester with a gelating group **45** (Figure 8.2), since esters readily undergo enzymatic hydrolysis at physiological conditions³³ and enhance drugs' overall lipophilicity.

2.3.1.1 Liquid phase strategy

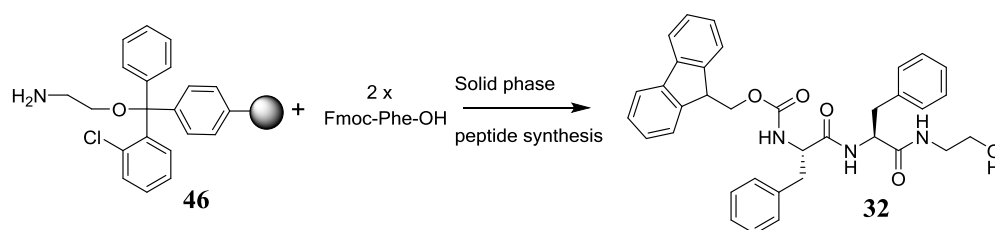
The choice of the gelating group to be covalently bound to MTX was chosen having analysed the most successful and common gelation motifs and also taking into account the characteristics of the drug (log P, molecular structure). In fact, an adequate balance between hydrophilicity and hydrophobicity is crucial for gel formation.²⁰



Scheme 1.2: Synthetic route to MTX derivative **36**. Reagents and conditions: i) Z-Glu-OtBu, EDC, HOBT, DMAP, CH₂Cl₂. ii) 10% Pd/C, H₂, HCl 0.1 M, MeOH. iii) APA, HATU, DIPEA, DMSO. iv) 50% TFA in CH₂Cl₂.

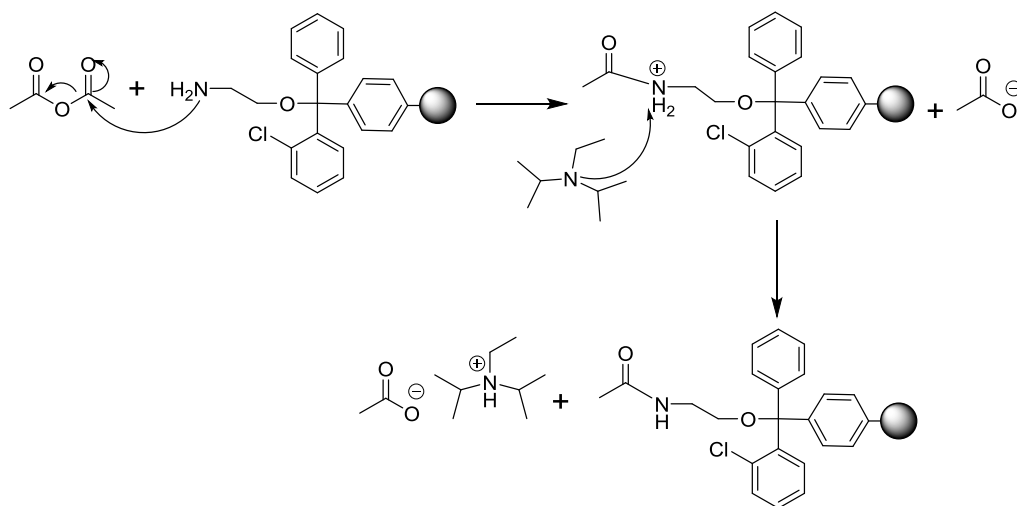
The protected-dipeptide Fmoc-Phe-Phe was selected as the gelating portion to be attached to the native drug via an ester bond.^{11, 12, 14} It was then decided to insert an ethylene linker between the MTX and the dipeptide in order to promote the release of the active compound from the prodrug.

The first synthetic route for the designed compound **36** involved initially the synthesis of a Fmoc-protected dipeptide via solid phase peptide synthesis (SPPS), then conjugation with a suitably protected glutamic acid, which represents a moiety of MTX, and finally coupling with APA, in order to minimise costs and toxicity related to this compound (Scheme 1.2).



Scheme 2.2: Synthesis of Fmoc-protected ethanolamine-linked dipeptide **32**

Synthesis of the protected dipeptide **32** was initially carried out. It was performed on a glycinol 2-chlorotrityl resin **46** (Scheme 2.2). This resin was chosen because it allows the release of the ethanolamide-linked peptide following cleavage.³⁴



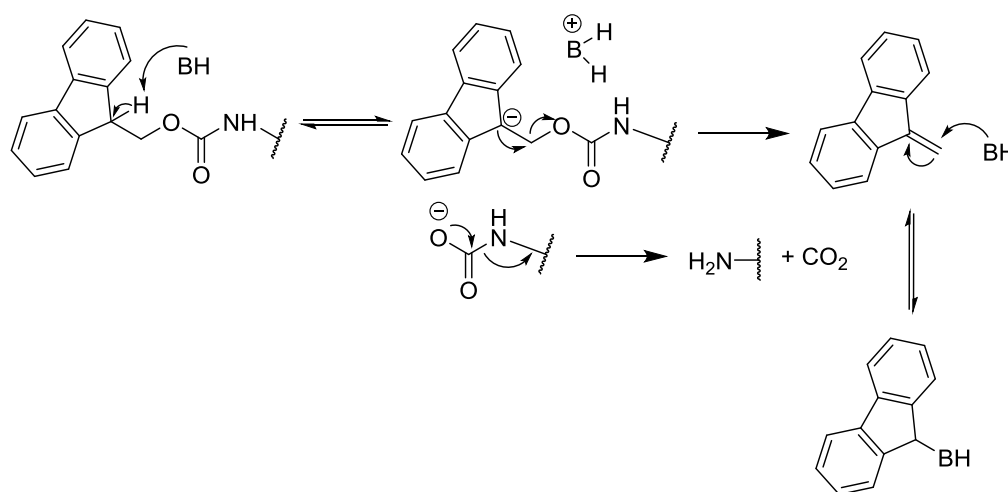
Scheme 3.2: N-acetylation of resin by acetic anhydride and DIPEA

Following attachment of the first amino acid to the resin, using HBTU and HOBT as coupling reagents in the presence of NMM as base, an N-terminal acetylation of the unreacted amine sites ('capping' of the resin) in presence of an excess of acetic anhydride and DIPEA was carried out, in order to prevent future side reactions (Scheme 3.2).

A base-mediated N-Fmoc-deprotection of the resin-bound first amino acid was then accomplished using 20% v/v piperidine in DMF. Fmoc is a base labile protecting group, which consists of an electron withdrawing fluorene ring system that renders the C⁹ proton acidic and thus, susceptible to abstraction by weak bases. This cleavage occurs via an E1cb mechanism, resulting in formation of a stabilised dibenzocyclopentadienyl anion; elimination of the carbamic acid generates the dibenzofulvene intermediate that forms stable adducts with excess amine cleavage agents (Scheme 4.2).

Elongation of the peptide, through coupling the second amino acid Fmoc-Phe-OH, was achieved in the same manner. The second Fmoc deprotection was intentionally omitted to achieve the gelating motif Fmoc-Phe-Phe. The final compound **32** was then obtained after cleaving the resin under acidolytic conditions (TFA 95% in water) in a 65% yield.

One of the main challenges in the design of this strategy was the choice of a suitable orthogonal protecting system for the compounds to be coupled. In order to keep the Fmoc-protection necessary for gelation on compound **32**, a protecting group removable by non-basic conditions was required for glutamic acid.



Scheme 4.2: Base-mediated Fmoc-deprotection

Benzyl carbamate (Cbz or Z) protection was therefore chosen, since it is removed by a catalytic hydrogenolysis and Fmoc-protection is relatively stable to these conditions with careful monitoring.³⁵

The double protected benzyl carbamate/tert-butyl ester Z-Glu-(OH)-OtBu was conjugated to **32** in presence of EDC, HOBt and catalytic DMAP to afford **33**. This reaction was achieved following evaluation of different coupling reagents and conditions (Table 1.2).

Reaction	Reagent	Reagent	Reaction time	Outcome
FC022	DCC	HOBt	15 h	Starting material
FC023	DCC	-	15 h	Starting materials
FC025	EDC	-	15 h	33 + corresponding N-acylurea
FC032	EDC	HOBt	3 days	33

Table 1.2: Different coupling reagents tested for synthesis compound **33**

The first attempts involved the use of DCC, alone or in presence of HOBt, and DMAP as base in a catalytic amount, but no products were synthesised after an overnight reaction. It was then decided to use the water soluble EDC (**47**, Figure 9.2) as alternative coupling reagent and the overnight reaction yielded **33**, but detection of the N-acylisourea as side product in the crude reaction mixture was also revealed, due to the absence of HOBt. As a result, in the last attempt, HOBt (**48**, Figure 9.2) was introduced in the reaction mixture, to avoid the formation of the side product N-acylisourea (Scheme 5.2). Despite the successful conversion to product **33**, this reaction was considered the limiting step of the whole synthetic route to final MTX peptide conjugate **36**.

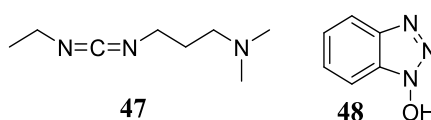
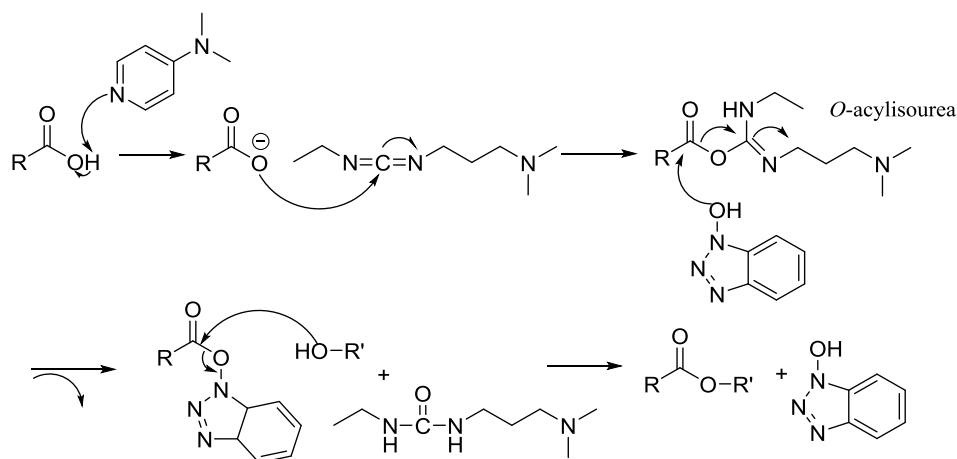
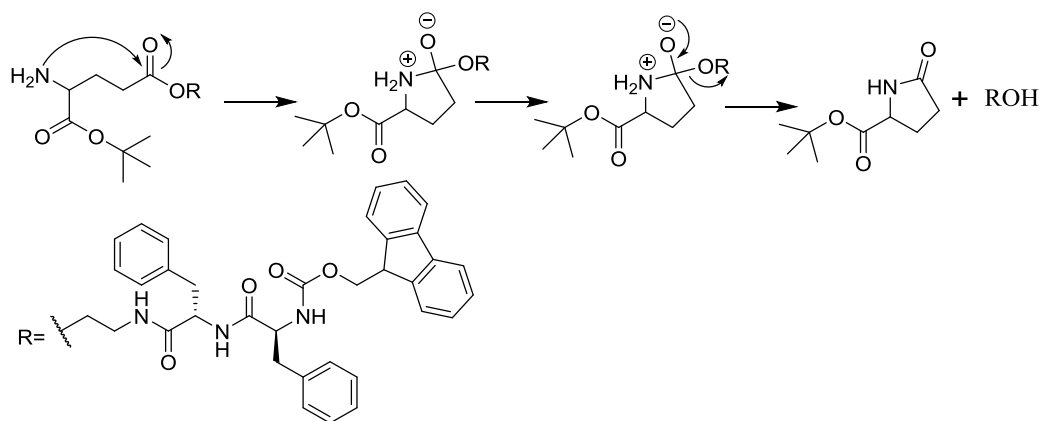


Figure 9.2: Molecular structures of EDC (**47**) and HOBt (**48**)



Scheme 5.2: Mechanism of esters formation EDC/HOBt mediated

Afterwards, the selective removal of protecting Cbz group of **33** was carried out by hydrogenolysis with 10% palladium on carbon, hydrogen and a solution of HCl 0.1 M, to yield **34** as hydrochloride salt. The main drawback during the deprotection of the amine of glutamic acid moiety is the risk of cyclisation that leads to formation of pyroglutamic acid as side product, due to the nucleophilic attack of the free amine to the γ -carboxylic function of glutamic acid (Scheme 6.2).²⁸ Synthesis of the hydrochloride salt of **34** (not the free amine form) prevented the formation of pyroglutamic side product.



Scheme 6.2: Mechanism of pyroglutamic acid derivative formation

The Fmoc-protected tripeptide **34** was then coupled with APA in the presence of HATU, to afford the α -tert-butyl-MTX conjugate **35**.

HBTU (**49**) and HATU (**50**) (Figure 10.2) are iminium based reagents used in amide coupling reactions. They activate the carboxylic group to allow the nucleophilic attack of the amino group of another amino acid and result in amide bond formation (Scheme 7.2).

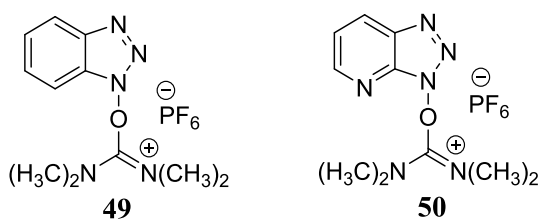
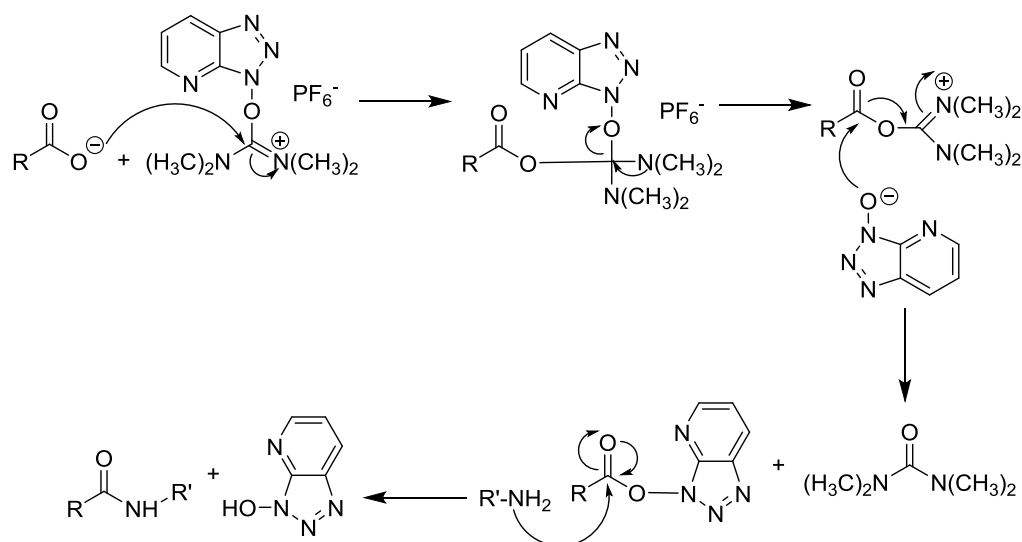


Figure 10.2: Molecular structure of HBTU (**49**) and HATU (**50**)



Scheme 7.2: Mechanism of HATU in amide-bond formation

HATU has a similar structure to HBTU but reacts faster with less epimerisation during coupling and, consequently, it is preferred to HBTU in most difficult coupling protocols.

The last step of the synthetic route involved removal of the tert-butyl group using TFA in CH_2Cl_2 , to yield the final compound **36**.

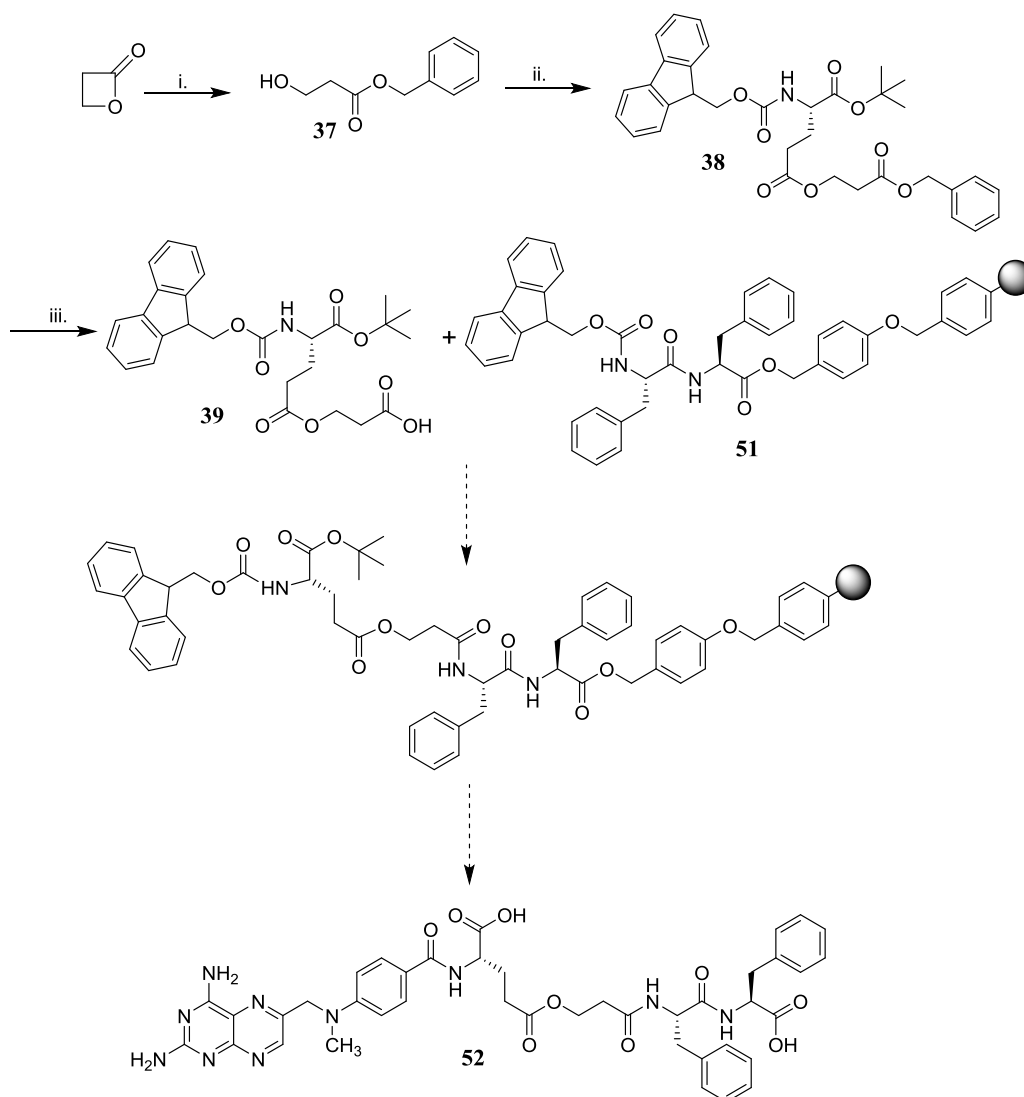
Despite this successful synthetic route, the main issue of the overall synthesis was represented by the low yields of intermediate products. Indeed, in order to test the compound for gelation, an adequate scaling up process was performed, with the attempt of increasing the yield of compound **33**, whose synthesis

represented the main limiting step of this synthetic route. A Mitsunobu reaction (diisopropyl azodicarboxylate, DIAD, and triphenyl phosphine) was also attempted to effect the required esterification between the protected dipeptide **32** and glutamic acid, without any success. Final compound **36** was afforded in crude quantities of 1-2 mg, which were not suitable for a first gelation screening. Therefore, this strategy was not considered effective for the synthesis of gelators on a suitable scale and an alternative synthetic route was investigated.

2.3.1.2 Solid phase strategy

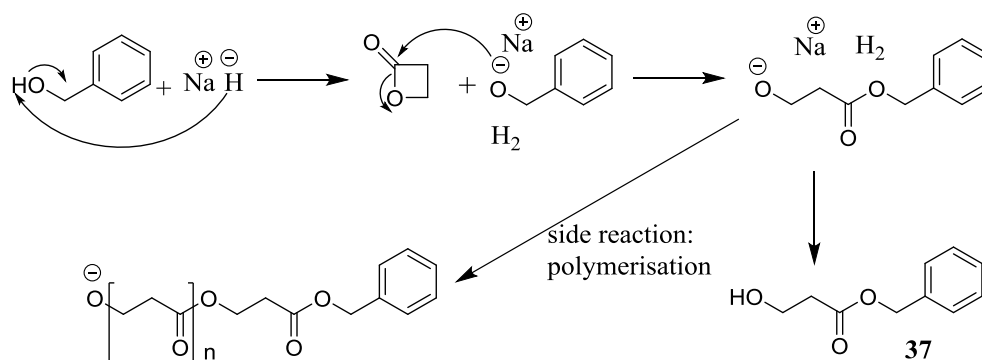
A solid phase synthetic strategy was then carried out in order to compare the efficiency and the feasibility of MTX conjugates synthesis in liquid and solid phase.

MTX derivative **52** (Scheme 8.2) was designed as the targeted compound to be synthesised. It has a similar chemical structure to **36**, differing in the absence of the Fmoc protection of the gelating motif Fmoc-Phe-Phe, which was omitted for synthetic reasons.



Scheme 8.2: Solid phase synthetic strategy for compound **52**. Reagents and conditions: i) Benzyl alcohol, NaH, -10°C. ii) Fmoc-Glu-OtBu, DCC, HOBt, DMAP, CH₂Cl₂. iii) 10% Pd/C, H₂, MeOH.

This solid phase strategy firstly involved a liquid phase synthesis of the compound to be reacted with a Phe-Phe pre-loaded resin, followed by the addition of APA in the last step of the reaction. This solid phase synthesis followed an Fmoc/tert-butyl orthogonal protecting group strategy, with the C-terminal residue (Phe) anchored to the resin. The first step of the synthetic route consisted in the opening of a β -propiolactone ring in the presence of sodium hydride and benzyl alcohol, in order to achieve a benzyl-protected hydroxypropionic acid, which represents the ethylene linker that connects the Phe-Phe moiety to MTX. The benzyl group was chosen as protection for the hydroxypropanoic acid according to the orthogonal protection system. Benzyl 3-hydroxypropanoate (**37**) was synthesised modifying a previously reported method,³⁶ by reacting an excess of benzyl alcohol with sodium hydride and β -propiolactone. In order to reduce the risk of polymerisation, caused by nucleophilic attack of the alcoholate to the lactone ring, a quick reaction at low temperature ($-10\text{ }^{\circ}\text{C}$, 30 min) favoured the ring opening without risk of side product formation (Scheme 9.2).

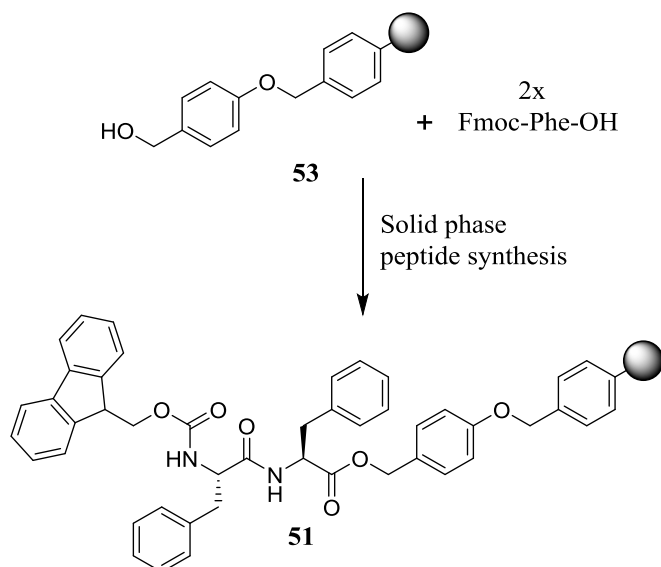


Scheme 9.2: Opening of β -propiolactone ring base-mediated and polymerisation side-reaction.

The following step involved the conjugation, via an ester bond formation, of the double protected Fmoc-Glu-(OH)-OtBu with the linker **37** in the presence of DCC and HOBt, to afford **38**.

Then, the selective removal of the Cbz protecting group of **38** was carried out by hydrogenolysis with 10% palladium on carbon and hydrogen to achieve **39**.

Synthesis of the Fmoc-Phe-Phe moiety (**51**) was performed on a Wang resin (**53**), which was chosen because its use has been previously reported in solid phase syntheses of MTX derivatives.^{27, 37}



Scheme 10.2: Synthesis of Fmoc-Phe-Phe moiety **51**

Synthesis of the Fmoc-Phe-Phe moiety (**51**) was carried out as previously reported, using HBTU and DIPEA and obtaining a 72% resin loading (0.65 mmol/g). After the synthesis of compound **39** in liquid phase and **51** in solid phase, a Fmoc deprotection of the Fmoc-Phe-Phe moiety was carried out, to allow the following coupling between the C-terminal of the linker and the N-terminal of Phe-Phe. Despite a double coupling was performed, a low loading of the resin was obtained (Table 2.2).

Reaction	HATU	DIPEA	Reaction time	Resin loading after 1 st coupling	Resin loading after 2 nd coupling
FC036	1.2 eq	2.4 eq	1.5 h	0.13 mmol/g	-
FC037	1 eq	2 eq	1 h	0.13 mmol/g	-
FC038	1 eq	4 eq	1.5 h	0.15 mmol/g	0.21 mmol/g

Table 2.2: Attempts of coupling **39** with different reaction conditions

A mini-cleavage of the compound from the resin was then carried out to analyse the product generated by liquid chromatography-mass spectrometry (LC-MS), which did not detect the presence of the main product **54** (Figure 11.2), but revealed formation of product **55**.

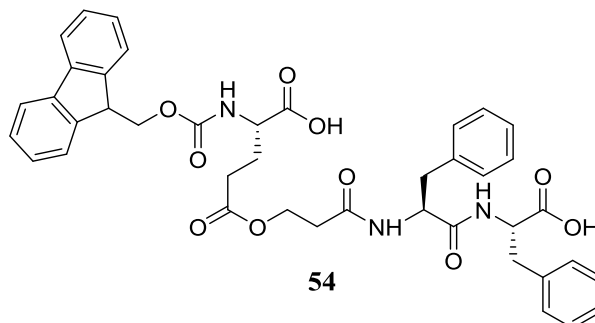
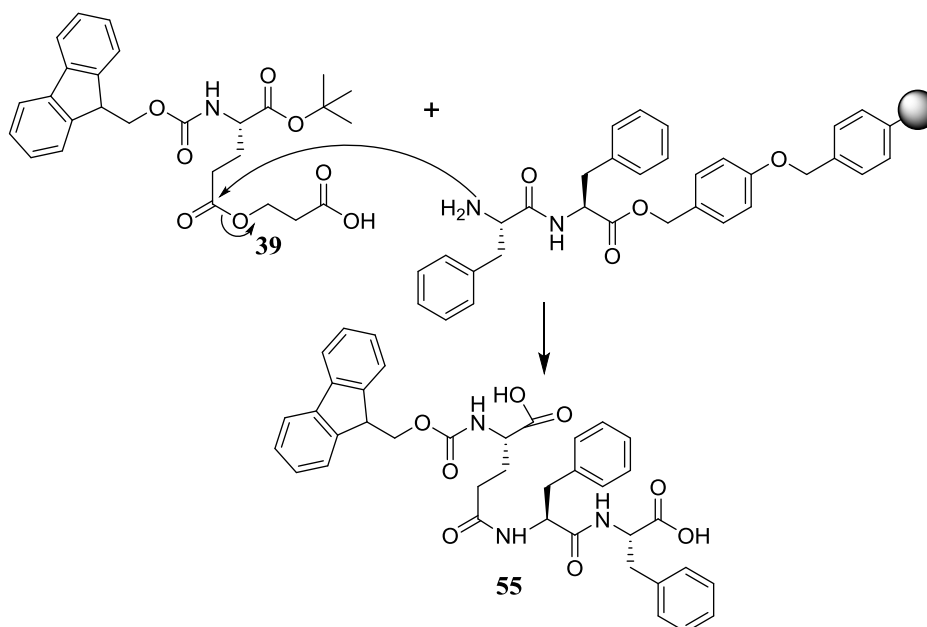


Figure 11.2: Molecular structure of expected compound **54** after mini cleavage

Formation of compound **55** could potentially be explained by aminolysis of the ester bond of compound **39**: the N-terminal of the Phe-Phe moiety makes a nucleophilic attack on the carboxylic group of **39**, resulting in an amide bond formation between the C-terminal of the resulting compound and the N-terminal of the Phe-Phe moiety (Scheme 11.2).



Scheme 11.2: Aminolysis reaction of **39**

This hypothesis was also confirmed by a test reaction in liquid phase, in which **39** was coupled with propylamine in presence of HATU and DIPEA as coupling

reagents. Also in this case, aminolysis occurred, thus proving formation of the side product in the previous reaction.

Therefore, this alternative synthetic route in solid phase demonstrated to be ineffective for the synthesis of MTX peptide conjugates due to occurrence of aminolysis side reaction during the coupling step.

Since both liquid and solid phase strategies used to produce potential self-assembling MTX conjugates were revealed to be unsuccessful, due to low yields and side products formation, the synthesis of a different class of MTX derivatives was investigated.

2.3.2 Design and synthesis of MTX acyl derivatives

Since the two previously reported synthetic procedures were shown to be ineffective with respect to production of MTX conjugates, the design of a new group of derivatives was attempted, with the aim of introducing a different self-assembling motif in the MTX structure compared to the protected dipeptide used before.

Therefore, the synthesis of MTX conjugates bearing short or long alkyl chains and aromatic groups on both α and γ carboxylic acid of MTX (diesters) or to the α moiety alone (monoester) were investigated.

2.3.2.1 Synthesis of MTX dodecyl monoester

Compound **42** (Figure 12.2) was rationally designed to have a dodecyl alkyl chain covalently conjugated to the α carboxylic acid moiety of the glutamic acid of MTX. In this case, an α functionalisation was carried out to render the γ terminus free, in order to assess whether the free alkyl chain of the glutamic acid could have promoted the self-assembly process, due to formation of hydrophobic forces.

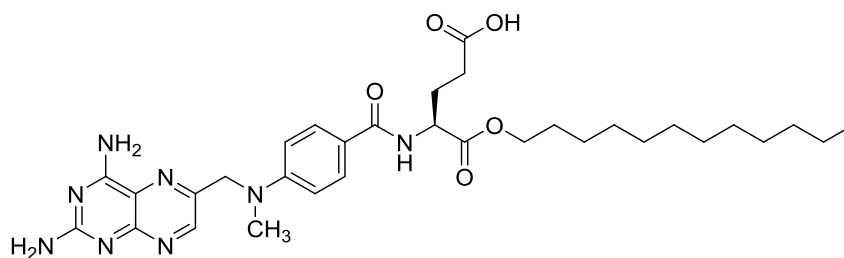
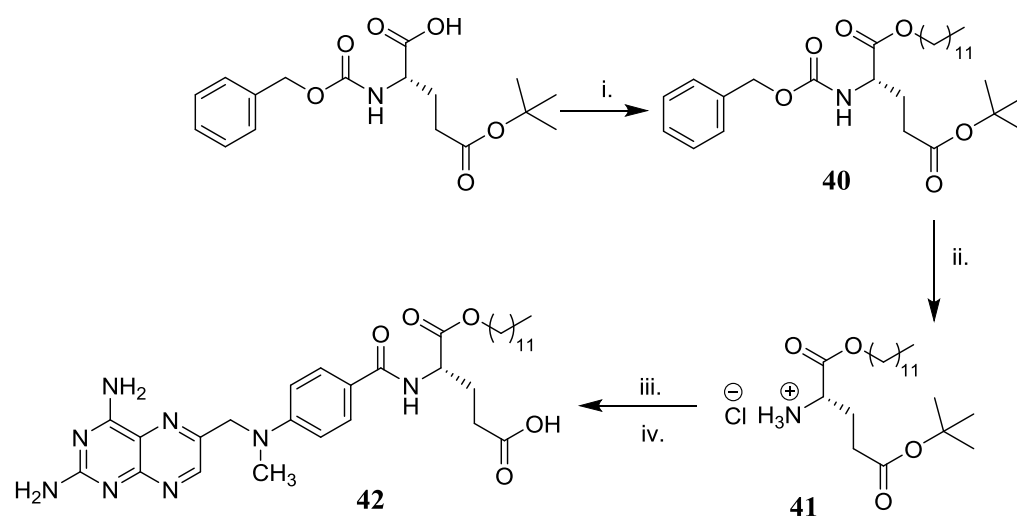


Figure 12.2: Molecular structure of MTX monoester conjugate **42**.

The length of the alkyl chain on the α terminus was chosen based on the successfully reported gelators developed in our group, in which cytidine derivatives possessing acyl chains of different lengths formed LMWGs in binary systems of water and organic solvents. In particular, the conjugate having a dodecyl chain demonstrated a higher propensity for gelation compared to other analogues with different chain lengths.³⁸

The synthetic route employed (Scheme 12.2) required a Z-Glu-OtBu-(OH) as starting material, which was coupled to dodecan-1-ol in the presence of DCC and DMAP, to afford the ester glutamic acid dodecyl derivative **40**. Then, selective removal of the Cbz protecting group was carried out by hydrogenolysis with 10% palladium on carbon and hydrogen to achieve **41**. The final step involved firstly the coupling of **41** with APA using HOBt and PYBOP (**56**, Figure 13.2) as coupling reagent and DIPEA as base; followed by removal of the tert-butyl group, carried out in acidic conditions using TFA in CH₂Cl₂, to yield the final compound **42**.



Scheme 12.2: Synthetic route to MTX conjugate **42**. Reagents and conditions: i) Dodecan-1-ol, DCC, DMAP, CH₂Cl₂. ii) 10% Pd/C, H₂, MeOH. iii) APA, PYBOP, DIPEA, DMSO. iv) 50% TFA in CH₂Cl₂.

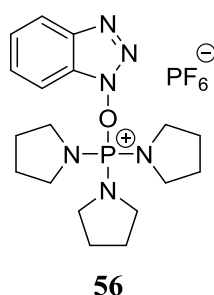
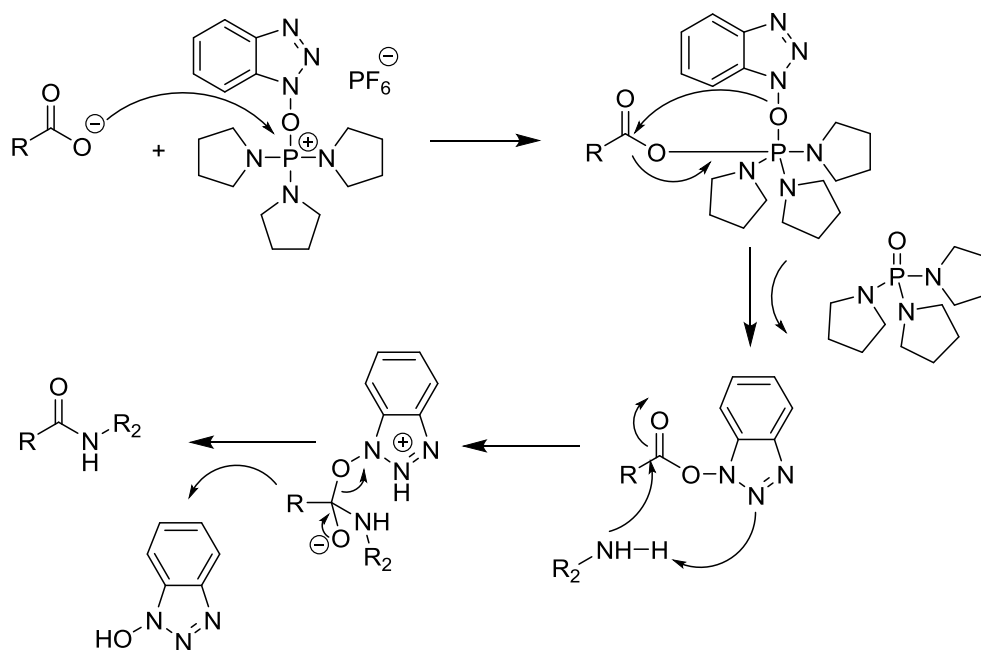


Figure 13.2: Molecular structure of coupling reagent PYBOP.

In an attempt to form an amide bond between APA and **41**, HATU was initially adopted as the coupling reagent, without giving full conversion of the starting

material. Then, PyBOP (**56**) was employed, by modifying a previously reported method,³⁹ affording compound **42** in 31% yield after purification by preparative RP-HPLC.

PYBOP is a phosphonium coupling reagent (Scheme 13.2), used as an alternative to HATU or EDC, which was developed to substitute BOP, in order to prevent generation of the toxic byproduct hexamethylphosphoramide (HMPA).



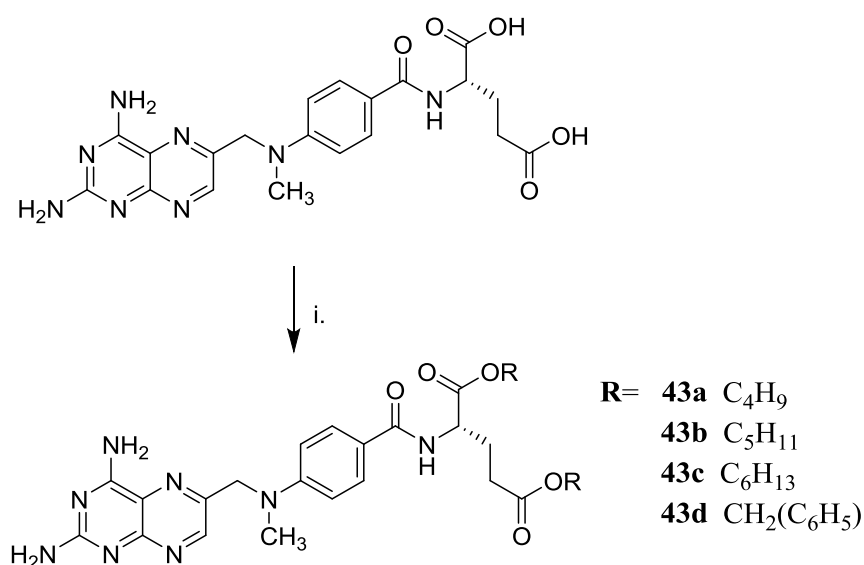
Scheme 13.2: Mechanism of PYBOP in amide bond formation

2.3.2.2 Synthesis of MTX diesters

A series of MTX diester derivatives were also synthesised to evaluate the effect of the double functionalisation of the α - and γ - carboxylates of glutamic acid on the self-assembly of MTX. In particular, MTX was conjugated via an ester bond to alcohols of different chains lengths and the addition of aromatic moieties was also investigated, with the aim of introducing non-covalent interactions (i.e. π - π stacking, hydrophobic and van der Waals interactions) to promote the self-assembly.

Moreover, as previously reported by Rosowsky and his group, addition of long aliphatic chains at the α and γ glutamic acid carboxylic terminal would improve the overall lipophilicity compared to the native MTX, with the possibility of increasing MTX uptake into cells via membrane passive diffusion.⁴⁰

Compounds **43a** to **43d** were synthesised by reaction of MTX with an excess of the corresponding alcohols using EDC as coupling reagent and DIPEA as base in DMF (Scheme 14.2). All the compounds were purified by preparative RP-HPLC and were obtained in yields between 48% and 61%.



Scheme 14.2: Synthetic route to MTX conjugates **43a** to **43d**. Reagents and conditions: i) DIPEA, HOBt, EDC, DMF, 0°C, 5 min, then selected alcohols, DMAP, r.t.

2.3.3 Preliminary gelation tests of MTX acyl derivatives

Preliminary gelation tests were carried out on MTX acyl conjugates to evaluate the self-assembling properties of these compounds, using a vial inversion test as initial screening to define gel formation. A solvent and temperature switch method (method A, section 2.2.1.5) was adopted for product **42** and the series of MTX diesters **43a** to **43d**, using DMSO or ethanol as organic solvents in binary mixtures with water.

The use of DMSO as organic solvent to promote gelation in addition to water has been extensively reported because of its known ability to solubilise gelators prior to the self-assembling process, especially for poorly soluble compounds. Indeed, complete solubility is firstly required for gelation; then, addition of water induces a change in gelator solubility and acts as nucleation step for gelation.⁴¹ In terms of drug delivery, DMSO has been reported to enhance topical penetrations of drugs at concentrations of 15% and it is currently used as an excipient for several applications, i.e. for the sustained delivery of polymer-based formulations.⁴² DMSO has a low systemic toxicity but can cause localised side effects, therefore it should be carefully used for targeted drug delivery purposes; however, some studies showed its ability to reduce growth of human-derived ovarian and pancreatic cancer cell lines and to promote differentiation of leukaemic cells.⁴³⁻⁴⁵ Therefore, DMSO can be considered a suitable organic solvent to be employed as cancer drug delivery platform.

Ethanol, often used as co-solvent for enhancing solubilisation of poorly soluble drugs, has also been reported to be a gelating solvent.³⁸ Moreover, intra-tumoural ethanol injections have been recommended as a method for tumour ablation in oesophageal and gastric cancers.⁴⁶ Therefore, this organic solvent can also be used for preparation of LMWGs for localised cancer therapy.

Vial inversion tests performed on compound **42** and the series of MTX diesters **43a** to **43d**, determined formation of precipitates, solutions and viscous solutions (Table 3.2). In particular, results from these preliminary tests showed a similar trend for all the compounds tested, with formation of precipitates at low solvent concentrations (expressed as the volume fraction of the solvent, ϕ_{SOL} , from 0.1 to 0.5), whilst clear solutions were formed by increasing the

organic solvent-to-water ratios ($\phi_{\text{SOL}} > 0.6$). This could be explained by the higher solubility of compounds in organic solvents compared to water, due to their high lipophilic nature.

Samples **43b**, **43c** and **43d**, possessing longer alkyl chains and benzyl moieties, formed viscous solutions in the binary mixtures with DMSO after the cooling process, at the highest solvent-to-water ratios (ϕ_{DMSO} from 0.7 to 0.9). However, because of their macroscopic aspect, more similar to solutions rather than gels (Figure 14.2), further analyses were not carried out, as the samples did not possess the suitable stiffness of a gel required for drug delivery purposes. Indeed, the ability of the gel to remain localised in a body tissue is directly related to its stiffness.¹⁰

Finally, results showed that mono- or di-functionalisation of carboxylic acid terminals of MTX did not provide any differences in terms of self-assembling properties.

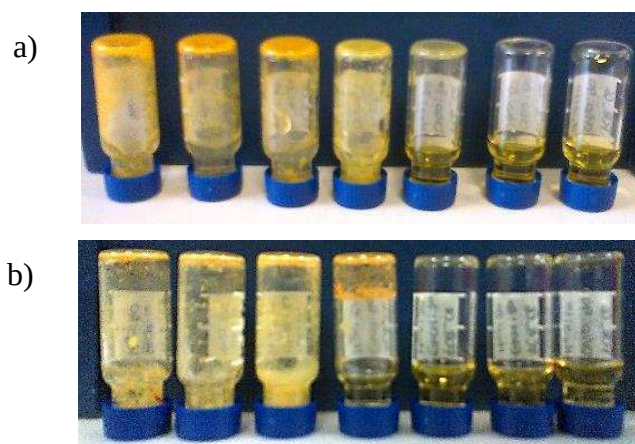


Figure 14.2: Representative vial inversion tests of MTX acyl derivatives (compound **43c**) using a solvent-switch method in binary mixtures of water and DMSO (a) or ethanol (b). Vials are presented from low solvent volume fraction ϕ_{SOL} on the left (ϕ_{SOL} of 0.1) to higher solvent concentrations ϕ_{SOL} on the right (ϕ_{SOL} of 0.9).

	42		43a		43b		43c		43d	
ϕ_{SOL}	DMSO	Ethanol	DMSO	Ethanol	DMSO	Ethanol	DMSO	Ethanol	DMSO	Ethanol
0.1	P	P	P	P	P	P	P	P	P	P
0.2	P	P	P	P	P	P	P	P	P	P
0.3	P	P	P	S	P	P	P	P	P	P
0.5	P	P	S	S	P	P	P	P	P	P
0.7	S	S	S	S	S	S	S	S	VS	S
0.8	S	S	S	S	VS	S	VS	S	VS	S
0.9	S	S	S	S	VS	S	VS	S	VS	S

Table 3.2: Data table of vial inversion tests of compounds **42** and **43a-d** in binary mixtures of DMSO or ethanol and H₂O ($0.1 \leq \phi_{\text{SOL}} \leq 0.9$). P=precipitate, S= solution and VS=viscous solutions.

2.4 Conclusions

In this chapter, different approaches for the synthesis of potential self-assembling MTX conjugates have been reported. Furthermore, preliminary gelation tests have been carried out on MTX acyl derivatives.

The first strategy involved the synthesis in liquid phase of a MTX derivative by conjugation of MTX at the γ moiety to a Fmoc-Phe-Phe gelating group via an ester bond (compound **36**). Unfortunately, several attempts of scaling up the final compound for gelation tests resulted in failure or low yields, therefore it was not possible to carry out preliminary self-assembling studies.

A second synthetic route was then investigated and compound **52** was designed as the targeted MTX derivative to be prepared in solid phase. Although the synthesis of the linker **39** was carried out efficiently, attempts to couple it to the pre-loaded Phe-Phe resin were unsuccessful, probably due to the occurrence of an aminolysis side reaction during the coupling step.

Since both liquid and solid phase synthetic strategies used to produce potential self-assembling MTX conjugates were unsuccessful, a new group of derivatives were synthesised by conjugation of MTX to alkyl chains of different lengths and aromatic groups. The monoester **42** consisted of MTX linked to a dodecyl alkyl chain at the α moiety of the glutamic acid via an ester bond. MTX diesters **43a** to **43d** were synthesised by conjugation of MTX to alcohols with different alkyl chain lengths and a benzyl group.

Preliminary gelation studies carried out on the synthesised MTX acyl derivatives via a solvent-switch method demonstrated lack of self-assembling properties of these compounds, with formation only of viscous solutions of **43b-43d** at high DMSO-to-water ratios (ϕ_{DMSO} from 0.7 to 0.9); thus these derivatives were not suitable for drug delivery applications. Therefore, derivatisation of MTX with self-assembling groups did not promote formation of self-assembled conjugates of this anticancer drug, confirming the previous reported lack of gelation behaviour of MTX.⁷

As a consequence, the design and synthesis of novel potential self-assembling derivatives of another anticancer candidate were evaluated and the results will be described in the following two chapters.

2.5 References

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Chapter 3 : Self-assembling amide prodrugs of benzothiazole 5F 203

In this chapter, a brief review of LMWGs based on benzothiazoles and amino acids derivatives will be described. Then, the synthesis, self-assembling properties and in vitro growth inhibitory activity of two novel series of amide prodrugs based on the antitumour drug candidate 5F 203 will be evaluated.

3.1 Introduction

Phortress, the L-lysylamide benzothiazole prodrug of 5F 203 which progressed to Phase I clinical trial, displayed toxicities to lungs and liver when administered intravenously in patients.¹ Therefore, in order to avoid systemic benzothiazole exposure and toxicity, a formulation of prodrugs of 5F 203 as LMWGs for localised drug delivery at tumour resection sites was investigated.²

3.1.1 LMWGs based on benzothiazoles derivatives

Benzothiazoles are aromatic heterocyclic compounds that consist of a benzene ring fused to a five-membered 1,3-thiazole ring (**57**, Figure 1.3). Their aromatic structure confers the π - π stacking required for directional self-association.

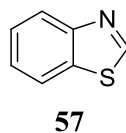


Figure 1.3: Chemical structure of benzothiazole (**57**)

Up to date, only Gao and his group reported two examples of low molecular weight organogelators based on benzothiazole derivatives.

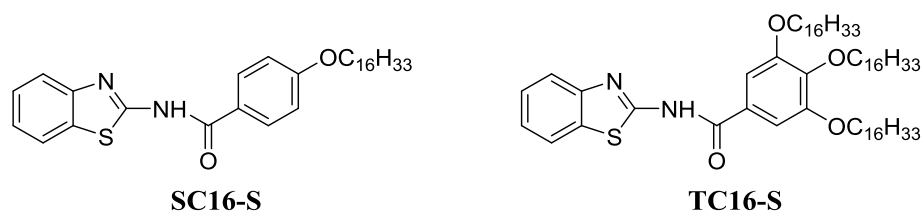


Figure 2.3: Structures of **SC16-S** and **TC16-S**.

They firstly synthesised benzimidazole and benzothiazole amide derivatives bearing different substituted alkyl chains. Compounds SC16-S and TC16-S, with a single and three alkoxy chains on the phenyl moiety respectively, showed different gelation behavior in organic solvents depending on the molecule substituents (Figure 2.3). In particular, TC16-S was able to form organogels in a wider range of solvents (i.e. isopentanol, nitrobenze, DMF, ethyl acetate) compared to its counterpart SC16-S, meaning that the number of alkyl substituents makes a difference in gelation, due to increased strength of intermolecular hydrophobic force between alkyl substituents chains. The group also confirmed the organogelator nature of the benzothiazole derivatives by SEM and AFM, showing formation of different morphologies, such as wrinkles or lamellae, depending on the solvent used. They also demonstrated by FTIR the pivotal role in self-assembly of the intermolecular hydrogen bonds between amide functional groups.³

More recently, the same group investigated the gelation behaviour of binary mixtures of aminobenzimidazole/aminobenzothiazole (**58**) with benzoic acid derivatives with alkyl substituents (**59**). The binary mixtures of the benzothiazole and the mono-substituted benzoic acid formed gels only in ethanolamine, nitrobenzene and aniline (Figure 3.3). Increasing the number of alkyl chains in the benzoic acid structure led to formation of organogels in more different solvents, such as 1,4-dioxane and DMF, demonstrating again the importance of the alkyl chain substitution in the self-assembling process due to increase of the intermolecular hydrogen bonds, confirmed also by FTIR.⁴ To date, the organogelators reported above are the only examples of LMWGs based on benzothiazoles' derivatives and there are currently no examples of hydrogelators based on this class of compounds.

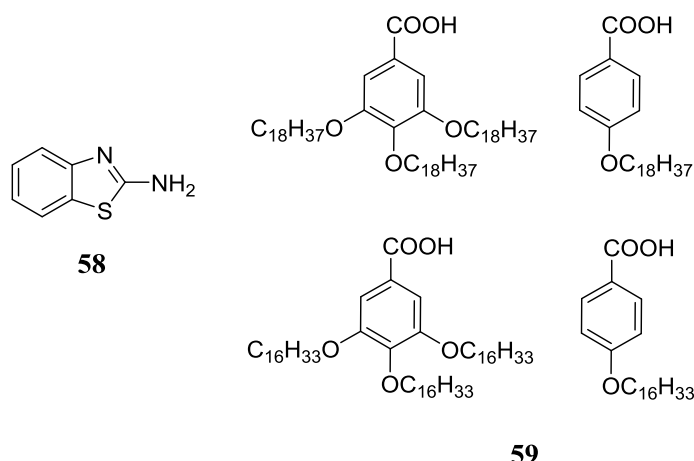


Figure 3.3: Structures of amino-benzothiazole (**58**) and mono/multialkyl substituted benzoic acid derivatives (**59**) used in the binary mixtures.

3.1.2 LMWGs based on amino acids derivatives

Amino acids derivatives have been extensively reported in the literature to possess both hydrogelation and organogelation properties.

In the field of hydrogelators, gels based on the amino acid L-cystine have been described: Menger and Caran demonstrated the self-assembling ability of a family of aroyl-cystine derivatives at low concentrations (0.25 mM, 0.01% w/w).⁵ Bradley et al. reported a combinatorial library of cysteine-based compounds capable of forming hydrogels using water and low concentrations of DMSO (10-20%).⁶

Hanabusa and Suzuki pioneered the field of hydrogelators based on L-lysine by synthesising amphiphilic derivatives of this amino acid conjugated to positively charged alkyl chains of different lengths (**60**).^{7, 8} A Fmoc-protected pentafluorophenylalanine amino acid (**61**) has also been reported by Nilsson and co-workers to form self-assembling entangled nanofibrillar structures in water at a concentration of 0.1 % w/t. The mechanism of self-assembly of the molecule was attributed to π - π stacking of the phenyl and Fmoc moieties and the hydrogen bonds of the fluorines, which promote the so-called “fluorous effect” (Figure 4.3).⁹

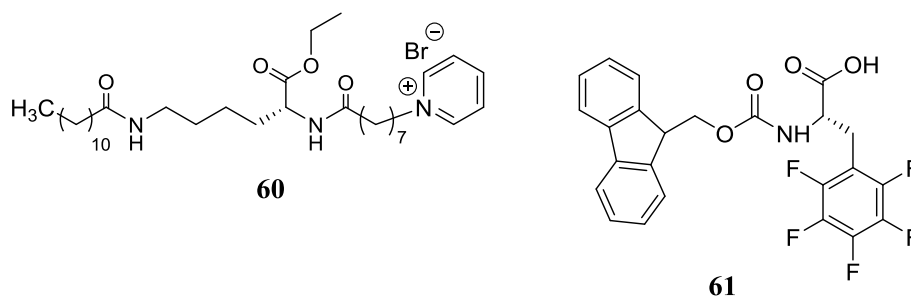


Figure 4.3: Examples of amino-acid based hydrogelators L-lysine derivative (**60**) and Fmoc-pentafluorophenylalanine (**61**).

In terms of organogelation, fewer examples are reported in the literature; Hanabusa et al. investigated on the self-assembling properties of L-leucine derivatives in nonpolar solvents, as hexane, octane, decane and dodecane.¹⁰ More recently, Li and his group developed an organogel based on N stearoyl-L-alanine methyl ester, which formed gels in different vegetable oils, such as soybean, olive and sunflower oil. They also demonstrated that this gel can act as an in situ forming implant for drug delivery, due to its biocompatibility and biodegradability properties.¹¹

These studies proved the propensity of amino acid-based compounds to self-assemble into supramolecular structures and their potential applications in drug delivery and tissue engineering.¹²

3.1.3 LMWGs with a terminal carboxylic acid functional group

Carboxylic acids are an example of polar groups known to form aggregates in water and organic solvents, due to their hydrogen bond donor and acceptor moieties which promote the self-assembly. In particular, the sol-gel transition of gelators containing a free carboxylic acid functionality can be easily triggered by modifying the pH, with a pH-switch method. With this approach, the acidic compound forms transparent solutions and colloidal dispersions at high pH; on lowering the pH, protonation of the carboxylate function occurs, leading to self-assembly and gel formation.¹³

As reviewed by Estroff, any structural modification of the molecule (i.e. different chain length) produces a change in the final pKa of the compound and, consequently, a different final pH required for gelation.¹⁴

Imae and his group described one of the first examples of N-acyl-L-aspartic acids bearing different alkyl chains which formed “gel-like solutions” by varying pH and temperature. They demonstrated the self-assembling behaviour of these amphiphilic compounds by showing formation of helical nanofibres by TEM.¹⁵ Since then, numerous groups have developed LMWGs taking advantage of the tunable properties of gels containing carboxylic units because of their dependence on pH.

Kim and co-workers synthesised biotin-amino acid conjugates (**62**) by introducing an amide bond in the biotin structure to increase the hydrogen bonds but retaining the free carboxylic acid groups to make the gels pH-dependent (Figure 5.3). They formed hydrogels in a broad range of pHs (from 2 to 9) and showed formation of distinct types of gels morphologies (fibres and lamella) depending on different substituent amino-acids on the biotin molecule, having long linear or branched and bulky alkyl chains.¹⁶

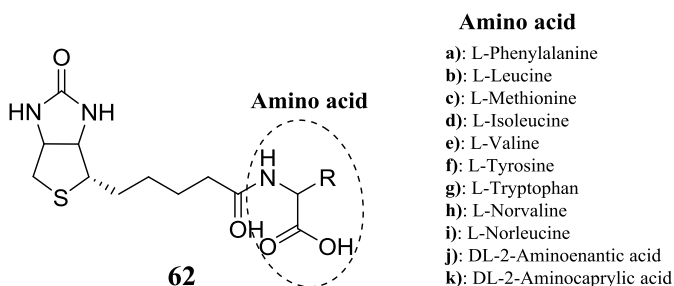


Figure 5.3: Structures of biotin-amino acid conjugate hydrogelators (**62**) bearing long linear or branched alkyl chains. Taken from Ref. 16.

Most di- and tripeptides reported in the literature that form hydrogelators have self-assembled via a pH trigger, because of their free carboxylic acid terminus.^{13, 17, 18} However, in terms of gel formation, one of the main challenges is to maintain a controlled homogeneity throughout all the self-assembly process, to allow for production of consistent and reproducible materials, particularly for drug delivery and tissue culture purposes. Addition of aqueous acid, usually HCl, to a basic solution of compound was the most common approach to lower the

pH and promote gel formation. However, this resulted in a drastic and rapid pH change, with the presence of non-homogeneous gel parts mixed with denser regions (Figure 6.3).

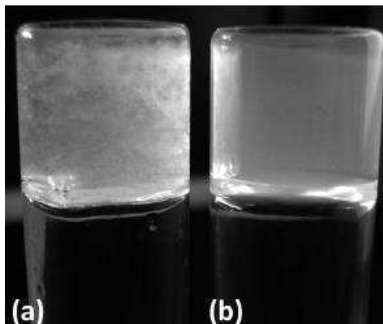


Figure 6.3: Pictures of hydrogels of Fmoc-leucine–glycine, both having a final pH of 3.9. a) pH changed using HCl. Gel shows formation of turbid non-homogeneity. b) pH changed using GdL. A homogeneous and transparent gel is formed. Adapted from Ref. 19.

As a consequence, Adams and his group introduced for the first time the use of glucono- δ -lactone (GdL), a hydrophilic cyclic ester which slowly hydrolyses in water into gluconic acid, lowering the pH. The slow rate of hydrolysis of GdL results in a uniform and controlled decrease in pH and, consequently, more visibly homogeneous gel formation.¹⁹ Since then, the GdL approach has been extensively adopted in pH-triggered methods for hydrogelation of many molecules bearing a free carboxylic acid functionality.

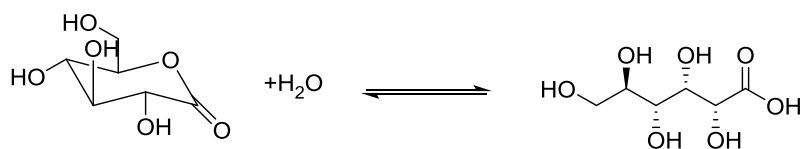


Figure 7.3: Hydrolysis of glucono- δ -lactone (GdL) into gluconic acid.

3.2 Materials and Methods

3.2.1 General procedures

3.2.1.1 Materials

Chemicals were purchased from commercial suppliers and were used without further purification. All solvents used were either analytical reagent or HPLC grade and were supplied by Fisher Scientific. Dry CH₂Cl₂, DMF and THF were purchased by Acros Organics. Deuterated solvents were purchased from Cambridge Isotope Laboratories Inc. and Sigma Aldrich.

3.2.1.2 General methods

Reactions were performed according to general methods described in Chapter 2, section 2.2.1.2.

3.2.1.3 Instrumentation

NMR, LC-MS, HRMS, melting points, lyophilisation and analytical RP-HPLC were carried out as described in Chapter 2, section 2.2.1.3.

Optical rotation was measured on a Bellingham + Stanley Ltd. ADP 220 polarimeter, using a polarimeter tube of 0.25 dm. $[\alpha]_D$ values are reported, with D indicating that the wavelength of the light used is the sodium D line (589 nm). Concentration (c) is in g/100 mL.

Preparative HPLC was performed using a Waters 2767 sample manager, Waters 2525 binary gradient module and visualised at 254 nm with a Waters 2487 dual wavelength absorbance detector. Spectra were analysed using MassLynx. Preparative RP-HPLC was performed using a Phenomenex Luna 10 μ m C8 (2) 100 Å column (150 mm x 30.00 mm) at a flow rate of 20 mL/min and UV detection at 254 nm using a preparative RP-HPLC method of 0-16 min gradient of 40% to 80% solvent B in solvent A, 16-18 min held at 80% solvent B in solvent A, 18-20 min 80% to 40% solvent B in solvent A, 20-22 min held at 40%

solvent B in solvent A, with solvent A= 0.05 % TFA in H₂O and solvent B= 0.05 % TFA in 9:1 v:v CH₃CN:H₂O.

3.2.1.4 Gelation tests

Method A: solvent switch method

The compounds were weighed using an OHAUS semi micro-analytical balance into 1.5 mL sample vials. DMSO was added to create a stock solution at 10% w/v. The solution was then sonicated in a Sonicator Ultra-sonic bath for one minute and successively heated at 60 °C to help full dissolution of the compound. An aliquot (25 µL) of the stock solution was transferred in another 1.5 mL vial and the remaining DMSO and pre-heated (60 °C) ultra-purified water were added. Samples were then allowed to cool down to room temperature. Vial inversion tests were subsequently carried out as preliminary screens for gelation. All samples prepared had a final concentration of 0.5% w/v.

Method B: pH-switch method for acidic compounds

The compounds (5 mg) were weighed into 1.5 mL sample vials. Ultra-purified water (0.5 mL) was added and the suspension was sonicated for 1 min. A 0.5 M solution of NaOH (30 µL) was then added and the mixture was sonicated until a clear solution was obtained. Glucono-δ-lactone (GdL, 0.7 molar equivalents) was added to the solution which was vortexed for ~1 min (HatiRotamixer). Samples were then allowed to stand for 12 h. All samples prepared had a final concentration of 1 % w/v.

Method C: pH-switch method for basic compounds

The compound (2.5 mg) was weighed into 1.5 mL sample vials. Ultra-purified water (0.5 mL) was added. The suspension was sonicated for one minute and a solution of 0.1 M HCl (5 µL) was added. After this addition, the solution was vortexed for 1 min, until a clear solution was obtained. Different aliquots of 0.5 M NaOH (from 5 to 15 µL) were then added and mixing was achieved by sonication for approximately 10 sec. The samples prepared had a final concentration of 0.5% w/v.

For methods B and C, pH was measured using a pH H18424 bench-top pH meter (Hanna Instruments) with a SLS Electrode pH glass semi microprobe. The pH meter was calibrated using pH 4, pH 7 and pH 10 buffer solutions. The probe was inserted into the solution and pH readings were recorded 3 times.

3.2.1.5 Transmission electron microscopy (TEM)

TEM was performed on a JEOL JEM-2100 using an accelerated voltage of 100 kV. A 10 μ L micropipette was used to load a small amount of gel on to a graphene oxide lacey carbon hexagonal copper grid (300 mesh, Agar). The grid was subjected to high vacuum prior to imaging. ImageJ software was used to analyse TEM images.

3.2.1.6 Rheological experiments

Rheological studies were carried out using an Anton Paar MCR302 Modular Compact Rheometer. A four-bladed vane geometry was used, with a diameter of 8.5 mm and length 8.5 mm in a cup with a diameter of 14.5 mm. Preparation of the hydrogels was carried out in a 7 mL Sterilin plastic sample vial with gelation method B (section 3.2.1.4), to make a 2 mL gel (1% w/v). After gelation was considered complete (12 h), the vial containing the gel was mounted in the lower plate of the rheometer (cup) and then the vane (attached to the upper part) was lowered into place. This arrangement gave a sample depth of approximately 16 mm in the 14.5 mm diameter cup which allowed positioning of the vane in the centre of the sample. Measurements of the storage modulus (G') and loss modulus (G'') as function of strain γ , were carried out at a frequency of 10 rad/s. Oscillatory frequency sweeps were performed within the LVR of the gel sample, where G' and G'' moduli are independent of the strain amplitude, under a strain of 1%. All measurements were carried out at 37 °C.

3.2.1.7 Fluorescence spectroscopy

Fluorescence measurements were performed on a Varian Cary Eclipse Fluorescence Spectrophotometer, with λ_{ex} = 380 nm, λ_{em} = 424 nm and slit widths

of 5 nm. Gel samples (0.5 mL, 1% v/w) were prepared in fluorescence disposable cuvettes (Sigma Aldrich) with method B (section 3.2.1.4), using different concentrations of GdL.

3.2.1.8 ^1H -NMR spectroscopy

^1H -NMR spectra for monitoring hydrogels formation were recorded on a Bruker 400 Ultrashield at 400 MHz at 25° C. Samples were prepared according to the following method: in a 20 mL scintillation vial a solution of **68b** (7 mg) in D_2O (700 μL) was prepared by addition of NaOD (21 μL) to obtain full dissolution of the compound. GdL (2.44 mg) was then added and the solution was immediately transferred into specific NMR tubes (Wilmad 507-PP-7, previously dried under vacuum) and ^1H -NMR spectra were recorded at 15 min, 1 h, 2 h, 4 h and 24 h after addition of GdL using NS= 32.

In the case of time 0 (no GdL), the solution was transferred into the NMR tube without addition of GdL and ^1H -NMR spectrum was recorded.

3.2.1.9 General cell culture methods

All cell work was carried out in a class II microbiological safety cabinet using aseptic techniques.

Human-derived cell lines were serially sub-cultured in 25 or 75 cm^2 tissue culture flasks incubated at 37 °C in a humidified atmosphere comprising 5% CO_2 in a Sanyo LEEC incubator. Human-derived cancer cells (MCF-7 breast, IGROV-1 and OVCAR-4 ovarian) were grown in RPMI 1640 (Sigma Aldrich) cell culture medium supplemented with 10% v/v foetal bovine serum (FBS, Sigma Aldrich).

Normal lung fibroblasts (MRC-5 human-derived) were grown in Eagle's minimum essential medium (MEM, Sigma Aldrich) supplemented with 10% v/v FBS, 5% v/v penicillin/streptomycin solution (Sigma Aldrich), 5% v/v L-glutamine (200 nM, Sigma Aldrich), 5% v/v non-essential amino acids (Sigma Aldrich), 5% v/v hepes solution (1 M, Sigma Aldrich) and 5% v/v sodium

bicarbonate solution (7.5%, Sigma Aldrich). Experiments were set up when cultures reached approximately 70-80% confluence.

Trypsinisation was used as a method to detach the cells from tissue culture flasks as follows: once cells had reached ~70-80% confluence, media was aspirated and the cells trypsinised. An adequate amount (1-2 mL) of 1x trypsin/EDTA solution (1 in 10 dilution with sterile PBS, from a 10x stock solution) was added to completely cover the cell monolayer. The flask was placed in the incubator until the cells became detached from the flask. Cells were then re-suspended in 5-7 mL of fresh medium and were counted using a Neubauer haemocytometer.

Cryopreservation was performed in order to replenish the cell stock and to minimise phenotypic/genotypic drift and contamination. It was carried out as follows: medium was firstly aspirated and 1x trypsin/EDTA (1-2 mL) was added to completely cover the cell monolayer. After 1 min, it was removed and the flask returned to the incubator until the cells became detached from the flask. The cells were then resuspended in cryopreservation medium (5% DMSO in FBS, 1 mL per 25 cm² flask) and transferred to cryovials (1 mL per vial). Cryovials were then gradually frozen over 3 days, starting from -20 °C, they were transferred to -80 °C, and finally they were placed to -180 °C in the liquid nitrogen vapour cell bank for long term storage.

Thawing was carried out by rapidly transferring the cryovials in a water bath at 37 °C and pipetting dropwise the contents into a 25 cm² flask containing 9 mL of pre-warmed cell culture medium. Cells were allowed to attach overnight, and then the medium was replaced with fresh medium after removal of DMSO. Cells were sub cultured at least once before being used for experiments.

3.2.1.10 Growth inhibitory assays (MTT assays)

5F 203 and prodrugs were prepared as 10 mM top stocks in DMSO. Cells were seeded at a density of 3×10^3 per well into 96-well microtiter plates and allowed to adhere for 24 h before test agent was introduced (final concentrations 1 nM – 10 μ M, n = 4). Serial dilutions were prepared in RPMI 1640 medium prior to each assay. At the time of agent addition and following 72 h exposure, 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well (final concentration 400 $\mu\text{g/mL}$). Incubation at 37 °C for 2.5 h allowed reduction of MTT by viable cells (mitochondrial dehydrogenases) to insoluble formazan crystals. Well supernatants were removed, and formazan solubilised by addition of DMSO (150 μL). Absorbance was read at 550 nm using a Perkin Elmer plate reader. Non-linear regression analysis was used to calculate compound concentrations required to inhibit 50 % of cell growth (GI_{50}).

3.2.1.11 Release studies

1% w/v hydrogels of **68b** (0.4 mL) were prepared according to method B (section 3.2.1.4). The liquid solution was pipetted into transwell polyethylene terephthalate (PET) membrane inserts (pore size 1.0 μm , Greiner Bio One) and allowed to gel for approximately 12 h. The transwells were then inserted into 24 well plates containing 1.2 mL of PBS (pH 7.4) and incubated in a Thermo Scientific MaxQ 4000 Shaker at 37 °C at 15 rpm. At each time point a sample (150 μL) was removed from each solution and replaced with the same amount of fresh PBS buffer.

Analyses were performed on a HP1050 RP-HPLC and visualised at 254 nm with a HP1050 variable wavelength absorbance detector. Spectra were analysed using Agilent. Analytical RP-HPLC was performed using a Phenomenex Luna 5 μm C8 (2) 100A column (150 mm x 4.60 mm) at a flow rate of 1 mL/min. Samples were analysed using an isocratic method: 70% solvent B in solvent A (solvent A= 0.05 % TFA in H_2O , solvent B= 0.05 % TFA in 9:1 v: v $\text{CH}_3\text{CN}:\text{H}_2\text{O}$). Quantification of known concentrations of 5F 203 (Figure 8a.3) and **68b** (Figure 8b.3) was determined by UV-HPLC measurements and used to produce two standard curves, from which unknown concentrations of both compounds were determined.

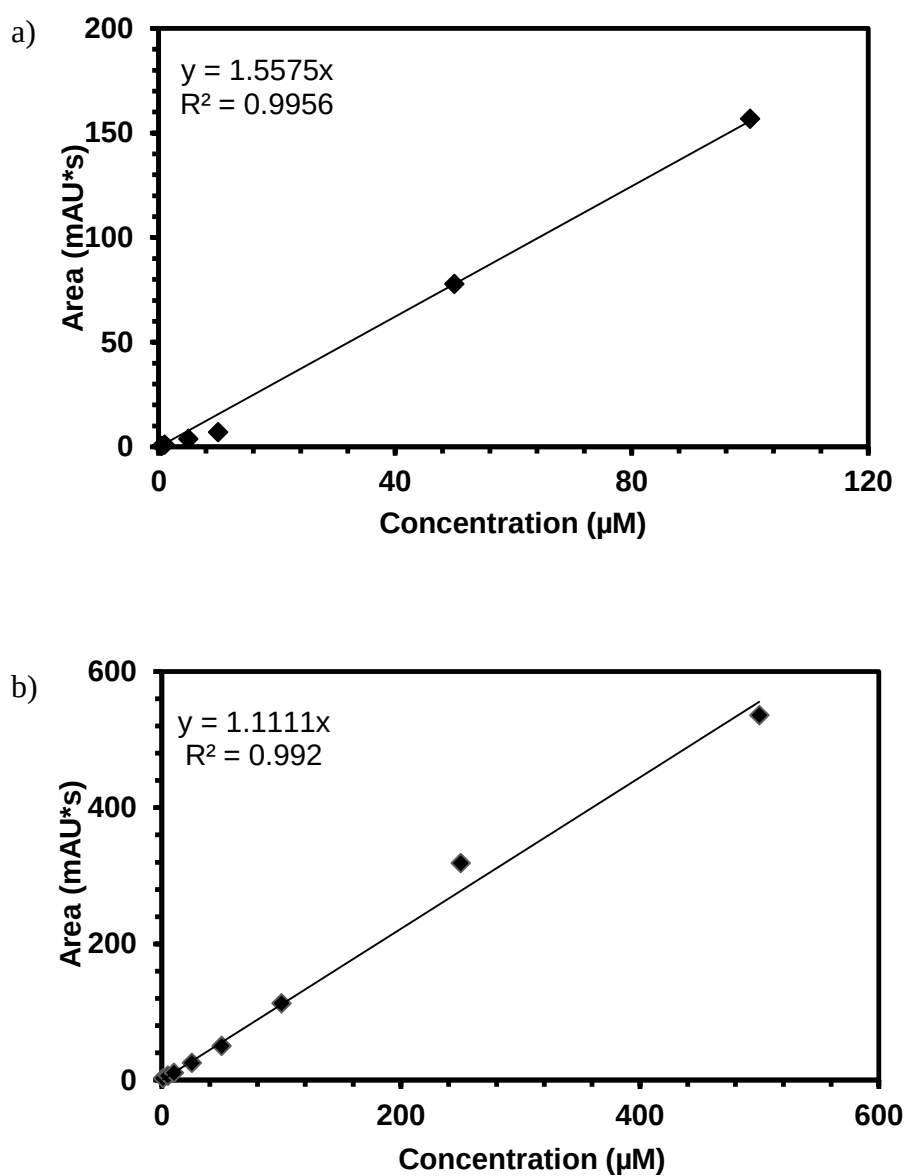
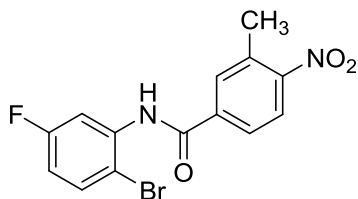


Figure 8.3: Standard curves of a) 5F 203 (**19**) and b) **68b** versus concentration.

3.2.2 Experimental data

N-(2-Bromo-5-fluorophenyl)-3-methyl-4-nitrobenzamide (63)

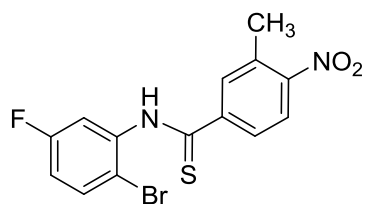


To a stirred suspension of 3-methyl-4-nitrobenzoic acid (5.00 g, 27.62 mmol) in dry CH_2Cl_2 (35.0 mL) at 0 °C, were added $(\text{COCl})_2$ (4.73 mL, 55.24 mmol) and DMF (0.25 mL) and the suspension was stirred at 0 °C for 10 min and then warmed up at r.t. for 3 h. The yellow solution was then concentrated under reduced pressure. The yellow residue was finally washed twice with THF to yield 3-methyl-4-nitrobenzoyl chloride as a yellow solid that was used immediately for the following coupling without further purification. 3-methyl-4-nitrobenzoyl chloride (5.49 g, 27.62 mmol), dissolved in 2 mL of dry pyridine, was added to a stirring solution of 2-bromo-5-fluoroaniline (5.25 g, 27.62 mmol) in dry pyridine (10.0 mL). The dark brown solution was heated under reflux for 2 h, then poured into water (30.0 mL), forming a white precipitate that was collected via vacuum filtration and washed with water and methanol, to afford a white solid (8.13 g, 23.16 mmol, 84% yield).

^1H NMR (DMSO- d_6): δ 10.38 (bs, 1H, NH-CO), 8.13 (d, J =8.6 Hz, 1H, H-5'), 8.07 (bs, 1H, H-2'), 8.00 (dd, J =2.3, 8.7 Hz, 1H, H-6'), 7.78 (dd, J =5.8, 9.0 Hz, 1H, H-3), 7.53 (dd, J =3.2, 9.9 Hz, 1H, H-6), 7.18 (td, J =8.7 Hz, 1H, H-4), 2.59 (s, 3H, CH_3).

^{13}C NMR (DMSO- d_6): δ 164.43, 162.78, 160.35, 151.29, 138.04, 134.35, 133.36, 132.57, 127.07, 125.10, 116.32, 116.08, 115.70, 19.80.

m/z: HRMS (TOF ES^+) $\text{C}_{14}\text{H}_{11}\text{BrFN}_2\text{O}_3^+ [\text{MH}]^+$ calculated 352.9932, 354.9912; found: 352.9959, 354.9938.

N-(2-Bromo-5-fluorophenyl)-3-methyl-4-nitrobenzothioamide (64)

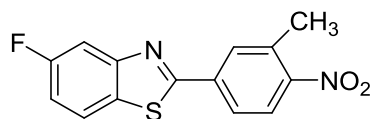
63 (8.13 g, 23.15 mmol) was dissolved in HMPA (12.3 mL) and then Lawesson's reagent (6.55 g, 16.20 mmol) was added and the reaction mixture was heated at 100°C for 16 h.

The dark solution was then poured into water (70.0 mL) and the product was extracted with diethyl ether (3 x 70.0 mL) and washed with water. The organic fractions were collected, dried over Na₂SO₄ and the solvent was removed under vacuum. Recrystallization from methanol gave thiobenzanilides as orange crystals (6.49g, 17.68 mmol, 76% yield).

¹H NMR (DMSO-*d*₆): δ 12.04 (b, 1H, NH-CS), 8.09 (d, *J*=8.5, 1H, H-5'), 7.93 (bs, 1H, H-2'), 7.88 (dd, *J*=2.1, 8.6 Hz, 1H, H-6') 7.83 (dd, *J*=6.0, 9.1 Hz, 1H, H-3), 7.50 (dd, *J*=3.2, 9.5 Hz, 1H, H-6), 7.27 (td, *J*=3.1, 8.8 Hz, 1H, H-4), 2.59 (s, 3H, CH₃).

¹³C NMR (DMSO-*d*₆): δ 197.86, 162.99, 160.55, 150.44, 144.83, 140.27, 134.67, 133.14, 131.92, 126.69, 124.92, 117.94, 117.50, 19.97.

***m/z*:** HRMS (TOF ES⁺) C₁₄H₁₁BrFN₂O₂S⁺ [MH]⁺ calculated 368.9704, 370.9683; found 368. 9701, 370.9681.

5-Fluoro-2-(3-methyl-4-nitrophenyl)benzothiazole (65)

Sodium hydride 60% dispersion in mineral oil (0.13 g, 3.25 mmol) was slowly added to a

stirring suspension of **64** (1.00 g, 2.71 mmol) in dry N-methyl-2-pyrrolidinone (2.6 mL) and the reaction mixture was heated at 140 °C until for 6 h. Water (50.0 mL) was then added to the dark brown solution and the precipitate was collected via vacuum filtration to give **65** as a light brown solid (0.66 g, 2.29 mmol, 84% yield).

¹H NMR (DMSO-*d*₆): δ 8.28 (dd, *J*=5.3, 8.9 Hz, 1H, H-5'), 8.22 (bs, 1H, H-2'), 8.15-8.18 (m, 2H, 1H, H-7, H-6'), 7.98 (dd, *J*=2.5, 9.8 Hz, 1H, H-4), 7.45 (td, *J*=2.7, 9.1 Hz, 1H, H-6), 2.64 (s, 3H, CH₃).

^{13}C NMR (DMSO- d_6): 168.20, 162.18, 150.73, 136.84, 134.64, 131.62, 126.45, 126.13, 124.51, 115.43, 115.19, 109.80, 109.57, 19.81.

m/z: HRMS (TOF ES $^+$) $\text{C}_{14}\text{H}_{10}\text{FN}_2\text{O}_2\text{S}^+$ $[\text{MH}]^+$ calculated 289.0442; found 289.0406.

4-(5-Fluorobenzothiazol-2-yl)-2-methylaniline (**19**)

65 (3.0 g, 10.42 mmol) was suspended in ethanol (500 mL) and then Tin (II) chloride dihydrate (11.75 g, 52.0 mmol) was added and the reaction mixture was heated under reflux for 3 h. The solvent was removed under reduced pressure to yield a brown oil. The residue was taken up into EtOAc (240.0 mL) and washed with 2 M NaOH (2 x 70.0 mL), H_2O (35.0 mL) and brine (10.0 mL). The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure to yield a brown solid. The crude product was purified by silica gel flash chromatography (gradient method, 0.5%-2% MeOH in CH_2Cl_2) to afford **5F 203 (19)** as a yellow solid (1.30 g, 5.04 mmol, 48 % yield).

^1H NMR (DMSO- d_6): δ 8.03 (dd, $J=5.5, 8.8$ Hz, 1H, H-7), 7.71 (dd, $J=2.5, 10.1$ Hz, 1H, H-4), 7.67 (d, $J=1.9$ Hz, 1H, H-2'), 7.63 (dd, $J=2.4, 8.3$ Hz, 1H, H-6'), 7.23 (td, $J=2.7, 9.1$ Hz, 1H, H-6), 6.70 (d, $J=8.4$ Hz, 1H, H-5'), 5.72 (bs, 2H, NH_2), 2.14 (s, 3H, CH_3).

^{13}C NMR (DMSO- d_6): 171.03, 162.51, 160.13, 154.89, 150.65, 129.23, 126.60, 123.06, 121.01, 120.06, 113.59, 112.45, 107.77, 17.31.

Mp: 188-190 $^\circ\text{C}$.

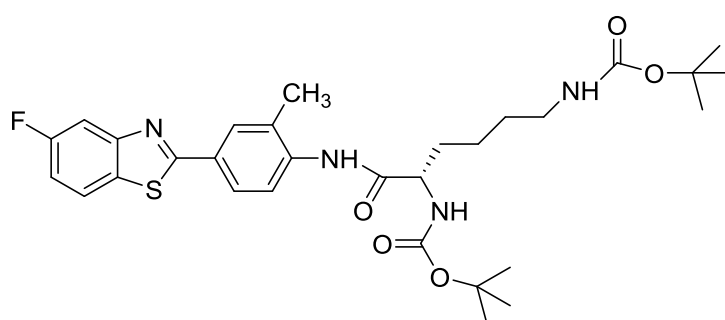
m/z: HRMS (TOF ES $^+$) $\text{C}_{14}\text{H}_{12}\text{FN}_2\text{S}^+$ $[\text{MH}]^+$ calculated 259.0700; found 259.0522.

HPLC t_R = 18.26 min.

General procedure for the synthesis of tert-butyl-protected 5F 203 amino acid prodrugs (66):

N-Boc-amino acid (1.1 eq) was dissolved in dry CH₂Cl₂ and HATU (1.5 eq) and DIPEA (3 eq) were added and the solution was stirred for 20 min. In a separate flask, 5F 203 (1 eq) was suspended in dry CH₂Cl₂ and stirred at 40 °C for 20 min. The N-Boc-amino acid/HATU solution was then added dropwise to the suspension containing 5F 203 and the reaction mixture was stirred under reflux for 24 h. The solvent was removed in vacuo to yield a yellow oil. The crude material was purified by silica gel flash chromatography (gradient elution 0.5-2% MeOH in CH₂Cl₂ or 10-30% EtOAc in petroleum ether 40-60 °C).

Di-tert-butyl (6-((4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)amino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (66a)



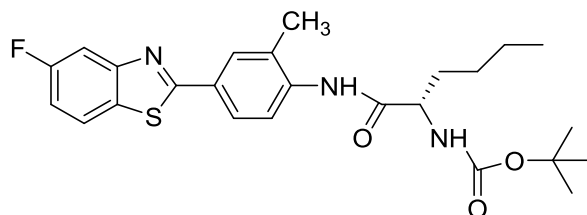
Synthesis of **66a** was performed according to the general procedure, using N², N⁶-bis (tert-butoxycarbonyl)-L-

lysine (0.25 g, 0.73 mmol), dry CH₂Cl₂ (7.5 mL), HATU (0.38 g, 0.99 mmol), DIPEA (0.35 mL, 1.98 mmol) and 5F 203 (0.17 g, 0.66 mmol). A yellow solid was afforded (0.26 g, 0.44 mmol, 67% yield).

¹H NMR (DMSO-d₆): δ 9.40 (bs, 1H, NH-H-4'), 8.18 (dd, J=5.6, 9.1 Hz, 1H, H-7), 7.96 (d, J=1.8 Hz, 1H, H-2'), 7.93-7.85 (m, 2H, H-4, H-6'), 7.73 (d, J=8.7 Hz, 1H, H-5'), 7.36 (td, J=2.4, 9.20 Hz, 1H, H-6), 7.10 (d, J=8.1 Hz, 1H, NH-CH), 6.79 (t, J=5.8 Hz, 1H, NH-CH₂), 4.13 (bs, 1H, CH-CH₂), 2.92 (bs, 1H, NH-CH₂), 2.33 (s, 3H, CH₃), 1.77-1.56 (m, 2H, CH-CH₂), 1.40 (s, 9H, t-C₄H₉), 1.37 (s, 9H, t-C₄H₉), 1.25 (t, J=6.4 Hz, 2H, CH₂-CH₂).

LC-MS: m/z C₃₀H₄₀FN₄O₅S⁺ [MH]⁺ calculated 587.3; found 587.3.

Tert-butyl (S)-(1-((4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)amino)-1-oxohexan-2-yl)carbamate (66b)



Synthesis of **66b** was performed according to the general procedure, using N-Boc protected L-norleucine

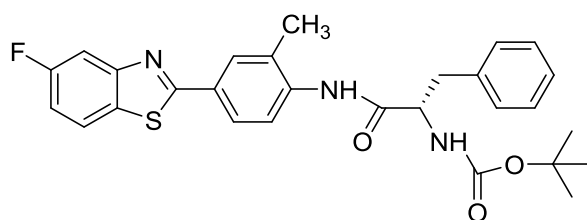
(0.15 g, 0.64 mmol), dry CH₂Cl₂ (6 mL), HATU (0.33 g, 0.87 mmol), DIPEA (0.30 mL, 1.74 mmol) and 5F 203 (0.15 g, 0.58 mmol). A yellow solid was afforded (0.16 g, 0.34 mmol, 59% yield).

¹H NMR (DMSO-d₆): δ 9.41 (bs, 1H, NH-H-4'), 8.18 (dd, J=5.5, 8.9 Hz, 1H, H-7), 7.96 (d, J= 1.4 Hz, 1H, H-2'), 7.89 (td, J=2.3, 9.4, 2H, H-5', H-6'), 7.72 (d, J=8.3 Hz, 1H, H-4), 7.36 (td, J=2.8, 9.2 Hz, 1H, H-6), 7.11 (d, J=7.6 Hz, 1H, NH-CH), 4.14 (dd, J= 7.9, 13.4 Hz, 1H, CH-CH_AH_B), 2.33 (s, 3H, CH₃-H-3'), 1.79-1.67 (m, 1H, CH_AH_B), 1.67-1.57 (m, 1H, CH_AH_B), 1.40 (s, 9H, t-C₄H₉), 1.36-1.27 (m, 4H, CH₂-CH₂), 0.89 (t, J=6.2 Hz, 3H, CH₂-CH₃).

¹³C NMR (DMSO-d₆): δ 171.73, 169.83, 162.70, 160.33, 155.67, 154.43, 139.61, 131.72, 130.29, 129.06, 125.35, 124.50, 123.64, 113.89, 113.64, 108.79, 107.72, 78.21, 55.03, 31.36, 28.23, 27.85, 22.05, 17.77, 13.88.

LC-MS: m/z C₂₅H₃₁FN₃O₃S⁺ [MH]⁺ calculated 472.2; found 472.2.

Tert-butyl(S)-(1-((4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (66c)



Synthesis of **66c** was performed according to the general procedure, using N-Boc protected phenylalanine (0.11 g, 0.40 mmol), dry CH₂Cl₂ (6

mL), HATU (0.20 g, 0.54 mmol), DIPEA (0.19 mL, 1.08 mmol) and 5F 203 (0.09 g, 0.36 mmol). A yellow solid was afforded (0.13 g, 0.26 mmol, 72% yield).

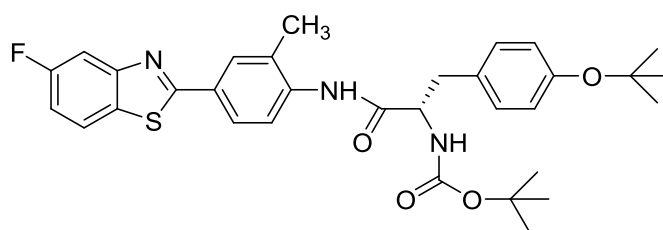
¹H NMR (DMSO-d₆): δ 9.48 (bs, 1H, NH-H-4'), 8.18 (dd, J=5.5, 9.0 Hz, 1H, H-7), 7.94 (d, J= 1.6 Hz, 1H, H-2'), 7.89 (td, J=2.5, 9.8, 2H, H-5', H-6'), 7.68

(d, $J=8.5$ Hz, 1H, H-4), 7.39-7.17 (m, 7H, H-2'', H-3'', H-4'', H-5'', H-6'', H-6, NH-CH), 4.49-4.40 (m, 1H, CH-CH_AH_B), 3.06 (dd, $J=5.6, 13.7$ Hz, 1H, CH_AH_B), 2.90 (dd, $J=9.5, 13.1$ Hz, 1H, CH_AH_B), 2.24 (s, 3H, CH₃), 1.34 (s, 9H, t-C₄H₉).

¹³C NMR (DMSO-d₆): δ 171.39, 170.25, 163.12, 160.72, 155.97, 155.06, 154.90, 139.79, 138.34, 132.37, 130.73, 129.79, 129.47, 128.58, 126.83, 125.73, 124.20, 124.09, 114.36, 114.12, 109.27, 109.03, 78.76, 37.73, 28.63, 22.99, 22.64, 18.08.

LC-MS: m/z C₂₈H₂₉FN₃O₃S⁺ [MH]⁺ calculated 506.2; found 506.3.

Tert-butyl (S)-(3-(4-(tert-butoxy)phenyl)-1-((4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)amino)-1-oxopropan-2-yl)carbamate (66d)



Synthesis of **66d** was performed according to the general procedure, using Boc-O-tert-butyl-L-tyrosine (0.26 g, 0.77

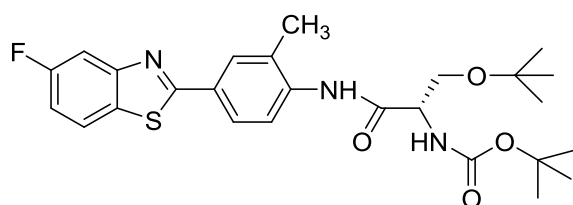
mmol), dry CH₂Cl₂ (6 mL), HATU (0.40 g, 1.04 mmol), DIPEA (0.36 mL, 2.09 mmol) and 5F 203 (0.18 g, 0.70 mmol). A yellow solid was afforded (0.24 g, 0.42 mmol, 60% yield).

¹H NMR (DMSO-d₆): δ 9.45 (bs, 1H, NH-H-4'), 8.17 (dd, $J=5.5, 9.1$ Hz, 1H, H-7), 7.93 (bs, 1H, H-2'), 7.91-7.84 (m, 2H, H-5', H-6'), 7.69 (d, $J=10.02$ Hz, 1H, H-4), 7.35 (td, $J=2.9, 9.3$ Hz, 1H, H-6), 7.23 (d, $J=7.3, 2$ Hz, H-2'', H-6''), 6.90 (d, $J=8.4, 2$ Hz, H-3'', H-5''), 6.70 (d, $J=9.3$ Hz, 1H, CH-NH), 4.50-4.40 (m, 1H, CH-CH_AH_B), 3.06-2.97 (m, 1H, CH_AH_B), 2.92-2.82 (m, 1H, CH_AH_B), 2.24 (s, 3H, CH₃), 1.35 (s, 9H, t-C₄H₉), 1.26 (s, 9H, t-C₄H₉).

¹³C NMR (DMSO-d₆): δ 170.94, 169.81, 162.57, 160.26, 155.44, 154.54, 153.51, 150.65, 139.35, 132.39, 131.70, 130.22, 129.80, 129.21, 128.98, 126.60, 125.25, 124.50, 123.70, 123.39, 120.98, 120.09, 113.85, 113.61, 108.65, 78.26, 77.63, 36.64, 28.60, 28.19, 17.61, 17.34.

LC-MS: m/z C₃₂H₃₇FN₃O₄S⁺ [MH]⁺ calculated 578.3; found 578.3.

Tert-Butyl (S)-(3-(tert-butoxy)-1-((4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)amino)-1-oxopropan-2-yl)carbamate (66e)



N-(tert-butoxycarbonyl)-O-(tert-butyl)-L-serine dicyclohexylamine (DCHA) salt (0.40 g, 0.90 mmol) was

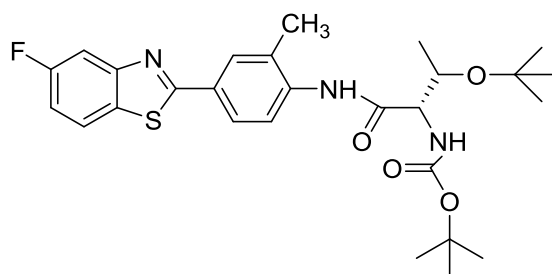
converted into the free amino acid. The DCHA salt was suspended into EtOAc and an aqueous solution of NaHSO₄ was added until reaching pH 2-3. The organic layer was washed with water and dried over Na₂SO₄ to yield Boc-O-tert-butyl-L-serine that was used for the reaction. Then synthesis of **66e** was performed according to the general procedure, using the N-Boc protected amino acid (0.16 g, 0.62 mmol), dry CH₂Cl₂ (6 mL), HATU (0.33 g, 0.86 mmol), DIPEA (0.30 mL, 1.71 mmol) and 5F 203 (0.15 g, 0.57 mmol). A yellow solid was afforded (0.18 g, 0.36 mmol, 63% yield).

¹H NMR (DMSO-d₆): δ 9.42 (bs, 1H, NH-H-4'), 8.17 (dd, J=5.4, 8.6 Hz, 1H, H-7), 7.95 (bs, 1H, H-4), 7.93-7.82 (m, 2H, H-5', H-6'), 7.72 (d, J=8.4 Hz, 1H, H-2'), 7.35 (dt, J=2.8, 8.8 Hz, 1H, H-6), 6.84 (d, J=7.7 Hz, 1H, NH-CH), 4.35-4.25 (m, 1H, CH-CH₂), 3.58 (d, J=5.6, 1H, CH₂-CH), 2.35 (s, 3H, CH₃-H-3'), 1.41 (s, 9H, t-C₄H₉), 1.14 (s, 9H, t-C₄H₉).

¹³C NMR (DMSO-d₆): δ 169.71, 169.52, 162.63, 160.25, 155.28, 154.42, 139.41, 131.71, 130.71, 130.20, 128.94, 125.29, 124.48, 123.68, 123.59, 113.82, 113.59, 108.75, 108.53, 78.40, 72.97, 61.79, 55.41, 28.15, 27.14, 17.72.

LC-MS: m/z C₂₆H₃₃FN₃O₄S⁺ [MH]⁺ calculated 502.2; found 502.3.

Tert-butyl ((2S,3R)-3-(tert-butoxy)-1-((4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)amino)-1-oxobutan-2-yl)carbamate (66f)



Synthesis of **66f** was performed according to the general procedure, using N-(tert-butoxycarbonyl)-O-(tert-butyl)-L-threonine (0.30 g, 1.10 mmol),

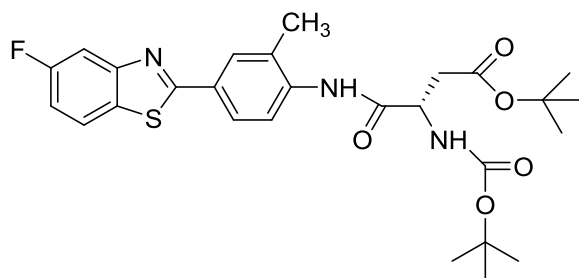
dry CH₂Cl₂ (8 mL), HATU (0.49 g, 1.28 mmol), DIPEA (0.45 mL, 2.55 mmol) and 5F 203 (0.22 g, 0.85 mmol). The reaction mixture was stirred for 70 h under reflux. A yellow solid was afforded (0.27 g, 0.52 mmol, 61% yield).

¹H NMR (DMSO-d₆): δ 9.21 (bs, 1H, NH-H-4'), 8.17 (dd, J=5.3, 8.8 Hz, 1H, H-7), 7.97 (d, J=1.9 Hz, 1H, H-2'), 7.95-7.89 (m, 2H, H-5', H-6'), 7.87 (dd, J=2.9, 10.1 Hz 1H, H-4), 7.36 (dt, J=2.5, 9.1 Hz, 1H, H-6), 6.44 (d, J=8.5 Hz, 1H, NH-CH), 4.29-4.24 (bsm, 1H, NH-CH), 4.05-3.97 (m, 1H, CH-CH₃), 2.37 (s, 3H, CH₃-H-3'), 1.43 (s, 9H, t-C₄H₉), 1.19 (s, 9H, t-C₄H₉), 1.11 (d, J=6.2 Hz, 1H, CH-CH₃).

¹³C NMR (DMSO-d₆): δ 169.73, 168.93, 162.61, 160.23, 155.31, 154.60, 154.46, 139.32, 130.20, 129.01, 128.47, 125.50, 123.71, 123.61, 122.85, 113.84, 113.54, 108.73, 108.47, 78.62, 74.30, 67.58, 59.68, 28.14, 27.99, 19.13, 17.79.

LC-MS: m/z C₂₇H₃₅FN₃O₄S⁺ [MH]⁺ calculated 516.2; found 516.3.

Tert-butyl (S)-3-((tert-butoxycarbonyl)amino)-4-((4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)amino)-4-oxobutanoate (66g)



Synthesis of **66g** was performed according to the general procedure, using Boc-L-aspartic acid 4-tert-butyl ester (0.19 g, 0.64 mmol), dry

CH₂Cl₂ (6 mL), HATU (0.33 g, 0.87 mmol), DIPEA (0.30 mL, 1.74 mmol) and 5F 203 (0.15 g, 0.58 mmol). The reaction mixture was stirred for 7 days under reflux. A yellow solid was afforded (0.15 g, 0.28 mmol, 49% yield).

¹H NMR (DMSO-d₆): δ 9.39 (bs, 1H, NH-H-4'), 8.16 (dd, J=5.5, 8.8 Hz, 1H, H-7), 7.94 (bs, 1H, H-2'), 7.91-7.83 (m, 2H, H-6', H-5'), 7.78 (d, J=8.8 Hz 1H, H-4), 7.44-7.28 (m, 2H, H-6, NH-CH), 4.53 (m, 1H, NH-CH), 2.78 (dd, J= 6.9, 16.1 Hz, 1H, CH_AH_B), 2.57 (dd, J= 8.1, 16.0 Hz, 1H, CH_AH_B), 2.32 (s, 3H, CH₃), 1.41 (d, J= 3.5 Hz, 18H, t-C₄H₉).

¹³C NMR (DMSO-d₆): δ 169.83, 169.73, 169.37, 162.63, 160.23, 155.52, 154.58, 154.45, 139.39, 131.22, 130.23, 129.00, 125.35, 123.87, 123.66,

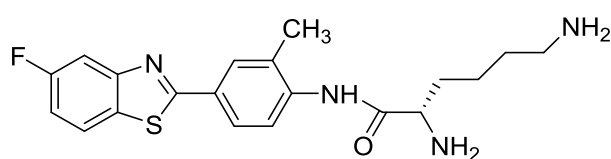
123.56, 113.84, 113.60, 108.76, 108.52, 80.22, 78.69, 51.62, 37.01, 28.14, 27.69, 17.55.

LC-MS: m/z $C_{27}H_{33}FN_3O_5S^+$ $[MH]^+$ calculated 530.2; found 530.1.

General procedure for the synthesis of 5F 203 amino acid prodrugs (67):

The N-Boc protected prodrug **66** was dissolved in mixtures of dry CH_2Cl_2 /TFA (1:1) or TFA/water/TIPS (95:2.5:2.5). The reaction mixture was stirred for 1 h. TFA was removed under a stream of nitrogen. TFA salts were then removed by Amberlyst® A21 ion exchange resin or with a basic aqueous workup.

(S)-2,6-diamino-N-(4-(5-fluorobenzothiazol-2-yl)-2-methylphenyl)hexanamide (67a)



Synthesis of **67a** was performed according to the general procedure, using **66a** (0.26 g, 0.44 mmol) in 3 mL

of CH_2Cl_2 /TFA. A pale yellow powder was afforded (0.11 g, 0.28 mmol, 66% yield).

^1H NMR (DMSO- d_6): δ 8.20 (dd, $J=5.3, 8.8$, Hz, 1H, H-7), 8.02 (bs, 1H, H-2'), 7.95 (dd, $J=1.9, 8.2$ Hz, 1H, H-6'), 7.89 (dd, $J=2.5, 9.9$ Hz, 1H, H-4), 7.85, (d, $J=8.5$ Hz, 1H, H-5'), 7.38 (td, $J=2.4, 9.1$ Hz, 1H, H-6), 3.99-3.91 (m, 1H, $\underline{CH}$ - CH_2), 2.81 (t, $J=7.3$ Hz, 2H, CH_2 - NH_2), 2.38 (s, 3H, $\underline{CH}_3$), 1.92-1.71 (m, 2H, CH_2), 1.55-1.65 (m, 2H, CH_2), 1.52-1.40 (m, 2H, CH_2).

^{13}C NMR (DMSO- d_6): δ 170.08, 163.61, 162.43, 160.96, 158.31, 154.78, 139.18, 132.42, 130.94, 129.77, 125.65, 124.47, 114.76, 109.17, 53.55, 36.24, 31.77, 27.37, 21.77, 18.24.

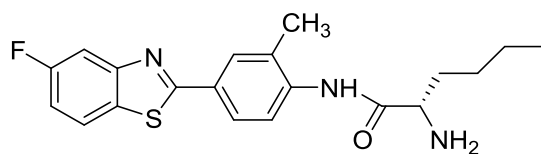
$[\alpha]_D^{25}$ = +14.41 ($c=0.28$, DMSO).

Mp: 134-136 °C.

m/z : HRMS (TOF ES^+) $C_{20}H_{24}FN_4OS^+$ $[MH]^+$ calculated 387.1649; found 387.1602.

HPLC t_R = 11.63 min.

(S)-2-amino-N-(4-(5-fluorobenzothiazol-2-yl)-2-methylphenyl)hexanamide (67b)



Synthesis of **67b** was performed according to the general procedure, using **66b** (0.16 g, 0.34 mmol) in

3 mL of CH₂Cl₂/TFA. A pale yellow powder was afforded (0.09 g, 0.24 mmol, 76% yield).

¹H NMR (DMSO-*d*₆): δ 8.19-8.14 (m, 2H, H-6', H-7), 7.98 (d, *J* = 2.1 Hz, 1H, H-2'), 7.93 (dd, *J* = 2.2, 8.5 Hz, 1H, H-5'), 7.87 (dd, *J* = 2.5, 9.7 Hz, 1H, H-4), 7.36 (td, *J* = 2.5, 9.0 Hz, 1H, H-6), 3.40 (dd, *J* = 4.8, 8.0 Hz, 1H, CH-CH_AH_B), 2.37 (s, 3H, CH₃-H-3'), 1.82-1.72 (m, 1H, CH_AH_B), 1.57-1.47 (m, 1H, CH_AH_B), 1.43-1.26 (m, 4H, CH₂-CH₂), 0.89 (t, *J* = 7.2 Hz, 3H, CH₂-CH₃).

¹³C NMR (DMSO-*d*₆): δ 173.99, 169.79, 162.58, 160.21, 154.57, 139.50, 130.10, 128.92, 127.83, 125.67, 123.51, 121.08, 113.46, 108.70, 55.24, 34.08, 27.46, 22.05, 17.26, 13.89.

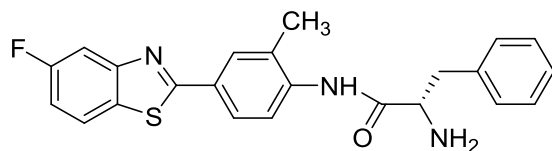
[α]_D²⁵ = +15.38 (*c* = 0.26, DMSO).

Mp: 138-141 °C.

***m/z*:** HRMS (TOF ES⁺) C₂₀H₂₃FN₃OS⁺ [MH]⁺ calculated 372.1541; found 372.8553.

HPLC *t_R* = 15.38 min.

(S)-2-amino-N-(4-(5-fluorobenzothiazol-2-yl)-2-methylphenyl)-3-phenylpropanamide (67c)



Synthesis of **67c** was performed according to the general procedure, using **66c** (0.13 g,

0.26 mmol) in 3 mL of CH₂Cl₂/TFA. A pale yellow powder was afforded (0.09 g, 0.22 mmol, 84% yield).

¹H NMR (DMSO-*d*₆): δ 8.17 (dd, *J* = 5.3, 8.9, 1H, H-7), 8.10 (d, *J* = 8.4 Hz, 1H, H-2'), 7.93 (d, *J* = 1.9 Hz, 1H, H-5'), 7.91 (dd, *J* = 2.0, 8.4 Hz, 1H, H-6'), 7.87 (dd, *J* = 2.4, 10.0 Hz, 1H, H-4), 7.35 (td, *J* = 2.7, 9.2 Hz, 1H, H-6), 7.31-7.19 (m, 5H, H-2'', H-3'', H-5'', H-6''), 3.70 (m, 1H, CH-CH_AH_B), 3.10 (dd,

$J = 5.0, 13.4$ Hz, 1H, $\text{CH}_\text{A}\text{H}_\text{B}$), 2.80 (dd, $J = 8.2, 13.5$ Hz, 1H, $\text{CH}_\text{A}\text{H}_\text{B}$), 2.23 (s, 3H, CH_3).

^{13}C NMR (DMSO- d_6): δ 173.20, 169.80, 162.59, 160.19, 154.56, 154.44, 139.32, 138.30, 130.15, 129.35, 129.20, 128.91, 128.18, 127.99, 126.27, 125.65, 123.63, 121.48, 108.70, 108.43, 56.70, 40.42, 17.15.

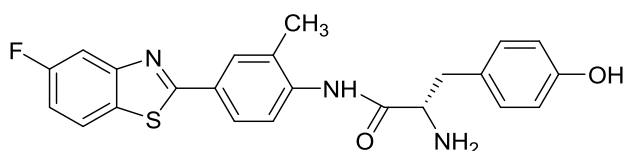
$[\alpha]_\text{D}^{25} = +17.39$ ($c = 0.23$, DMSO).

Mp: 152-154 °C.

m/z: HRMS (TOF ES $^+$) $\text{C}_{23}\text{H}_{21}\text{FN}_3\text{OS}^+$ $[\text{MH}]^+$ calculated 406.1384; found 406.1359.

HPLC $t_\text{R} = 15.49$ min.

(S)-2-amino-N-(4-(5-fluorobenzothiazol-2-yl)-2-methylphenyl)-3-(4-hydroxyphenyl) propanamide (67d)



Synthesis of **67d** was performed according to the general procedure, using

66d (0.24 g, 0.42 mmol) in 3 mL of $\text{CH}_2\text{Cl}_2/\text{TFA}$. TFA salts were then removed with an aqueous extraction: the compound was dissolved in water and CH_2Cl_2 was added. The aqueous phase was basified with 5% NaHCO_3 and the compound was extracted 3 times with CH_2Cl_2 . The organic layer was dried over NaSO_4 and the solvent was removed under vacuum. A yellow powder was afforded (0.12 g, 0.29 mmol, 66% yield).

^1H NMR (DMSO- d_6): δ 9.19 (bs, 1H, OH), 8.17 (dd, $J = 5.3, 8.8$ Hz, 1H, H-7), 8.12 (d, $J = 8.6$ Hz, 1H, H-4), 7.94 (bs, 1H, H-2'), 7.91 (dd, $J = 1.9, 8.4$ Hz, 1H, H-5'), 7.86 (dd, $J = 2.8, 10.1$ Hz, 1H, H-6'), 7.35 (td, $J = 2.7, 9.3$ Hz, 1H, H-6), 7.05 (d, $J = 8.5, 2\text{H}$, H-2'', H-6''), 6.67 (d, $J = 8.4, 2\text{H}$, H-3'', H-5''), 3.61 (dd, $J = 5.0, 8.1$, 1H, CH_H - $\text{CH}_\text{A}\text{H}_\text{B}$), 2.97 (dd, $J = 5.2, 13.5$ Hz, 1H, $\text{CH}_\text{A}\text{H}_\text{B}$), 2.69 (dd, $J = 7.8, 13.5$ Hz, 1H, $\text{CH}_\text{A}\text{H}_\text{B}$), 2.24 (s, 3H, CH_3 -H-3').

^{13}C NMR (DMSO- d_6): δ 173.35, 169.81, 161.63, 155.86, 154.61, 139.43, 130.24, 129.07, 128.91, 128.16, 127.90, 125.67, 123.63, 123.55, 121.38, 115.02, 113.77, 113.50, 108.71, 108.47, 56.91, 37.42, 17.16.

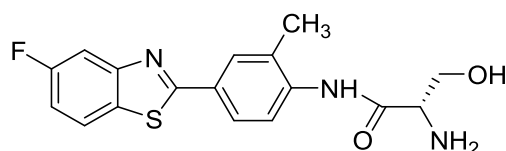
$[\alpha]_\text{D}^{25} = +13.79$ ($c = 0.29$, DMSO).

Mp: 190-192 °C.

m/z: HRMS (TOF ES⁺) C₂₃H₂₁FN₃O₂S⁺ [MH]⁺ calculated 422.1334; found 422.9607.

HPLC t_R = 14.17 min.

(S)-2-Amino-N-(4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)-3-hydroxypropanamide (67e)



Synthesis of **67e** was performed according to the general procedure, using **66e** (0.20 g, 0.40 mmol) in 3 mL of TFA/water/TIPS

(95:2.5:2.5). The reaction mixture was stirred for 4 h. TFA salts were then removed with a basic aqueous extraction: the compound was dissolved in water and CH₂Cl₂ was added. The aqueous phase was basified with 5% NaHCO₃ and the compound was extracted 3 times with CH₂Cl₂. The organic layer was dried over NaSO₄ and the solvent was removed under vacuum. A white powder was afforded (0.09 g, 0.26 mmol, 63% yield).

¹H NMR (DMSO-d₆): δ 8.23 (d, J=8.4 Hz, 1H, H-7), 8.17 (dd, J=5.5, 8.9, 1H, H-4), 7.97 (d, J=1.9 Hz, H-2'), 7.92 (dd, J=2.2, 8.6 Hz, 1H, H-6'), 7.86 (dd, J=2.5, 9.9 Hz, 1H, H-5'), 7.35 (td, J=2.7, 9.0 Hz, 1H, H-6), 4.97 (t, J=5.1 Hz, 1H, OH), 3.65 (t, J= 4.8 Hz, 2H, CH-CH₂), 3.46 (t, J= 5.1 Hz, 1H, CH-CH₂), 2.37 (s, 3H, CH₃).

¹³C NMR (DMSO-d₆): δ 172.30, 169.85, 154.58, 151.27, 139.50, 130.15, 128.93, 128.47, 127.77, 125.75, 123.64, 120.65, 113.73, 108.67, 63.60, 57.49, 17.23.

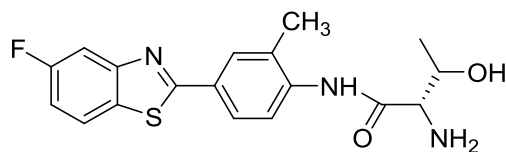
[α]_D²⁵ = +16.00 (c= 0.25, DMSO).

Mp: 170-173 °C.

m/z: HRMS (TOF ES⁺) C₁₇H₁₇FN₃O₂S⁺ [MH]⁺ calculated 346.1020; found 346.1019.

HPLC t_R = 12.67 min.

(2S,3R)-2-amino-N-(4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)-3-hydroxybutanamide (67f)



Synthesis of **67f** was performed according to the general procedure, using **66f** (0.23 g, 0.45 mmol) in 4

mL of TFA/water/TIPS (95:2.5:2.5). The reaction mixture was stirred for 3 hours. TFA salts were then removed with a basic aqueous extraction: the compound was dissolved in water and CH₂Cl₂ was added. The aqueous phase was basified with 5% NaHCO₃ and the compound was extracted 3 times with CH₂Cl₂. The organic layer was dried over NaSO₄ and the solvent was removed under vacuum. A white powder was afforded (0.09 g, 0.25 mmol, 50% yield).

¹H NMR (DMSO-d₆): δ 8.26 (d, J=8.5 Hz, 1H, H-7), 8.16 (dd, J=5.5, 8.9 Hz, 1H, H-4), 7.96 (d, J=2.0 Hz, 1H, H-2'), 7.92 (dd, J=2.1, 8.4 Hz 1H, H-6'), 7.86 (d, J=2.5, 9.9 Hz, 1H, H-5'), 7.34 (dt, J=2.5, 8.9 Hz, 1H, H-6), 4.87 (d, J= 5.1 Hz, 1H, OH), 4.15 (bs, 1H, CH-OH), 3.23 (d, J= 3.13 Hz, 1H, CH-NH₂), 2.36 (s, 3H, CH₃-H-3'), 1.66 (d, J= 6.6 Hz, 3H, CH₃-CH).

¹³C NMR (DMSO-d₆): δ 172.63, 169.85, 162.59, 160.20, 154.50, 139.58, 130.13, 128.94, 127.60, 125.74, 123.55, 120.44, 113.55, 108.52, 66.72, 60.68, 20.42, 17.24.

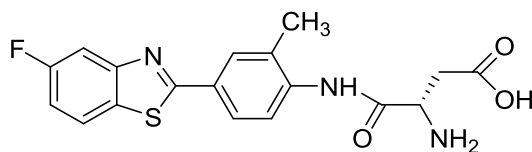
[α]_D²⁵= -25.55 (c= 0.36, DMSO).

Mp: 179-181 °C.

m/z: HRMS (TOF ES⁺) C₁₈H₁₉FN₃O₂S⁺ [MH]⁺ calculated 360.1177; found 360.1063.

HPLC t_R= 13.22 min.

(S)-3-amino-4-((4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)amino)-4-oxobutanoic acid (67g)



Synthesis of **67g** was performed according to the general procedure, using **66g** (0.13 g,

0.25 mmol) in 2 mL of CH₂Cl₂/TFA. A pale yellow powder was afforded (0.04 g, 0.11 mmol, 45% yield).

¹H NMR (DMSO-*d*₆): δ 8.58 (d, *J* = 4.2 Hz, 1H, H-7), 8.19 (dd, *J* = 5.5, 9.1 Hz, 1H, H-4), 8.00 (d, *J* = 1.8 Hz, 1H, H-2'), 7.95 (dd, *J* = 2.1, 8.4 Hz, 1H, H-6'), 7.88 (d, *J* = 2.5, 9.9 Hz, 1H, H-5'), 7.76 (d, *J* = 8.47, 1H, NH), 7.41-7.34 (m, 1H, H-6), 4.34 (t, *J* = 6.2 Hz, 1H, CH-CH_AH_B), 2.97 (dd, *J* = 5.4, 17.3 Hz, 1H, CH_AH_B), 2.85 (dd, *J* = 7.1, 17.9 Hz, 1H, CH_AH_B), 2.36 (s, 3H, CH₃-H-3').

¹³C NMR (DMSO-*d*₆): δ 170.99, 169.48, 167.05, 162.38, 160.45, 138.38, 132.17, 130.25, 129.55, 129.18, 125.36, 124.63, 123.66, 113.92, 108.64, 49.55, 35.63, 17.69.

[α]_D²⁵ = +15.38 (*c* = 0.26, 0.5 M NaOH solution).

Mp: 239-242 °C.

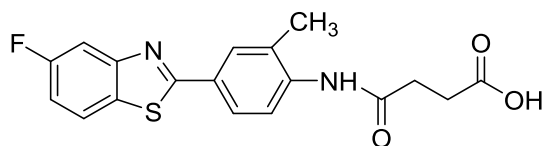
m/z: HRMS (TOF ES⁺) C₁₈H₁₇FN₃O₃S⁺ [MH]⁺ calculated 374.0969; found 374.0443.

HPLC t_R = 13.09 min.

General procedure for the synthesis of 5F 203 amide prodrugs (68):

5F 203 (1 eq) and the corresponding anhydride (24 eq) were suspended in dry pyridine and the reaction mixture was stirred at 60 °C to effect full dissolution and the solution was then maintained at this temperature for further 15 h. The cooled solution was evaporated in vacuo to afford a semi-solid material which was then triturated with water to aid removal of residual pyridine. The solid was then collected via vacuum filtration and purified using preparative RP-HPLC.

4-((4-(5-Fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)amino)-4-oxobutanoic acid (68a)



Synthesis of **68a** was performed according to the general procedure, using 5F 203 (0.50 g, 1.94 mmol), succinic anhydride

(4.65 g, 46.56 mmol) in dry pyridine (30 mL). A white solid was afforded (0.29 g, 0.81 mmol, 42% yield).

¹H NMR (DMSO-*d*₆): δ 12.15 (s, 1H, COOH), 9.45 (s, 1H, NH), 8.17 (dd, 1H, J= 5.4, 8.8 Hz, H-7), 7.94 (d, J= 1.7 Hz, 1H, H-4), 7.89-7.85 (m, 2H, H-2', H-6'), 7.76 (d, J= 8.7 Hz, 1H, H-5'), 7.36 (td, J= 2.5, 8.9 Hz, 1H, H-6), 2.66 (t, J= 6.5 Hz, 1H, CH₂-COOH), 2.55 (t, J= 6.3 Hz, 1H, CH₂), 2.34 (s, 3H, CH₃).

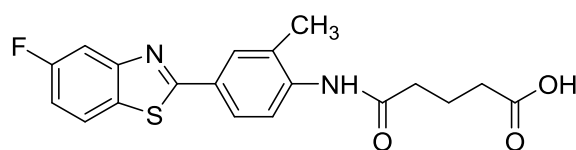
¹³C NMR (DMSO-*d*₆): 173.79, 170.49, 169.79, 162.58, 160.19, 154.54, 139.80, 130.16, 128.98, 125.15, 123.63, 113.77, 113.52, 108.70, 108.47, 30.78, 29.04, 17.82.

Mp: 222-224 °C.

m/z: HRMS (TOF ES⁺) C₁₈H₁₆FN₂O₃S⁺ [MH]⁺ calculated 359.0860; found 359.0938.

HPLC t_R= 17.16 min.

5-((4-(5-Fluorobenzo[d]thiazol-2-yl)-2-methylphenyl) amino)-5-oxopentanoic acid (68b)



Synthesis of **68b** was performed according to the general procedure, using 5F

203 (0.15 g 0.58 mmol,) glutaric anhydride (1.59 mg 13.92 mmol,) in dry pyridine (14 mL). A white solid was afforded (0.12 g, 0.32 mmol, 56% yield).

¹H NMR (DMSO-*d*₆): δ 12.10 (s, 1H, COOH), 9.40 (s, 1H, NH), 8.17 (dd, 1H, J= 5.4, 8.8 Hz, H-7), 7.94 (bd, J= 2.0 Hz, 1H, H-4), 7.90-7.85 (m, 2H, H-2', H-6'), 7.76 (d, J=8.6 Hz, 1H, H-5'), 7.35 (td, J=2.5, 9.0 Hz, 1H, H-6), 2.45 (t, J= 7.4 Hz, 1H, CH₂), 2.34-2.28 (m, 5H, CH₃, CH₂), 1.84 (quint, J= 7.5 Hz, 2H, CH₂-CH₂-CH₂).

¹³C NMR (DMSO-*d*₆): 174.13, 171.01, 169.78, 162.60, 160.20, 154.48, 139.77, 130.19, 128.97, 128.65, 125.13, 124.58, 123.59, 113.66, 108.62, 34.96, 32.97, 20.53, 17.85.

Mp: 219-222 °C.

LC-MS: m/z C₁₉H₁₈FN₂O₃S⁺ [MH]⁺ calculated 373.1; found 373.1.

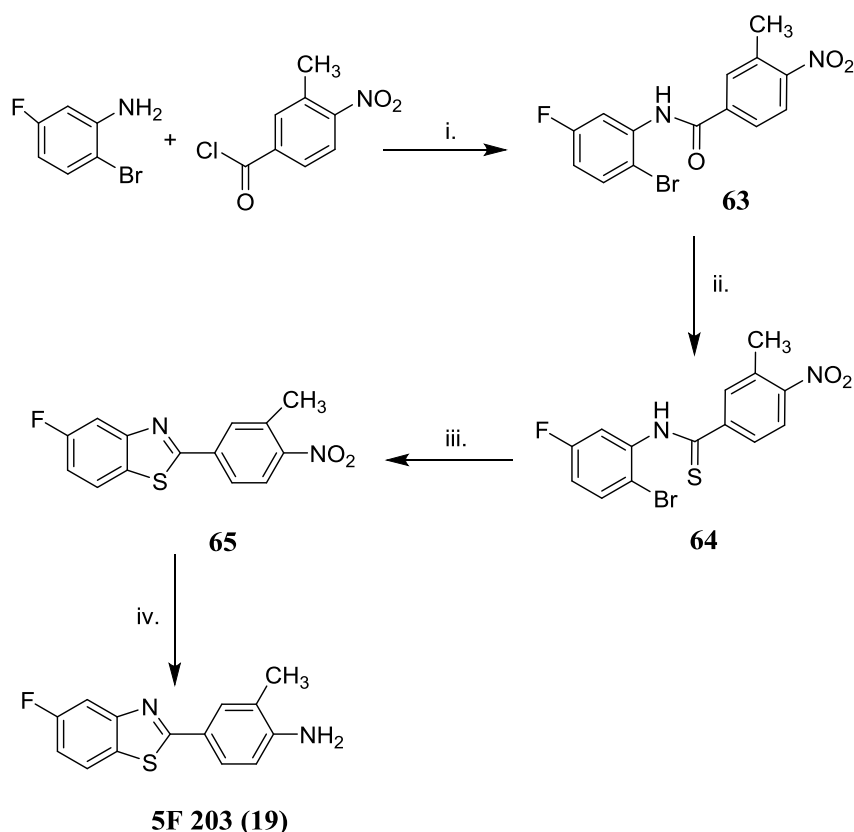
HPLC t_R= 17.57 min.

3.3 Results and Discussion

3.3.1 Design and synthesis of 5F 203 and amide prodrugs

The L-lysyl- and L-alanylamide prodrugs of the 2-(4-aminophenyl)-5-fluorobenzothiazole chemotherapeutic agent 5F 203 were developed with the aim of increasing drug's physicochemical characteristics and bioavailability for systemic delivery.²⁰ The design features and synthetic strategies of 5F 203 prodrugs for localised drug delivery have been adapted from the previous work reported for systemic delivery,²⁰ in order to produce amphiphilic self-assembling derivatives.

3.3.1.1 Synthesis of 5F 203

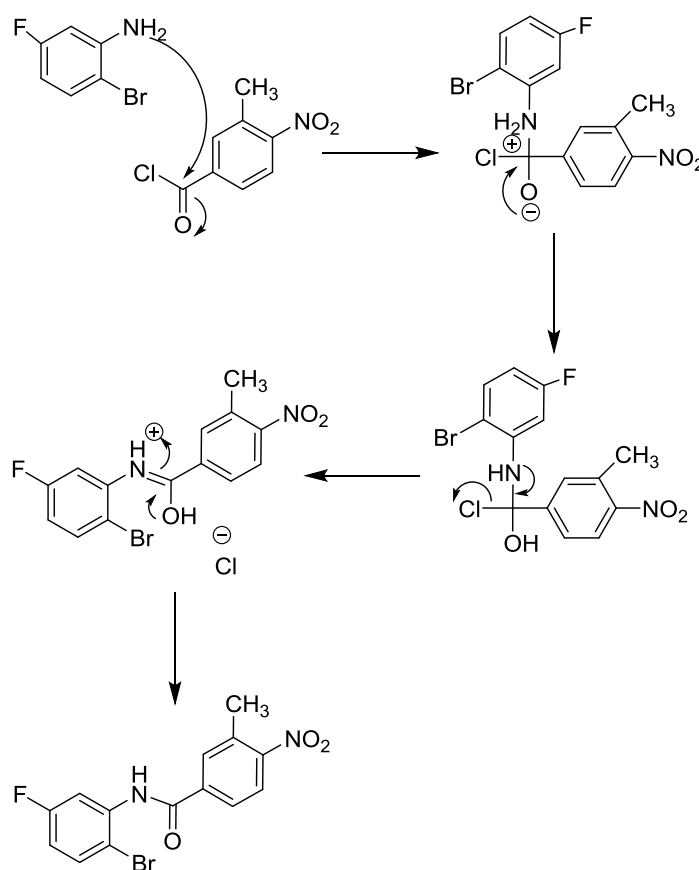


Scheme 1.3: Schematic representation of the synthetic route of 5F 203. Reagents and conditions: i) Pyridine, reflux. ii) Lawesson's reagent, HMPA, 100° C. iii) NaH, NMP, 140°C. iv) SnCl₂ (2H₂O), ethanol, reflux.

The first step of the synthetic route of the amide prodrugs involved synthesis of the active drug 5F 203, established from a previously reported method according to Hutchinson et al. (Scheme 1.3).²¹

The synthesis consisted of 4 steps, with formation of an initial benzanilide, followed by conversion to a thiobenzanilide; a subsequent bromo-atom directed ring closure yielded a nitro phenyl-benzothiazole, successively reduced to amino phenyl-benzothiazole, known as 5F 203 (**19**).

In the first step, 3-methyl-4-nitrobenzoyl chloride was reacted with 2-bromo-5-fluoroaniline to yield the 2-bromo-5-fluorobenzanilide (**63**). This method is frequently used to synthesise amide derivatives; the reaction proceeds via a nucleophilic substitution, with the amine acting as nucleophile, which displaces the chloride group and forms the amide bond (Scheme 2.3).



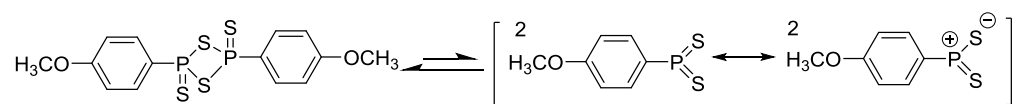
Scheme 2.3: Amide bond formation via acid chlorides

Conversion of **63** into the corresponding thiobenzanilide **64** was carried out with Lawesson's reagent using hexamethylphosphoramide (HMPA) as solvent, as previously reported.²¹ Due to HMPA high toxicity and known carcinogenicity, several reactions were carried out to avoid its use, testing different solvents, reaction times and a different thionating agent (phosphorus pentasulfide, P_4S_{10} , in combination with hexamethyldisiloxane, HMDO).²² However, not one of the reactions carried out gave complete conversion of the starting benzanilide into the corresponding thiobenzanilide (Table 1.3). Moreover, when a partial conversion was obtained, the starting material and the product proved difficult to separate by silica gel flash chromatography. Therefore, it was decided to use HMPA as solvent for the reaction, which allowed a complete conversion of the starting material into the corresponding product in an overnight reaction.

Reaction	Solvent	Reaction time	Reagent	Outcome
FC095	THF (under reflux)	15 h	Lawesson's	Starting material
FC096	Toluene (under reflux)	15 h	Lawesson's	Partial conversion
FC098	Toluene (under reflux)	48 h	Lawesson's	Partial conversion
FC103	Chloroform	48 h	$P_4S_{10}^+$ HMDO	Partial conversion

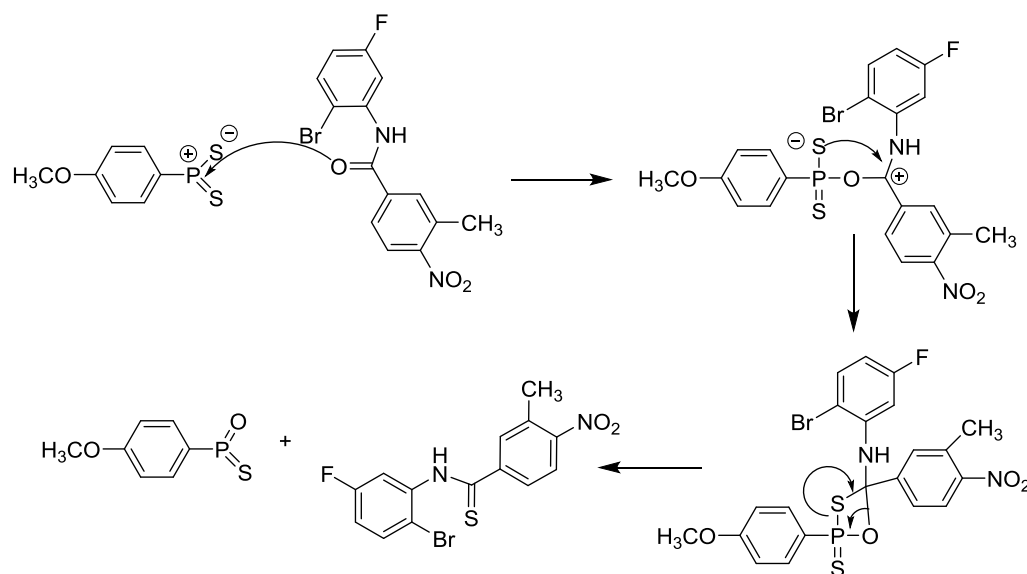
Table 1.3: Different reaction conditions to synthesise **64**

Lawesson's reagent is a thionating agent used to convert carbonyl groups i.e. ketones, amides or esters into the corresponding thioketones, thioamides and thioesters.



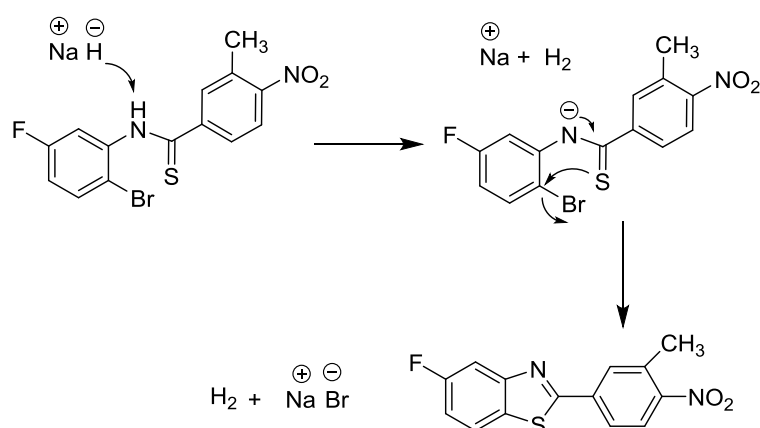
Scheme 3.3: Lawesson's reagent is in equilibrium with two more reactive dithiophosphine ylides

In solution Lawesson's reagent is in equilibrium with two more reactive dithiophosphine ylides (Scheme 3.3), which react with the carbonyl group to yield a thioxaphosphetane intermediate, followed by production of the thiocarbonyl derivative (Scheme 4.3).



Scheme 4.3: Lawesson's reagent mechanism in thioamide synthesis

Bromo atom-directed ring closure was performed by the following step: sodium hydride (NaH) in N-methylpyrrolidinone was used as a base to deprotonate the thioamide **64**, forming the thiolate anion and then promoting closure of the benzothiazole ring to obtain **65** (Scheme 5.3).



Scheme 5.3: Phenyl-benzothiazole synthesis via bromo atom-directed ring closure

Finally, reduction of the nitro group in the presence of Tin(II) chloride dehydrate in ethanol gave the desired amino phenyl-benzothiazole, known as 5F 203 (**19**).

3.3.1.2 Synthesis of 5F 203 amino acid-based prodrugs

The first series of LMWG benzothiazole prodrugs consisted of amino acid-based compounds: they were designed with the aim of creating 5F 203 amphiphilic derivatives, given by the π - π stacking of the phenyl-benzothiazole core together with the more polar group of the amino acid functionality.

The molecular design of 5F 203-amino acid self-assembling gelators was based on two main factors: i) the successfully previously reported L-lysyl- and L-alanyl- amide compounds, which increased the physicochemical characteristics of 5F 203; ii) the established gelator properties of some amino acids derivatives.^{6, 7, 9, 23} Therefore, these compounds were prepared modifying and optimising a previously reported synthetic procedure for Phortress.²⁴

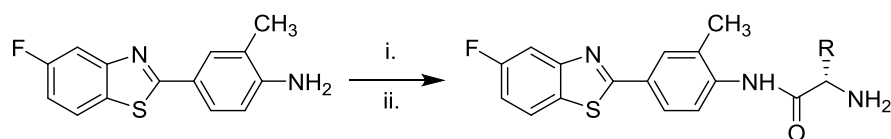
Reaction	Coupling reagent	Base	Reaction time	Outcome
FC102	EDC/HOBt	DIPEA	7 days	20% of final product
FC108	PyBOP	DIPEA	9 days	20% of final product
FC109	EDC/HOBt	Triethylamine	7 days	Only starting materials
FC114	TFFH	DIPEA	19 h	NOT applicable on a large scale
FC121	HATU	DIPEA	24 h	Only final product

Table 2.3: Different coupling reagents tested for synthesis of 5F 203 amino-acid prodrugs.

According to the literature, the N-Boc protected amino acid was covalently conjugated to the aniline of 5F 203 via an amide bond.

Hutchinson and co-workers used the water soluble reagent EDC for the reaction, and they reported a complete conversion of the starting material in 5 days. In order to optimise this reaction, different synthetic strategies were carried out (Table 2.3), and the coupling between Bis-Boc-Lysine and 5F 203 was used as a test reaction for optimisation. The first attempt of coupling involved the use of

EDC and HOBt as activating reagents and DIPEA as a base, but only 20% conversion into the final product was observed after 7 days of reaction, as detected by HPLC. Then, the phosphonium PyBOP was used as a coupling reagent and DIPEA as base, but still a small amount of starting material (20%) was converted into the final product. It was then decided to use the EDC/HOBt system and triethylamine as a base, but after 7 days no product was present. Using of tetramethylfluoroformamidinium hexafluorophosphate (TFFH) gave full conversion of starting materials into the final product after 19 h; however, when the reaction was tried on a larger scale, no complete reaction was observed. The tetramethyluronium HATU was finally found to be the most suitable reagent, giving complete conversion of 5F 203 into the correspondent N-Boc protected prodrugs in 24 h. Removal of the Boc group was carried out using TFA in CH_2Cl_2 (Scheme 6.3, Table 3.3). TFA salts were then deprotonated, such that prodrugs were in their freebase or neutral form, in order to be assessed for gelation studies.



Scheme 6.3: Synthesis of 5F 203 amino acid prodrugs. Reagents and conditions: i) Boc-amino acid, HATU, DIPEA, CH_2Cl_2 , reflux, 24 h. (ii) 50% TFA in CH_2Cl_2 or 95: 2.5: 2.5 TFA: TIPS: H_2O .

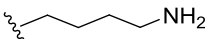
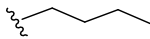
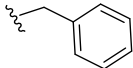
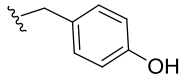
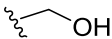
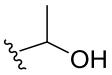
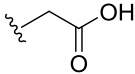
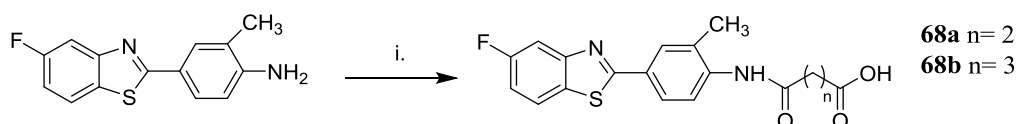
Compound	Amino acid	R group	Yield
67a	-lysine		66%
67b	-norleucine		76%
67c	-phenylalanine		84%
67d	-tyrosine		66%
67e	-serine		63%
67f	-threonine		50%
67g	-aspartic acid		45%

Table 3.3: 5F 203 amino acid prodrugs synthesised with relative yields.

3.3.1.3 Synthesis of 5F 203 prodrugs with an alkyl chain and a terminal carboxylic acid functional group

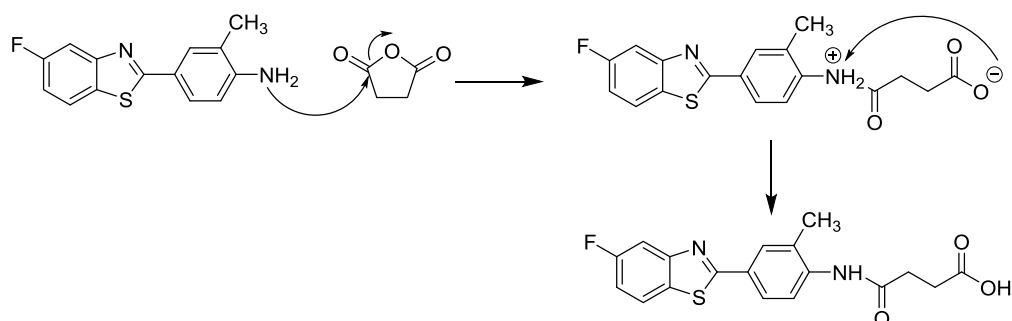
The second series of 5F 203 amide prodrugs was rationally designed to have an alkyl chain and a free carboxylic acid terminus, such that hydrophobic forces together with water solubility, given by a “pH switch”, could be used to promote the self-assembly process.

It was assumed that the phenyl-benzothiazole group of 5F 203 would provide the necessary π - π stacking interactions for initial self-assembly. Introduction of an alkanolic acid at the N-position of the native compound via an amide bond would create the hydrophobic forces and the hydrogen bond donor and acceptor groups fundamental for the self-assembly. 5F 203 derivatives were synthesised by reacting 5F 203 with succinic anhydride (**68a**) and glutaric anhydride (**68b**) in presence of pyridine (Scheme 7.3), by adapting previous literature ²⁵.



Scheme 7.3: Synthesis of 5F 203 prodrugs. Reagents and conditions (i): Anhydride, dry pyridine, 60 °C.

Formation of the amide bond was achieved by reaction between the nucleophilic aniline of 5F 203 and the corresponding cyclic anhydride (Scheme 8.3).



Scheme 8.3: Mechanism of amide bond formation via cyclic anhydride. As example, succinic anhydride is reported for synthesis of compound **68a**.

3.3.2 Preliminary gelation tests of 5F 203 amide prodrugs

Preliminary gelation tests were carried out to determine the self-assembling properties of the synthesised compounds, using a tabletop rheology technique, i.e. vial inversion test, as initial screening to define gel formation. Different gelation techniques were adopted for the two series of 5F 203 amide prodrugs. The amino acid derivatives were tested with a solvent and temperature switch method (method A, section 3.2.1.4). Vial inversion tests determined formation of metastable gels and precipitates (Table 4.3).

ϕ_{DMSO}	5F 203	67b	67c	67d	67e	67f	67g
0.05	MG	P	P	P	P	P	P
0.1	MG	P	P	P	P	P	P
0.2	P	P	P	P	P	MG	P
0.3	P	P	P	P	P	MG	P
0.4	P	P	P	P	P	MG	P

Table 4.3: Data table of vial inversion tests in DMSO/H₂O mixtures with ϕ_{DMSO} from 0.05 to 0.4, where MG= metastable gel, P= precipitate.

5F 203 (**19**) and 5F 203-threonine (**67f**) were found to be the only compounds which self-assembled to form metastable gels, whilst the other prodrugs resulted in precipitation. 5F 203 formed a gel at high water ratios (ϕ_{DMSO} of 0.05 and 0.1) during the initial 3-4 h; however, it slowly precipitated over the following 24 h. The threonine prodrug **67f** showed a similar gelation trend, with formation of stable gels at high water ratios (ϕ_{DMSO} from 0.2 to 0.4), and subsequent precipitation over 24 h (Figure 9.3). The initial self-assembly of 5F 203 and **67f** can be explained by formation of non-covalent bonds due to the π - π stacking interactions of the phenyl-benzothiazole group.³ Their subsequent precipitation may be a consequence of metastable states of the gels, characterised by limited stability and tendency to precipitate into crystals over time.²⁶ Indeed, the gel state can be considered as a trapped state that is not always kinetically favoured, and precipitation is the result of this instability.²⁷

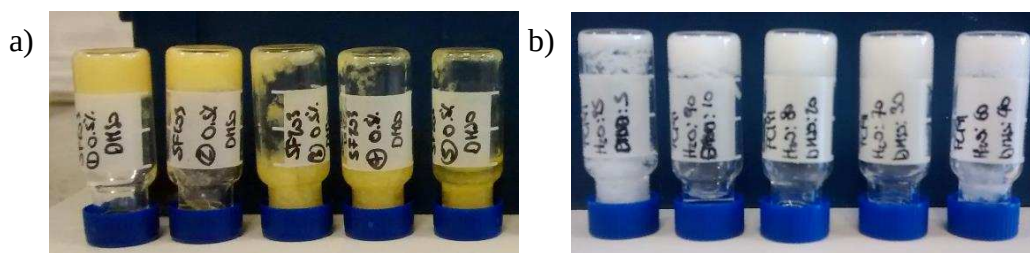


Figure 9.3: Pictures of vial inversion tests of 5F 203 (a) and 5F 203-threonine prodrug **67f** (b) using a solvent-switch method (water/DMSO). Vials are presented from low solvent volume fraction ϕ_{DMSO} on the left (0.05) to higher DMSO concentrations ϕ_{DMSO} on the right (0.4).

The gelation behaviour of **67a** was tested using a different method. This prodrug consists of a lysine functional group, whose amine on the alkyl chain has a pKa value of 10.2, that means it is positively ionised at neutral pH. Consequently, self-assembly can be hindered at a pH lower than 10.2 because of electrostatic repulsions between the positively charged molecules.^{14, 17} Gelation tests with a solvent switch method in water/DMSO mixtures resulted in clear solutions at each solvent ratio, meaning that the ionised form of the compound favoured the dissolution into the solvent system. For this reason, a pH switch method (method C, section 3.2.1.4) was performed according to the literature.^{17, 28} At pH 9.60 and 10.02 a cloudy solution was formed, at pH 11.06 and 11.19 a viscous liquid appeared and the system resulted in a yellow precipitate at pH 11.97 (Figure 10.3). These findings proved that **67a** does not possess the adequate self-assembling properties which allow gelation.

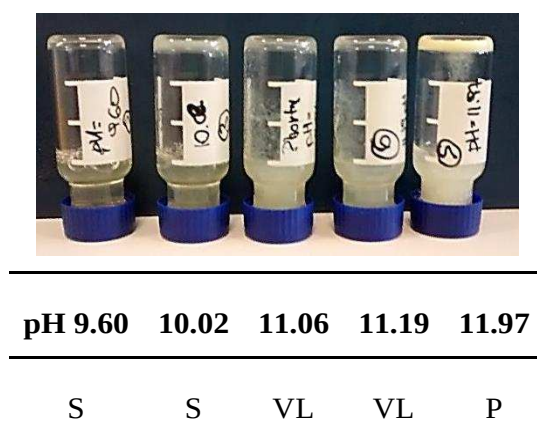


Figure 10.3: Picture of vial inversion test of **67a** using pH-switch method C: (from left to right) formation of solutions (S), viscous liquids (VL) and precipitate (P).

Gelation tests of the second series of 5F 203 amide prodrugs, consisting of a free carboxylic acid group (compounds **68a** and **68b**), were carried out using a pH switch method, as previously reported in the literature (method B, section 3.2.1.4).¹⁹ The acidic compounds were firstly dissolved in water, at pH ~ 11, after addition of a fixed volume of sodium hydroxide; gelation was then achieved by addition of the pH acidifier glucono- δ -lactone (GdL). For compound **68a**, different concentrations of GdL were tested and the most stable and translucent gels, determined by vial inversion test, were found to be the ones with 0.7 molar equivalents of GdL and a final pH of 7.2 after 12 h. Using the same method and amount of GdL, compound **68b** initially formed a clear gel in the first h; however, over 12 h, its turbidity increased and it finally precipitated into white crystals. The slightly higher hydrophobicity of **68b**, due to an additional methylene group in the alkyl chain, promotes compound precipitation as also described for other gelators, i.e. lysine derivatives bearing alkyl chains of different lengths.⁷ This minimal change in molecular structure can lead to disruption of gel formation, as the hydrogelation process is a result of a balance between association of the hydrophilic functional groups in water and aggregation of the hydrophobic counterparts in the gel. Indeed, precipitation of **68b** is an example of how the number of methylene groups in the molecule is a critical factor for determining the propensity of a LMWG to aggregate in water or organic solvents.^{12, 29}

3.3.3 Probing the self-assembly properties of 5F 203-succinic acid prodrug

Due to the metastable nature of 5F 203 and **67f** gels, it was not possible to carry out any characterisation for these gelators.

Amongst the second series of 5F 203 prodrugs, **68a** formed a macroscopically stable and transparent hydrogel. Therefore, its gelation process was studied and evaluated by different characterisation methods, usually adopted for analysing supramolecular gels.³⁰

3.3.3.1 Dynamic rheological studies

Rheology was used to gain information about the mechanical properties of **68a** gel. In particular, oscillatory measurements were carried out to evaluate the viscoelastic behaviour of the tested material.

The two parameters provided by the oscillatory tests, i.e. the storage modulus (G') and the loss modulus (G''), respectively define the elastic and the viscous behaviour of a sample. Another important parameter used to evaluate gel formation is the loss factor ($\tan \delta$), which is the ratio between the viscous and the elastic portions of a material (Equation 1.3).

$$\tan \delta = \frac{G''}{G'}$$

Equation 1.3: Loss factor ($\tan \delta$) calculated from oscillatory tests.

$\tan \delta < 1$ ($G' > G''$) in the case of gel-like state or solid state, whereas $\tan \delta > 1$ ($G'' > G'$) for fluid-like state; when $\tan \delta = 1$, the sol/gel transition point is reached.

Amplitude sweeps, oscillatory measurements performed at variables strains (γ) and constant frequency values (angular frequency, ω), are initially carried out to define whether a material is characterised by a viscoelastic behaviour. At low amplitudes, G' and G'' display a linear range, defined as linear viscoelastic region (LVR), in which both curves

have constant plateau values. Analysis of the LVR provide information about the viscoelastic behaviour of a sample. For gel-like samples, $G' > G''$, with the elastic behaviour dominating the viscous one.

Once determined the LVR range from the amplitude sweeps, frequency sweeps are then carried out within the LVE range, at variable frequencies and constant strain. Gel-like samples display $G' > G''$ across the whole frequencies tested and the curves often occur in the form of almost parallel straight lines, with $0.01 < \tan\delta < 0.1$.³¹

Oscillatory amplitude sweeps (Figure 11a.3) of **68a** gel (1% w/v), were performed over a strain from 0.5 to 100%, at a constant angular frequency of 10 rad/s and at 37 °C, to mimic the physiological conditions at which the gel would be used for drug delivery purposes. Results revealed the presence of a LVR, with only a slight decrease of the storage modulus at high strains. Moreover, G' values were higher than G'' values of at least one order of magnitude, as a representative characteristic of LMWGs.¹² A slight decrease of G'' at $\gamma = 8 - 10\%$ was also detected: this so-called 'wall slip' could be attributed to an inhomogeneous deformation behaviour occurring upon exceeding the linear viscoelastic range.

Frequency sweeps were performed over a frequency from 0.1 to 100 rad/s, using a constant strain of 1%. A frequency dependence of G' and G'' was displayed throughout the values tested, with G' and G'' curves displaying a gradual slope upwards with increasing frequencies. This characteristic has been attributed to a low degree of cross-linked network. Moreover, G' values were in the range of 20-60 Pa, which suggested weak mechanical properties of hydrogel **68a** (Figure 11b.3).

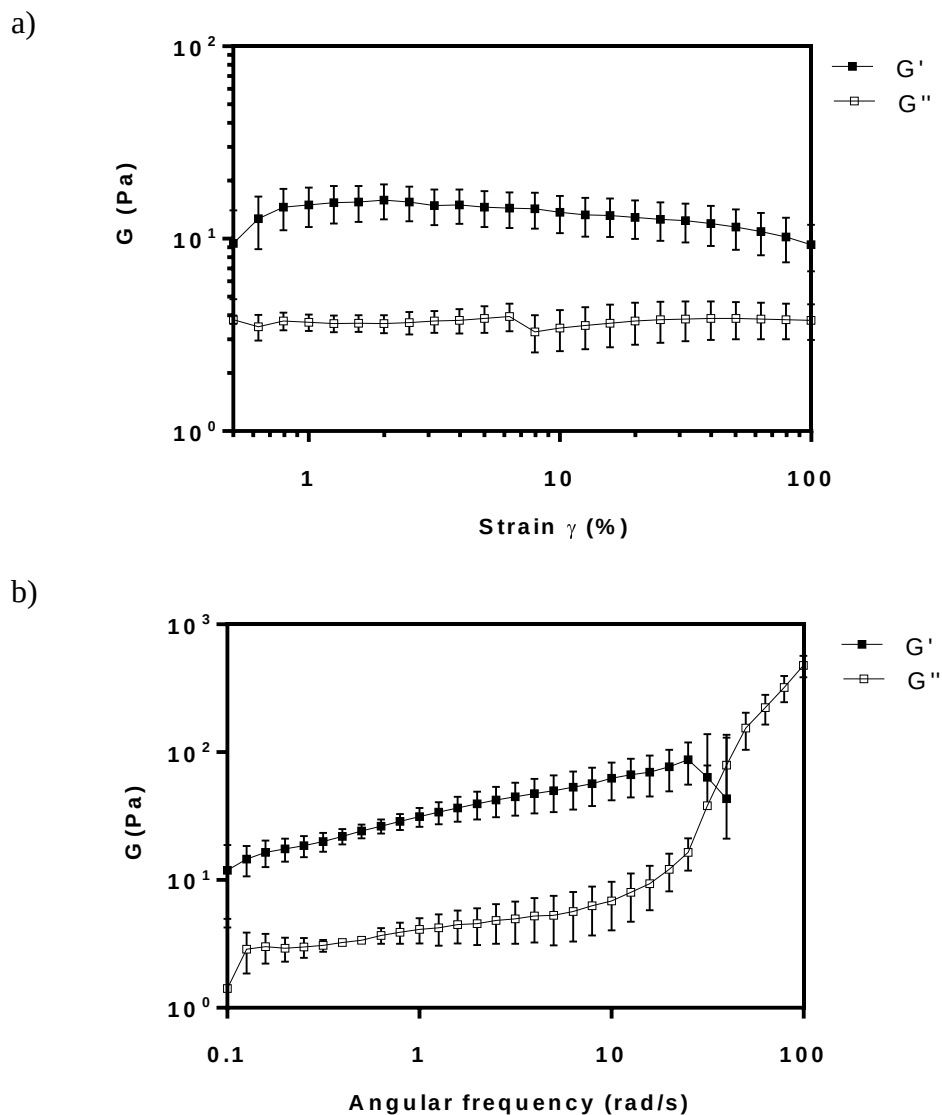


Figure 11.3: a) Amplitude sweep of **68a** (1% w/v) performed out at $\omega = 10$ rad/s and at 37 °C. b) Frequency sweeps of **68a** (1% w/v) performed at $\gamma = 1$ % and at 37°C. All measurements were carried out in triplicate. Error bars represent standard deviation.

3.3.3.2 Electron Microscopy analysis of hydrogel morphologies at different pHs

To gain further understanding of the self-assembling process of **68a** with changes in pH, the morphology of the samples at different pHs and at same concentration (1% w/v) was analysed by transmission electron microscopy (TEM). At pH 11, after addition of NaOH, **68a** was fully dissolved and a small population of fibres could be observed along with rod-shaped micelles (Fig. 12a.3).³² This can be explained by the fact that, at this pH, all molecules of **68a** are assumed to be ionised (calculated pKa of COOH= 4.25) giving higher solubility and not favouring self-assembly into fibres, due to the electrostatic repulsion between the negatively charged molecules.¹⁷ When 0.3 molar equivalents of GdL were added to the basic solution of **68a**, a final pH of 7.8 was obtained after 12 h.

At pH 7.8 the population of fibres increased, confirming the self-assembling process, and this was also corroborated by formation of a weak translucent gel. Nanofibres displayed widths ranging from 20 nm to 35 nm and were several microns long (Fig. 12b.3).

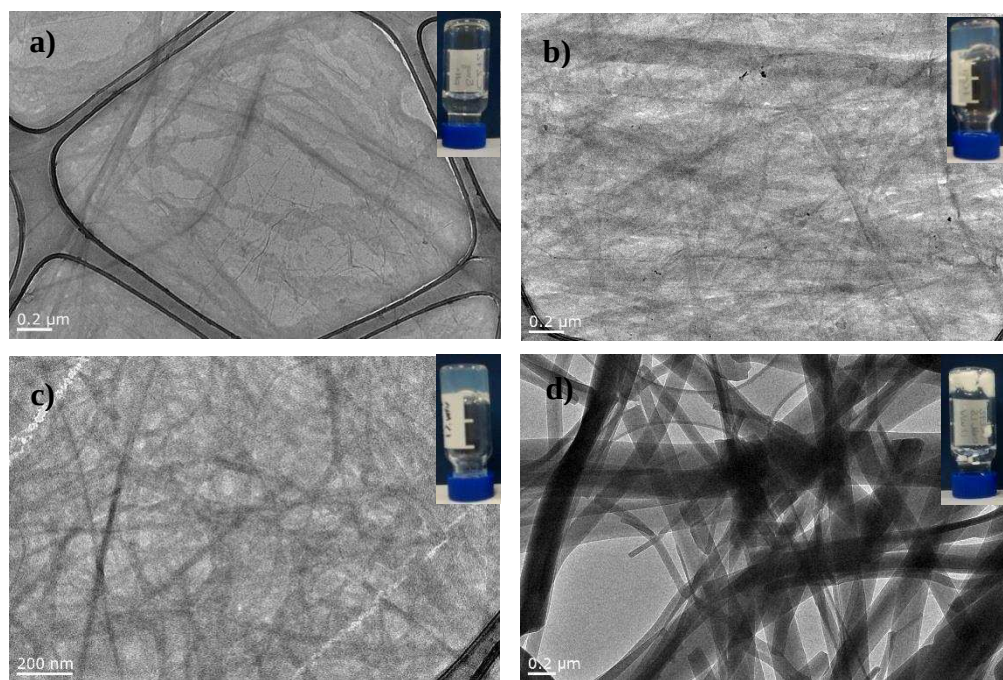


Figure 12.3: Transmission electron micrographs and vial inversion test showing macroscopic appearance of sample **68a** (1% w/v) a) at pH 11.0, with no GdL; b) at pH 7.8, with 0.3 molar equivalents of GdL; c) at pH 7.2, 0.7 eq. of GdL; d) at pH 4.5, with 1.5 eq. of GdL.

Addition of GdL determined a decrease of the pH; however, at pH 7.8 almost all the molecules of **68a** (i.e. 99.96 %, as calculated from Reaxys software) are still deprotonated. Therefore, the self-assembled fibres are thought to have incorporated a large amount of ionised compound. A slight increased thickening of the fibres was observed after addition of 0.7 molar equivalents of GdL to the basic solution of **68a**, which led to a more stable gel, confirmed also by the vial inversion test, with a final pH of 7.2. TEM micrographs showed a more entangled network compared to the previous samples at higher pH, with a prevalence of wider and more defined fibres with diameters in the region of 35-45 nm (Fig. 12c.3). At pH 7.2, 99.84 % of molecules are still calculated to be in their ionised form, suggesting that a small proportion of neutralised molecules are sufficient to promote the self-assembling process, with the negatively charged molecules being localised on the surface of the fibres, according to a model developed by Smith et al.¹⁷ When the pH was reduced to 4.5, near the calculated pKa of **68a**, using 1.5 molar equivalents of GdL, a marked change in the fibres' morphology was observed, with wider and longer fibres. These fibres displayed a variety of widths ranging from 80 nm to 450 nm but with similar lengths of a few micrometers (Fig. 12d.3). From a macroscopic aspect, a white precipitate appeared in the first 12 h and phase separation occurred later when the sample was left standing for more than two days, showing a white precipitate at the bottom and a liquid phase at the top. This phenomenon could be attributed to a metastable gel state, characterised by initial aggregation and subsequent collapse, due to weakening of the physical interactions that led to precipitation.^{27, 33}

3.3.3.3 Fluorescence spectroscopy studies of hydrogels at different pHs

Fluorescence spectroscopy was also used as a tool to further evaluate the gelation of **68a** with changes in pH. Indeed, benzothiazoles possess a fluorescent chromophore which can be detected by fluorescence spectroscopy.

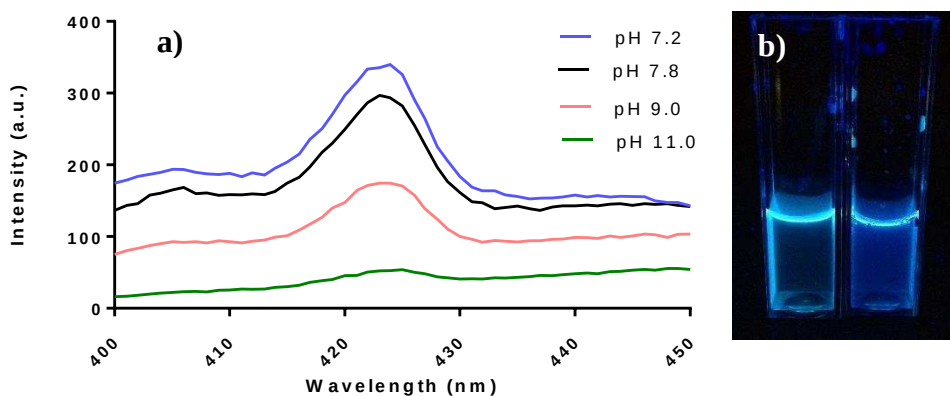


Figure 13.3: a) Fluorescence emission spectra ($\lambda_{\text{ex}} = 380$ nm) of **68a** (1% w/v) at pH 11.0, with no GdL (green line), pH 9.0 (pink line), pH 7.8 (black line) and pH 7.2 (blue line). b) Solution (pH 11, left) and gel (pH 7.2, right) of **68a** under UV light at 365 nm.

Binding of benzothiazoles derivatives to β amyloid fibres, involved in the Alzheimer's disease, has been reported to induce an increase of fluorescence intensity.³⁴ The self-assembly of **68a** was therefore monitored by carrying out fluorescence analysis on the samples as a function of pH (Fig. 13a.3). As reported by Kitts et al.,³⁵ who investigated the spectroscopic properties of a phenyl-benzothiazole derivative having a similar structure to 5F 203, a maximum emission peak at 424 nm was detected when the samples of **68a** were excited at 380 nm. As the pH was acidified from 11 to 7.2, a clear increase of the emission intensity was observed, with a final fluorescence intensity enhancement of 6 times from the solution state at pH 11 to the gel state at pH 7.2. This increase in emission intensity could be attributed to formation of higher ordered aggregates during the gelation process, as a result of π - π stacking interactions between the phenyl-benzothiazole rings that give the characteristic fluorescence peak at 424 nm.^{18, 36} Detection of this band proved the pivotal role of the phenyl groups in the self-assembling process of hydrogel **68a**. This was also visually confirmed: when the samples of **68a**, in the solution state at pH 11 and gel state at pH 7.2, were observed under the UV light at 365 nm, they displayed two different colours, respectively light blue and violet, confirming the change in emission as function of pH (Figure 13b.3). Furthermore, the increase in emission intensity between high and low pH can also be attributed to the change in the

morphology, as already demonstrated by TEM micrographs, i.e. from micelle-like structures to fibres.³²

3.3.3.4 Time-dependent ¹H-NMR spectroscopy studies

In order to study the gelation process of **68a** after addition of GdL, ¹H-NMR spectroscopy was also used to monitor self-assembling over time and to understand the functional groups involved in gel formation. This phenomenon, widely reported for LMWGs, can be probed by ¹H-NMR spectroscopy as the self-assembly leads to a broadening of the peaks associated with functional groups responsible for the physical interactions that promote gelation e.g. π - π stacking interactions between phenyl rings.³⁰ The ¹H-NMR spectra of **68a** (1% w/v) was therefore monitored before and after addition of GdL (Figure 14.3). Compound **68a** was initially solubilised in D₂O after addition of sodium deuterioxide 0.5 M (NaOD) and the first NMR spectrum showed three distinctive peaks in the aliphatic region (1.9-2.7 ppm), characteristic of the alkyl chain and the -CH₃ on the phenyl-benzothiazole ring, and the peaks in the aromatic region also associated with the phenyl- benzothiazole ring (6.8-7.4 ppm). When GdL was added, after 15 min, an initial broadening of both aliphatic and aromatic peaks was noticed. After 24 h, when gelation was considered complete, the peaks almost disappeared, meaning that both functional groups of the gelator are involved in the self-assembling process and that both the π - π stacking of the phenyl-benzothiazole ring and the hydrophobic interactions plus hydrogen bonding of the alkyl chain equally contribute to hydrogel formation. On the contrary, the signals from GdL did not attenuate over time, demonstrating that this molecule is not incorporated into the gel matrix and acts only as pH acidifier by hydrolysis into gluconic acid.

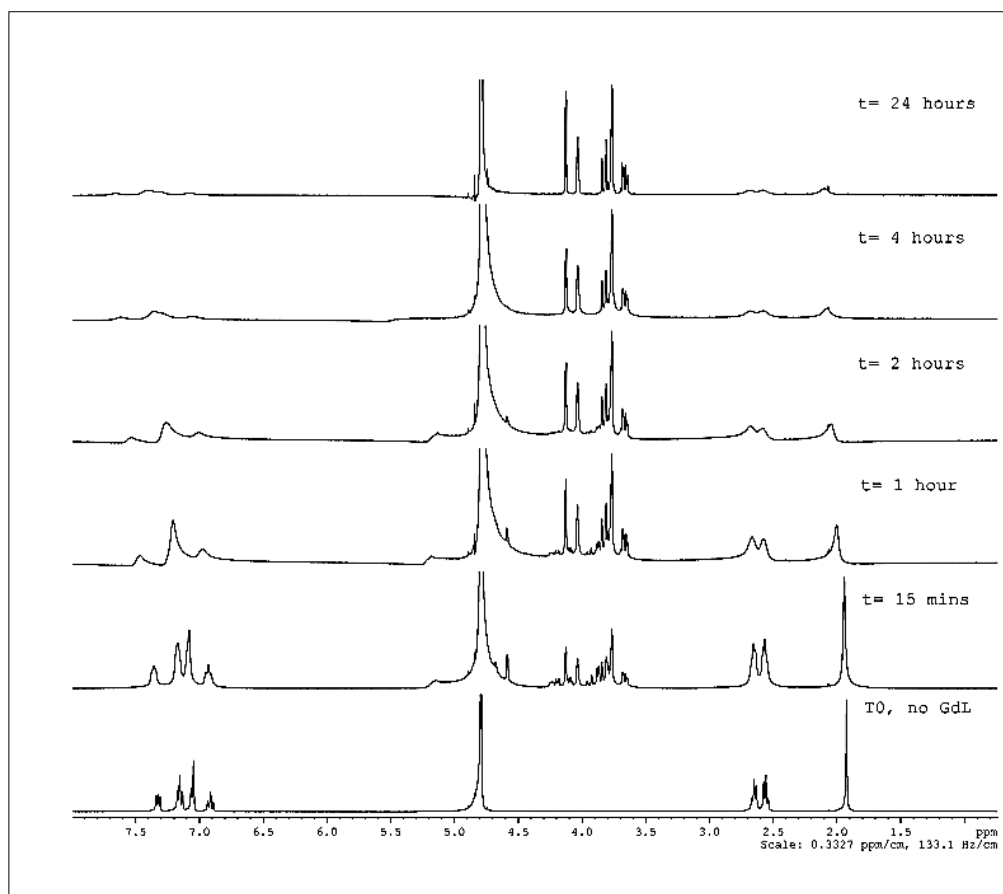


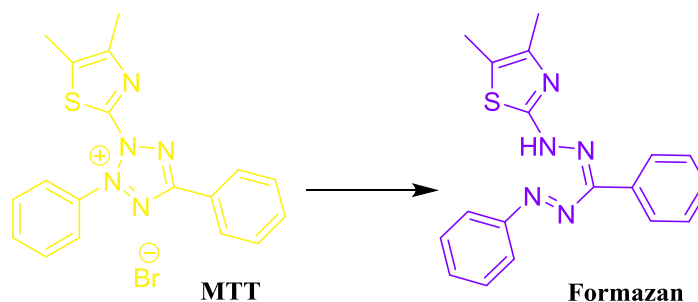
Figure 14.3: ^1H -NMR spectra of **68a** in D_2O without GdL (bottom spectrum) and during gelation (until 24 h) after addition of GdL.

3.3.4 In vitro growth inhibitory activity of 5F 203 amide prodrugs

The in vitro activity of 5F 203 amide prodrugs against human cancer cells lines was investigated, to assess whether the derivatives were able to maintain the growth inhibitory activity of the active drug 5F 203. Prodrugs were tested against MCF-7 breast and IGROV-1 ovarian carcinoma cell lines – 2 models which had previously shown sensitivity to 5F 203 and Phortress in vitro and in vivo.^{37, 38} Furthermore, activity against human ovarian adenocarcinoma cell line OVCAR-4 was also tested, to study whether prodrugs displayed potent growth inhibitory properties against this cell lines as 5F 203 did, or weak activity, as Phortress revealed. In vitro assays against the MRC-5 normal human embryonic lung fibroblast cell line were also carried out, to investigate the selectivity of these prodrugs against cancer cell lines, since MRC-5 fibroblasts were highly resistant to 5F 203.³⁹

3.3.4.1 The MTT assay

For all growth inhibitory experiments the MTT assay was used. This assay, reported for the first time in early 1980s, is based on the reduction of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Scheme 9.3) and measures the effect of a drug on cells' growth and viability. MTT is a yellow water-soluble dye which is reduced by living cells into purple formazan crystals insoluble in water. In this assay, cells are exposed to a test agent when they are in their exponential phase of growth. After a precise time of exposure (72 h in experiments reported herein), the medium containing the test agent is removed and the cells are exposed to MTT for a time (2.5 h) that allows the proliferating cells to reduce the dye into the formazan product, which is then solubilised with an organic solvent (usually DMSO). As a result, living cells are able to reduce the MTT and high concentrations of formazan products are formed. Detection of the amount of formazan produced is determined spectrophotometrically.



Scheme 9.3: Reduction of MTT into formazan by mitochondrial oxidoreductase

3.3.4.2 Growth inhibitory effects on MCF-7 breast carcinoma cell line

When MCF-7 cancer cells were exposed to 5F 203 amide prodrugs, all derivatives retained activity against this cell line (Table 5.3). The GI_{50} values of 5F 203 amide prodrugs reported in the following tables refer to the growth inhibitory effect of 5F 203 on cells, deriving from the hydrolysis of 5F 203 derivatives into the native drug.

Compound	GI_{50} mean $\pm$ SD (nM) in MCF-7
5F 203	38.82 $\pm$ 7.23
67a	234.34 $\pm$ 134.67
67b	61.53 $\pm$ 20.96
67c	513.18 $\pm$ 193.06
67d	717.22 $\pm$ 222.50
67e	622.77 $\pm$ 168.15
67f	513.27 $\pm$ 117.16
67g	619.58 $\pm$ 406.31
68a	4754.80 $\pm$ 1902.91
68b	8139.61 $\pm$ 1171.99

Table 5.3: Growth inhibitory activity of 5F 203 and amide prodrugs in MCF-7 carcinoma cells lines. Cells were exposed to experimental agents for 72 h before viability was assessed by MTT assay (n = 4 per experiment; ≥ 3 independent trials were performed).

In particular, estimated mean GI_{50} values $< 1 \mu\text{M}$ were calculated for 5F 203 and amino acid prodrugs, with 5F 203 and prodrug 5F 203-norleucine (**67b**) revealing the lowest GI_{50} values of 38.82 nM and 61.53 nM respectively. In addition, compounds showed to dose-dependently inhibit cancer cell growth (Figure 15.3).

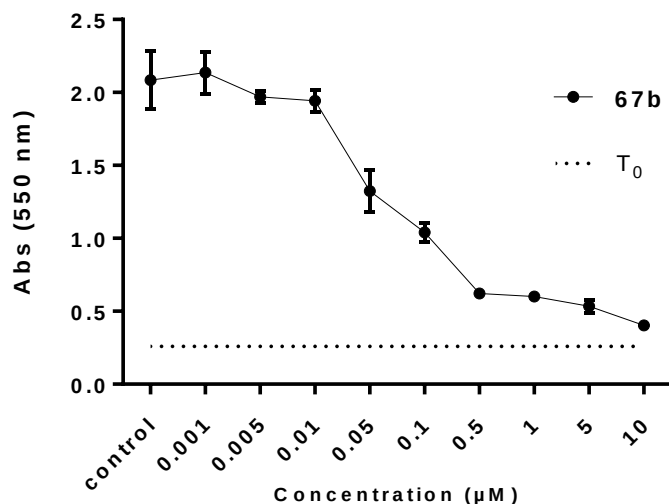


Figure 15.3: Example of dose-response profile of **67b** against MCF-7 cell line at different concentrations (1 nM to 10 μM, n=4). T_0 represents the absorbance of non-treated cells at the time of drug addition.

Prodrugs of the second series displayed higher GI_{50} values. Specifically, both prodrugs retained activity at micromolar concentrations. Compared to the active drug 5F 203, mean GI_{50} values of 4.75 μM and 8.14 μM for the succinic acid (**68a**) and the glutaric acid (**68b**) prodrugs respectively were obtained. The lower potency of this class of prodrugs could be attributed to a slower hydrolysis of the active drug from the prodrugs compared to the amino acid derivatives. As a consequence, less 5F 203 is available to bind to the AhR and trigger the CYP1A1 induction cascade that leads to cell death via DNA adducts' formation.

3.3.4.3 Growth inhibitory effects on IGROV-1 and OVCAR-4 ovarian carcinoma cell lines

MTT assays of 5F 203 amide prodrugs against IGROV-1 and OVCAR-4 cells showed greater activity against the first ovarian carcinoma cell line, revealing a trend of GI_{50} values similar to the ones previously reported for MCF-7 cells. Again, 5F 203 and all the amino acid derivatives displayed GI_{50} values < 1 μM with exception of 5F 203-serine (**67e**) which revealed a GI_{50} value of 1.05 μM. The active drug 5F 203 was still the most potent compound with a GI_{50} value of 138.24 nM. However, the threonine derivative (**67f**) revealed a similar activity,

with a GI_{50} value of 173.20 nM. Prodrugs of the second series again showed micromolar activity against IGROV-1: as for the MCF-7 cell line, the succinic acid derivative (**68a**) demonstrated higher growth inhibitory activity compared to glutaric acid (**68b**) prodrug, with GI_{50} values of 5.32 μ M and 9.21 μ M respectively (Table 6.3).

Compound	GI_{50} mean $\pm$ SD (nM) in IGROV-1
5F 203	138.24 $\pm$ 87.99
67a	546.75 $\pm$ 149.77
67b	391.24 $\pm$ 60.61
67c	446.60 $\pm$ 63.04
67d	741.56 $\pm$ 224.03
67e	1049.91 $\pm$ 100.65
67f	173.20 $\pm$ 112.21
67g	942.36 $\pm$ 409.31
68a	5325.85 $\pm$ 2231.67
68b	9212.59 $\pm$ 269.10

Table 6.3: Growth inhibitory activity of 5F 203 and amide prodrugs in IGROV-1 carcinoma cells lines. Cells were exposed to experimental agents for 72 h before viability was assessed by MTT assay (n = 4 per experiment; ≥ 3 independent trials were performed).

Also in this cell line, prodrugs revealed dose-dependent inhibition of cancer cell growth (Figure 16.3). Furthermore, it was interestingly noticed that sensitivity to 5F 203 and its prodrugs was lost when IGROV-1 cells had a passage higher than 10, probably due to loss of CYP metabolising enzyme inducibility accompanying phenotypic drift, as previously reported specifically for this cell line.³⁸

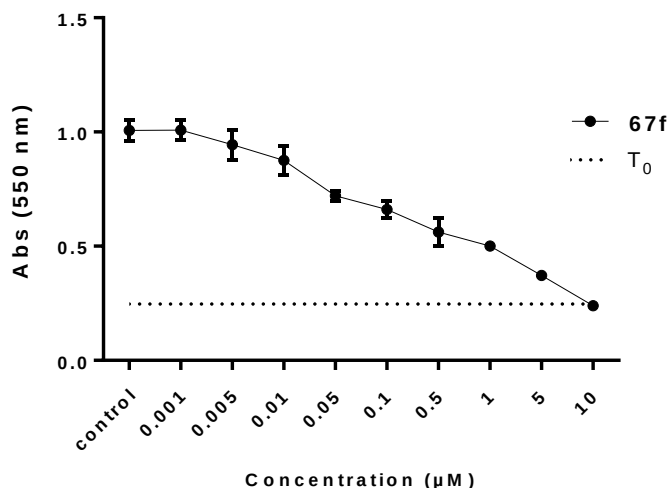


Figure 16.3: Example of dose-response profile of **67f** against IGROV-1 cell line at different concentrations (1 nM to 10 μM, n=4). T_0 represents the absorbance of non-treated cells at the time of drug addition.

When the compounds were tested against the ovarian cell line OVCAR-4, they showed different results compared to the ones reported in the literature.⁴⁰ In fact, 5F 203 was previously shown to induce expression of CYP1A1 enzyme and nanomolar activity in the OVCAR-4 cell line.²¹ Phortress, in contrast, revealed decreased potency against this cell line.³⁷

Results demonstrated different behaviour of 5F 203 and amide prodrugs against OVCAR-4: the active drug did not inhibit cancer cells' proliferation at the maximum concentration tested (10 μM); similarly, the second series of amide derivatives, i.e. the succinic (**68a**) and glutaric acid (**68b**) prodrugs also displayed negligible activity. Nevertheless, all the amino acid prodrugs revealed inhibitory activity against OVCAR-4 with GI_{50} values between 414.21 nM for the aspartic acid derivative (**67g**) and 3932.19 nM for **67a** (Table 7.3).

The opposing behaviour of 5F 203 against OVCAR-4 may be attributed to CYP1A1 inducibility, as previously experienced for IGROV-1. Since the same batch of cells was used for all the experiments carried out, it might be that this particular batch displayed a minimal concentration of cytosolic AhR. Benzothiazole compounds bind to this receptor inducing the signalling cascade which results in CYP1A1 induction. Due to the lipophilic nature of

5F 203, it passively diffuses in and out across the cell membrane and its uptake would be reduced in the case of low concentrations of AhR.

Compound	GI ₅₀ mean $\pm$ SD (nM) in OVCAR-4
5F 203	>10000
67a	3932.19 $\pm$ 443.15
67b	439.36 $\pm$ 87.32
67c	666.65 $\pm$ 200.78
67d	1708.44 $\pm$ 953.11
67e	1137.51 $\pm$ 247.76
67f	624.64 $\pm$ 281.12
67g	414.21 $\pm$ 6.24
68a	>10000
68b	>10000

Table 7.3: Growth inhibitory activity of 5F 203 and amide prodrugs in OVCAR-4 carcinoma cells lines. Cells were exposed to experimental agents for 72 h before viability was assessed by MTT assay (n = 4 per experiment; ≥ 3 independent trials were performed).

This would explain the lack of growth inhibitory properties due to an absence of signal transduction. Also the second series of amide prodrugs did not showed activity against this cell line: again, this could be due to a slow hydrolysis of the active 5F 203, which already elucidated the reduced potency in the two previous cell lines, i.e. MCF-7 and IGROV-1.

The reason why amino acid prodrugs were more active in inhibiting OVCAR-4 cell growth could be attributed to their physicochemical properties: they favour a higher uptake of 5F 203 into the cells and, consequently, more substrate is available for the AhR to induce the CYP1A1 cascade. Amino acid membrane transporters may also facilitate a more rapid intracellular uptake of amino acid prodrugs promoting increased cytoplasmic accumulation of 5F 203. This hypothesis could have been tested by incubating the cells for a longer time with the drugs, to assess whether longer exposure would have favoured the binding of 5F 203 to the receptor.

3.3.4.4 Growth inhibitory effects on MRC-5 normal lung fibroblast cell line

One of the ideal properties of chemotherapeutic agents is selectivity between specific cancer cell lines and non-malignant cells. MRC-5 embryonic lung fibroblasts were used as a model of normal cells to evaluate the selectivity of benzothiazole agents. As previously reported, MRC-5 cells were inherently resistant to 5F 203, demonstrating its selective growth inhibitory activity.³⁹

In the MTT assays carried out, 5F 203, the threonine (**67f**) and aspartic acid (**67g**) prodrugs and the compounds of the second series (**68a** and **68b**) showed lack of growth inhibition properties at the maximum concentration tested (10 μ M). The remaining prodrugs of the amino acid series inhibited MRC-5 cells' growth at micromolar concentrations, with mean GI₅₀ values up to ten times higher than those obtained for MCF-7 and IGROV-1 (the most sensitive to 5F 203 and its amide prodrugs; Table 8.3). The difference in GI₅₀ values between cancer and normal cell lines (Figure 17.3) may suggest the selectivity of the benzothiazole derivatives against malignant cell lines.

Compound	GI ₅₀ mean $\pm$ SD (nM) in MRC-5
5F 203	>10000
67a	5754.02 $\pm$ 1049.31
67b	5509.20 $\pm$ 721.87
67c	5642.96 $\pm$ 430.07
67d	4816.02 $\pm$ 638.21
67e	8340.49 $\pm$ 1409.04
67f	>10000
67g	>10000
68a	>10000
68b	>10000

Table 8.3: Growth inhibitory activity of 5F 203 and amide prodrugs in MRC-5 fibroblast cells lines. Cells were exposed to experimental agents for 72 h before viability was assessed by MTT assay (n = 4 per experiment; ≥ 3 independent trials were performed).

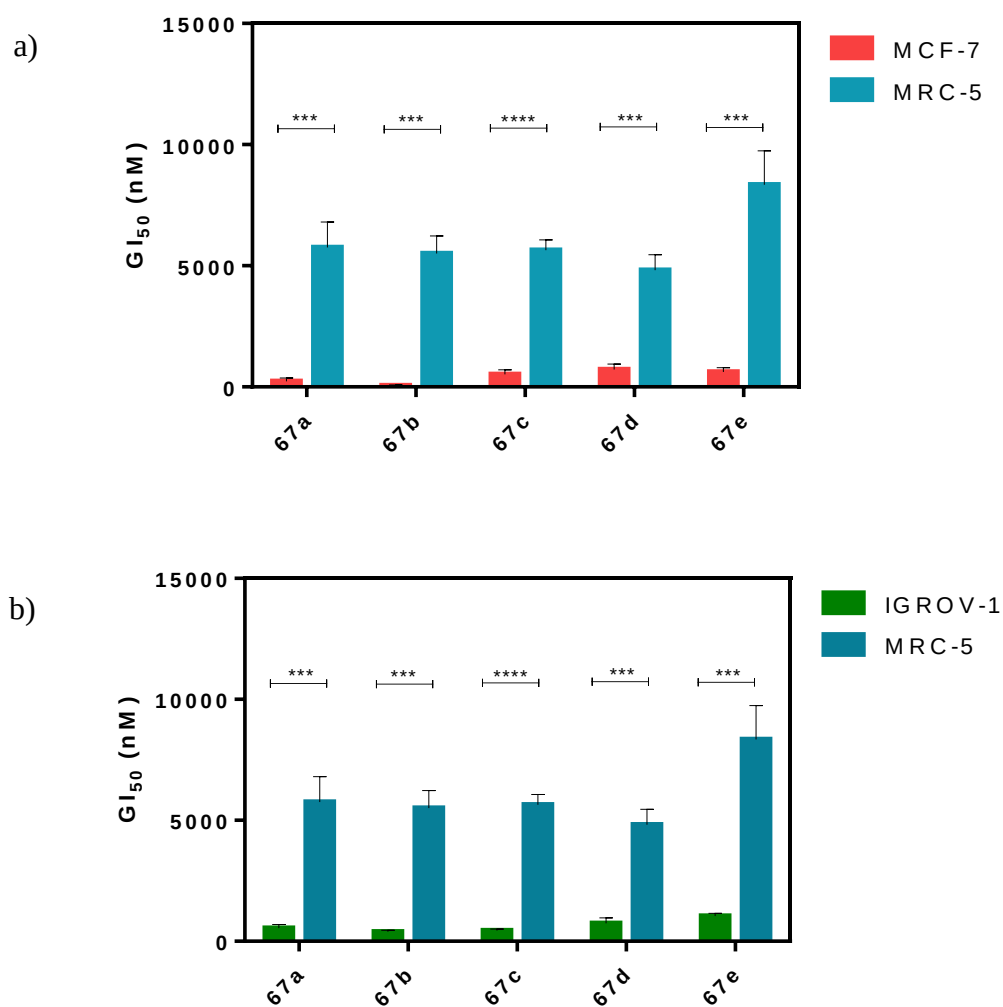


Figure 17.3: Paired t-test analyses of 5F 203 amino acid prodrugs (67a-67e) on MCF-7, IGROV-1 and MRC-5 cell lines (***) $p \leq 0.001$, **** $p \leq 0.0001$).

3.3.5 5F 203-succinic acid prodrug hydrogel in vitro release properties

The ability of 5F 203-succinic acid prodrug (**68a**) to form a self-assembling hydrogel matrix suggested that it might exhibit controllable release properties. Therefore, the release profile of the active drug 5F 203 and **68a** from the gel matrix was assessed. These studies were performed to verify the ability of hydrogel **68a** to act as a depot for the sustained release of the therapeutic agent for localised drug delivery. Release studies were carried out under physiological conditions at 37°C. The gels were prepared on transwell inserts at a concentration of 1% w/v and then incubated in 24 well plates containing PBS at pH 7.4. Samples were taken from the wells at specific time points and analysed by HPLC. Studies were performed over 80 h as longer times led to precipitation of the gel.⁴¹ Results showed that 48% of prodrug **68a** was released within 80 h (Fig. 18.3).

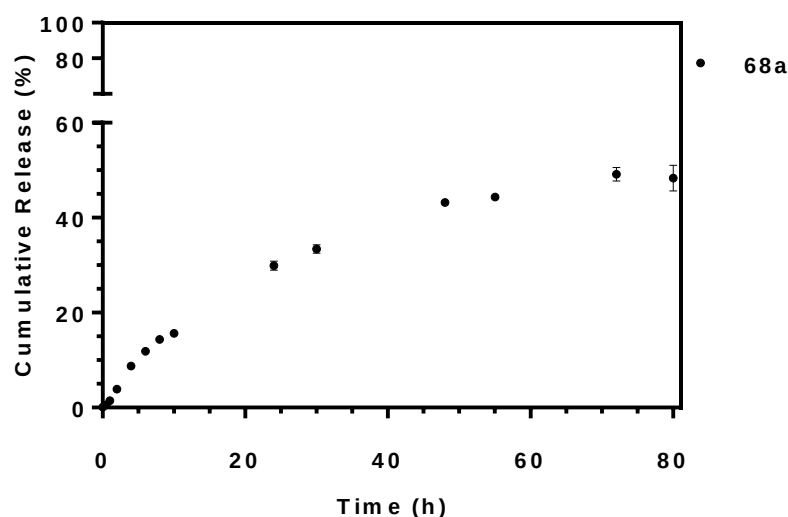


Figure 18.3: Cumulative release profile of **68a** from hydrogel of **68a** (1% w/v) in PBS (pH 7.4, 37°C). All measurements were carried out in triplicate. Error bars represent standard deviation.

Interestingly, detection of the active drug 5F 203 after 48 h was also revealed. It was hypothesised that the presence of 5F 203 in PBS buffer could derive from the slow hydrolysis of the prodrug already in solution, thus explaining the detection of the active drug after 2 days and ultimately proving the prodrug nature of **68a** (Figure 19.3). Moreover, no other products were observed in the incubation buffer.

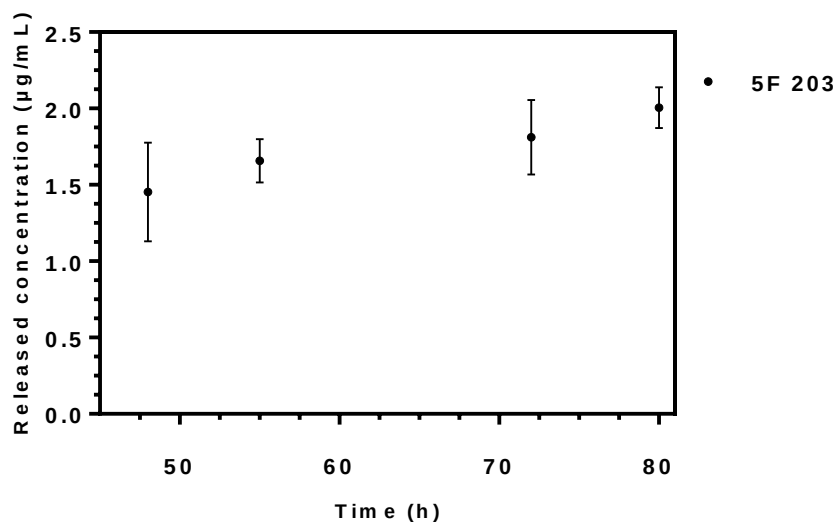


Figure 19.3: Accumulative release profile of 5F 203 (after 48 h) from hydrogel of **68a** (1% w/v) in PBS (pH 7.4, 37°C). All measurements were carried out in triplicate. Error bars represent standard deviation.

Finally, LC-MS analyses of the gel content, carried out on the gel in the transwell insert after 24 h from incubation in PBS buffer, detected only **68a** in the gel matrix, thus confirming that the hydrolysis of the prodrug into the active drug occurred only after its dissolution in PBS buffer.

3.4 Conclusions

In this chapter, two new prodrugs series of the antitumour benzothiazole agent 5F 203 have been designed and synthesised, with the aim of developing self-assembling gelators. Firstly, an optimisation of the reaction for the synthesis of the amino acid derivatives was carried out, finding a suitable coupling reagent (HATU) able to reduce the reaction times and increase the overall yields of the final compounds.

Then, preliminary gelation tests were performed, showing that the amino acid prodrugs of 5F 203 were able to form metastable organogels with a solvent-switch method: in particular the lead drug 5F 203 and the threonine prodrug (**67f**) self-assembled in mixtures of water and DMSO. Of the second series of prodrugs, the succinic acid derivative (**68a**) formed a hydrogel via a pH-triggered method. It was also demonstrated that minor structural changes in the prodrug gelator, i.e. addition of a methylene group in the molecule, affected the self-assembling process.

The gelation process of **68a** was then studied and evaluated by different characterisation methods. Dynamic oscillatory rheological studies on the hydrogel confirmed its LMWG nature. The self-assembling process was also studied by SEM and TEM imaging, and fluorescence spectroscopy, showing the importance of pH in the hydrogelation. In particular, it was demonstrated that a change from basic to neutral pH determines the formation of cross-linked fibres and that a drop to acidic conditions leads to precipitation of the gel and thickening of the fibrillar structures. Moreover, fluorescence studies showed the crucial role of π - π stacking interactions, derived from the phenyl-benzothiazole ring, in gel formation. Additionally, time-dependent ^1H -NMR spectroscopy studies elucidated the importance also of the alkyl chain in the self-assembly process.

The in vitro activity of 5F 203 and its amide prodrugs against breast, ovarian carcinoma and fibroblast cell lines was also tested, showing the selectivity of compounds in inhibiting cancer cell proliferation, particularly for MCF-7 breast and IGROV-1 ovarian carcinoma cells. Resistance of the non-malignant fibroblast cell line MRC-5 to these compounds proved the selectivity for tumour

over `normal` cell lines. In addition, the hydrogel based on the succinic acid derivative (**68a**) gave release of prodrug **68a** over three days and showed also release of the active drug 5F 203 after 48 h, as a result of the slow prodrug hydrolysis in the incubation medium.

These results proved the prodrug nature of **68a** and validated the use of this hydrogel as a depot formulation for the localised delivery of 5F 203 at the tumour resection sites.

3.5 References

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Chapter 4: Self-assembling carbamate prodrugs of benzothiazole 5F 203

The following chapter will briefly describe LMWGs having carbamate groups and carbamate-based prodrugs as a strategy for the preparation of derivatives of amines. Then, the synthesis, the self-assembling properties and the in vitro antitumour activity of a novel series of 5F 203 carbamate-based prodrugs will be investigated.

4.1 Introduction

Although 5F 203-succinic acid prodrug (**68a**) was revealed to be the most successful LMWG amongst the amide prodrugs of 5F 203, it possessed a diminished growth inhibitory activity against human-derived cancer cell lines, compared to the other compounds of the amide series, due to a slow hydrolysis of the amide bond. Therefore, in order to combine the self-assembling properties of 5F 203 derivatives and increase the rate of conversion of the prodrugs into the parent amine, the carbamate bond was selected as the linking group for the synthesis of a new class of 5F 203 prodrugs. This group was chosen because of the reported LMWGs based on carbamate bonds and the good chemical stability and susceptibility to plasma enzymes of carbamate-based prodrugs.

4.1.1 LMWGs with carbamate bonds as linking group

The role of the carbamate bond in the self-assembling process has been studied by different authors, who also investigated the contribution that the deriving hydrogen bonds provide to gelation.

Moniruzzaman and Sundararajan were the first authors to report a family of LMWGs based on carbamates bearing alkyl chains of different lengths. They developed organogels in benzonitrile with a temperature-switch method and demonstrated the gelation ability of the compounds with a maximum number of

12 carbons in the side chain, since crystallisation occurred with a higher number of carbons, due to the prevalence of van der Waals interactions. Analyses of the gels' morphologies proved the formation of nanofibres, with the thickness of the fibres depending on the chain length.¹

Cicchi et al. described the organogelation ability of 1-benzyl-pyrrolidine-3,4-diol derivative (**69**) linked to two long carbon chains via a carbamate bond (Figure 1.4). In their work, the authors pointed out the role of intermolecular hydrogen bonding, deriving from the carbamate units, in the formation of supramolecular structures, as revealed by IR studies. Moreover, X-ray crystallography results showed the presence of multiple hydrogen bonds between the carbamate groups of the benzylpyrrolidine-carbamate central unit, arranged into two antiparallel chains.²

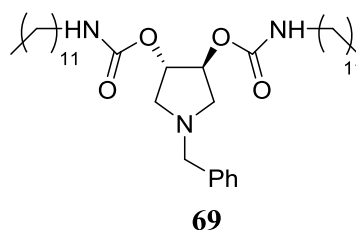


Figure 1.4: Molecular structure of 1-benzyl-pyrrolidine-3,4-diol derivative (**69**).

Wang and his group reported a series of LMWGs based on a family of monosaccharides linked to alkylnyl, aryl, and alkyl halide moieties via a carbamate bond. In a previous study, the same authors synthesised three series of D-glucopyranoside derivatives bearing ester and carbamate bonds and investigated their gelation properties, showing that the carbamate derivatives, having an additional hydrogen bond donor compared to the ester counterparts, formed more robust gels due to more non-covalent interactions involved in the self-assembly.³ In this study, they synthesised O-linked (**70**) and N-linked carbamate (**71**) derivatives (Figure 2.4) to study in detail the structural requirements for gelation, finding that the first series of compounds were efficient gelators of binary mixtures of DMSO/ethanol and water. The N-linked series formed organogels in a wider variety of solvents (i.e. hexane, isopropyl alcohol) and the ethyl and isobutyl derivatives were also able to gel pure water, demonstrating to be efficient LMWGs displaying a potential for drug delivery

and other biological purposes. In general, this study revealed the importance of a balance between hydrophobic forces and hydrogen bonds, especially for the self-assembly of gelators in polar solvents.⁴

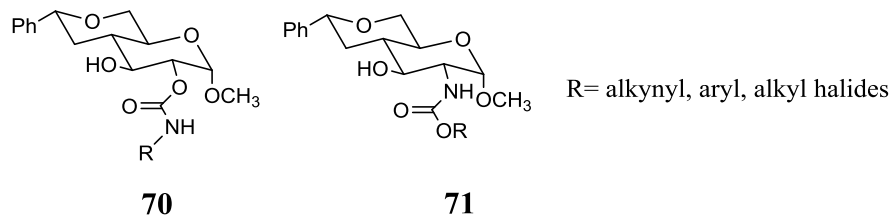
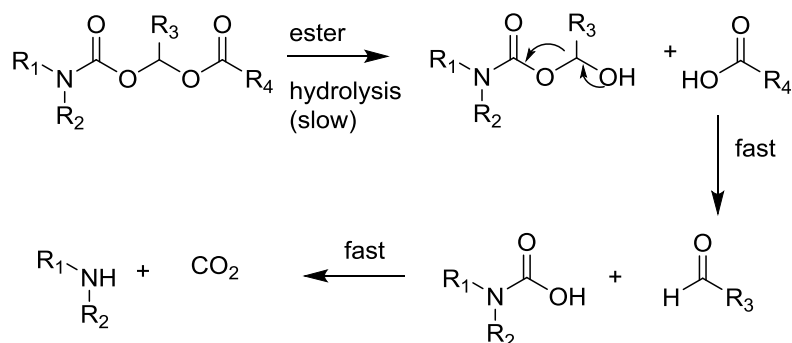


Figure 2.4: Molecular structures of sugar-based carbamates **70** (O-linked) and **71** (N-linked).

In spite of the examples reported above about LMWGs based on carbamates as linking group, there are currently no examples of self-assembled carbamate prodrugs in the literature.

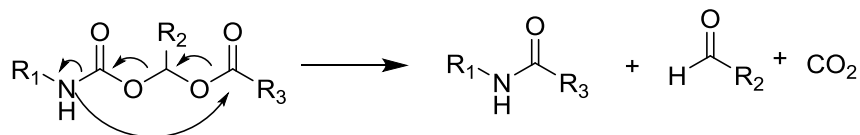
4.1.2 Carbamate-based prodrugs

Carbamate-based esterase-sensitive prodrugs are often used as strategy for the preparation of derivatives of amines.⁵ A particular approach, pioneered by Alexander and co-workers, consists of the synthesis of modified carbamates, i.e. acyloxyalkoxycarbonyl derivatives of amines, possessing an enzymatically hydrolysable ester function.⁶ The mechanism of release of the parent amine from its prodrug involves a first ester hydrolysis, which gives the unstable intermediate hydroxyalkoxycarbonylamine, followed by release of the carbamic acid derivative and the parent drug (Scheme 1.4).



Scheme 1.4: Mechanism of ester-mediated hydrolysis of N-acyloxyalkoxycarbonyl derivatives into parent amines.

In the case of primary amines, a specific intramolecular acyl transfer mechanism has been suggested for N-acyloxyalkoxycarbonyl derivatives, in which a nucleophilic attack from the carbamate nitrogen occurs to the carbonyl carbon, with formation of a 6-membered transition state (Scheme 2.4).⁵



Scheme 2.4: Suggested intramolecular acyl transfer in N-acyloxyalkoxycarbonyl prodrugs of primary amines

In his first study, Alexander et al. analysed the degradation kinetics of N-acyloxyalkoxycarbonyl derivatives of model drugs (i.e. 4-aminobenzenesulfonic acid, aniline) in aqueous media, as a function of pH and temperature. Results showed that at $1 \leq \text{pH} \leq 9$ acyloxyalkoxycarbonyl derivatives of primary amines exhibited constant rates of degradation, with stability at $\text{pH} \leq 4$ being dependent upon stability of the ester function and at $\text{pH} \geq 5$ upon both the ester function and the occurrence of the intramolecular acyl transfer reaction (Scheme 2.4). Moreover, these prodrugs had estimated shelf lives > 3 years, especially when formulated as solids or as liquids.⁶

In a second study, the authors described the release of parent amines after incubation of the acyloxyalkoxycarbonyl derivatives with human plasma, to assess their sensitivity to esterase activity. Results showed that human plasma esterases catalysed the release of parent drugs from the corresponding prodrugs. In particular, primary amines were not released in a quantitative manner, due to formation of the side product N-acetylated amine; therefore, although promising, authors suggested that the utility of these prodrugs could be specifically evaluated only after in vivo studies. On the contrary, acyloxyalkoxycarbonyl derivatives of secondary amines showed quantitative release of the parent amine at a higher rate in plasma compared to buffer at $\text{pH}=7.4$, thus demonstrating their suitability as prodrugs for secondary amines.⁷

In a further study, they also reported that acyloxyalkoxycarbonyl prodrugs of β -blocker drugs, such as atenolol, pindolol and propranolol, induce an increase of permeation through some biological barriers (i.e. skin).⁸

As a result of the successful reports of Alexander et al., numerous studies involving the synthesis and biological evaluation of acyloxyalkoxycarbonyl derivatives of primary and secondary amines have been carried out.

Cundy and co-workers reported the development of an acyloxyalkoxycarbonyl prodrug of the anticonvulsant agent Gabapentin, by detailing the synthetic route, the enzymatic conversion and in vivo bioavailability of XP13512 (Figure 3.4).^{9, 10} Gabapentin is characterised by a short half-life and lack of dose proportionality, i.e. increases in the administered dose of the drug do not lead to proportional increases in measures of exposure of the drug (such as maximal blood concentration or area under the curve, AUC). XP13512 was developed with the aim of increasing the pharmacokinetic properties of the parent amine. In vivo studies in monkeys revealed an increase in oral bioavailability from 25% to 84% when Gabapentin was administered as prodrug XP13512.

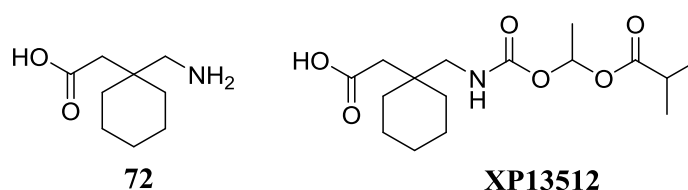


Figure 3.4: Molecular structure of acyloxyalkoxycarbonyl Gabapentin prodrug **XP13512** of the anticonvulsant agent **72**.

In another study, Meng and co-workers developed acyloxyalkoxycarbonyl prodrugs of the secondary amine SQ109 (Figure 4.4), a second-generation antituberculosis agent which underwent Phase I clinical trial. Due to its poor oral bioavailability, mainly caused by first-pass hepatic metabolism, formulation of this drug candidate as carbamate prodrugs was evaluated.

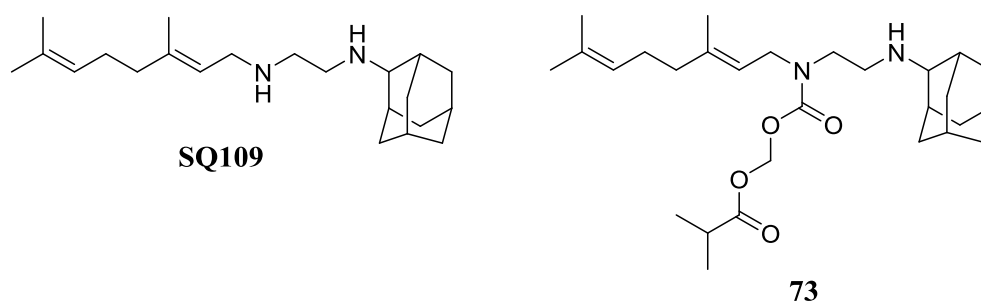


Figure 4.4: Molecular structure of antituberculosis drug SQ109 and its acyloxyalkoxycarbonyl prodrug **73**.

Pharmacokinetic studies in rats showed that carbamate prodrugs were rapidly metabolised into the parent drug when administered orally, giving higher plasma concentration of SQ109 compared to an equivalent dose of the parent drug. Results confirmed the successful approach of esterase-sensitive carbamate prodrugs developed by Alexander et al. Therefore, prodrug **73** (Figure 4.4) was selected as the lead candidate for further in vivo studies.¹¹

4.2 Materials and Methods

4.2.1 General procedures

4.2.1.1 Materials

Chemicals were purchased from commercial suppliers and were used without further purification. All solvents used were either analytical reagent or HPLC grade and were supplied by Fisher Scientific. Dry CH₂Cl₂ and THF were purchased by Acros Organics and Sigma Aldrich. Deuterated solvents were purchased from Cambridge Isotope Laboratories Inc. and Sigma Aldrich.

4.2.1.2 General methods

Reactions were performed according to general methods described in Chapter 2, section 2.2.1.2.

4.2.1.3 Instrumentation

NMR, HRMS, melting points and analytical RP-HPLC were carried out as described in Chapter 2, section 2.2.1.3.

4.2.1.4 Gelation tests

Gelation tests were performed according to a solvent-switch method. The compounds were weighed using an OHAUS semi micro-analytical balance into 1.5 mL sample vials. DMSO was added to create a stock solution at 10% w/v. The solution was then sonicated in a Sonicor Ultra-sonic bath for one minute and successively heated at 60 °C to help full dissolution of the compound. An aliquot (25 µL) of the stock solution was transferred in another 1.5 mL vial and the remaining DMSO and pre-heated (60 °C) ultra-purified water were added. Samples were then allowed to cool down to room temperature. Vial inversion

tests were subsequently carried out as preliminary screens for gelation. All samples prepared had a final concentration of 0.5% w/v.

4.2.1.5 Transmission electron microscopy (TEM)

TEM analyses were performed using a JEOL JEM-2100 at an accelerated voltage of 200 kV. Samples were prepared by dispersing a small amount of gel in 200 μ L of ultra-purified water and pipetted on to a graphene oxide lacey carbon hexagonal copper grid (300 mesh) using a 10 μ L micropipette. Excess sample was blotted with Whatman 50 filter paper. The grid was subjected to high vacuum prior to imaging. ImageJ software was used to analyse TEM images.

4.2.1.6 Rheological experiments

Samples for rheological measurements were prepared in 7 mL Sterilin plastic sample vials and gelation was promoted following the standard solvent-switch gelation method (section 4.2.1.4), to make gels of 2 mL volume (0.5% w/v).

Rheological studies were performed according to the procedure illustrated in Chapter 3, section 3.2.1.7.

For these gels, measurements of G' and G'' as function of strain γ , were carried out at a frequency of 10 rad/s. Oscillatory frequency sweeps were performed under a strain of 0.5%.

4.2.1.7 ^1H -NMR spectroscopy

Samples for ^1H -NMR spectroscopy analyses of gels were prepared in 20 mL scintillation vials according to the standard solvent-switch gelation method: the hot solutions (60 $^{\circ}\text{C}$, 600 μ L) of compounds in binary mixtures of DMSO- d_6 and D_2O were transferred into the NMR tubes (Wilma 528-PP-7, previously dried under vacuum) and the samples were left to stand for 12 h to allow cooling and gelation. ^1H -NMR spectra were then recorded on a Bruker Avance at 600 MHz at 25 $^{\circ}\text{C}$, 45 $^{\circ}\text{C}$, 65 $^{\circ}\text{C}$ and 75 $^{\circ}\text{C}$, using NS=2048. Pre-saturation was applied before the variation-temperature experiments to suppress the water signal.

4.2.1.8 Growth inhibitory assay

General cell culture methods and MTT assays were carried as described in Chapter 3, sections 3.2.1.9 and 3.2.1.10.

4.2.1.9 In vitro stability assays

Stability studies of **76a** were performed in PBS, rat and human plasma. All the experiments were carried out at 37 °C using a Thermo Scientific MaxQ 4000 orbital shaker at 200 rpm and were repeated in triplicate. In all the experiments, a stock solution of the prodrug (1.0 mM) in 100% CH₃CN was diluted to give a final concentration of 10 µM in PBS, rat and human plasma.

In PBS (pH=7.4), a stock solution (20 µL) of the compound was spiked into 1980 µL of PBS in pre-heated Fisher Scientific glass test tubes, and 100 µL of the samples were withdrawn at each specific time point.

Analyses were performed on a HP1050 RP-HPLC and visualised at 254 nm with a HP1050 variable wavelength absorbance detector. Spectra were analysed using Agilent. Analytical RP-HPLC was performed using a Phenomenex Luna 5 µm C8 (2) 100A column (150 mm x 4.60 mm) at a flow rate of 1 mL/min. Samples were analysed using an isocratic method: 70% solvent B in solvent A (solvent A= 0.05 % TFA in H₂O, solvent B= 0.05 % TFA in 9:1 v: v CH₃CN:H₂O). Quantification of known concentrations of 5F 203 (Chapter 3, section 3.2.1.12, Figure 8.3) and **76a** (Figure 5.4) was determined by UV-HPLC measurements and used to produce two standard curves, from which unknown concentrations of both compounds were determined.

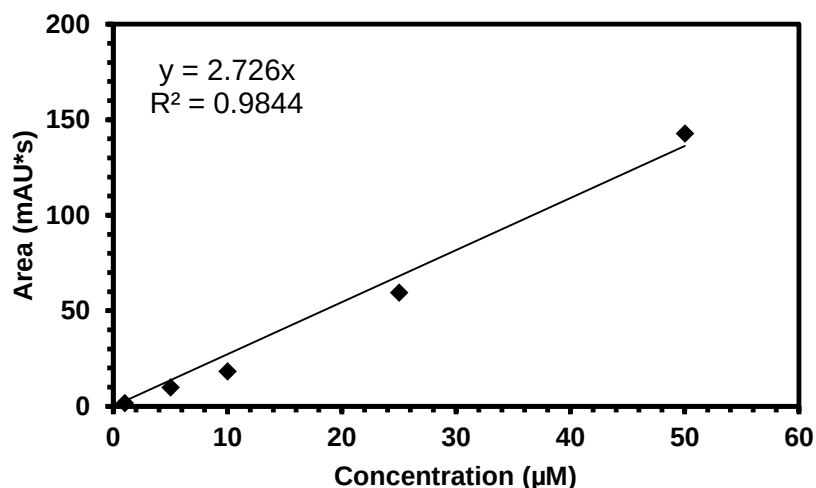


Figure 5.4: Standard curve of **76a** versus concentration stability studies in PBS at pH 7.4.

Stability studies in rat (Sera Laboratories) and human plasma (TCS Biosciences) were performed by spiking the stock solution of the compound (12 μ L, 1.0 mM) into 1188 μ L of pre-heated plasma (37 $^{\circ}$ C, 5 min). At each time point a sample (100 μ L) was withdrawn and transferred into test tubes containing 300 μ L of CH_3CN to allow for protein precipitation. Methyl-tert-butyl-ether (MTBE, 3 mL) was used to extract the samples, which were then vortex-mixed for 10 min and centrifuged at 1160 g for 10 min at 10 $^{\circ}$ C. The organic layer was transferred and evaporated to dryness under N_2 at 40 $^{\circ}$ C. The samples were reconstituted with 100 μ L of acetonitrile-water (40:60, v/v) and vortex-mixed for 10 min. Then they were transferred into HPLC vials for analysis.

Analyses were performed on a Waters 600 HPLC equipped of a Waters 717 Autosampler and Waters 2996 Photodiode Array Detector. Analytical RP-HPLC was performed using a Gemini 5 μ m C18 column (150 mm x 2.0 mm) equipped with a SecurityGuard 2 $\times$ 4 mm, 3 μ m (Phenomenex) at a flow rate of 0.2 mL/min. Samples were analysed using an isocratic method: 70% solvent B in solvent A (solvent A = H_2O , solvent B = CH_3CN). Chromatograms were observed at 347 and 325 nm for 5F 203 and **76a**, respectively.

Determination of in vitro $t_{1/2}$:

Calibration curves (Figure 6.4) were used to calculate the concentrations of the sample at each time point.

Half-life ($t_{1/2}$) was calculated using the following equation (Equation 1.4):

$$t_{1/2} = -\frac{0.693}{k}$$

Equation 1.4: Determination of half-life ($t_{1/2}$)

where k is the slope from the plot of natural log percentage of parent compound concentration versus incubation time.

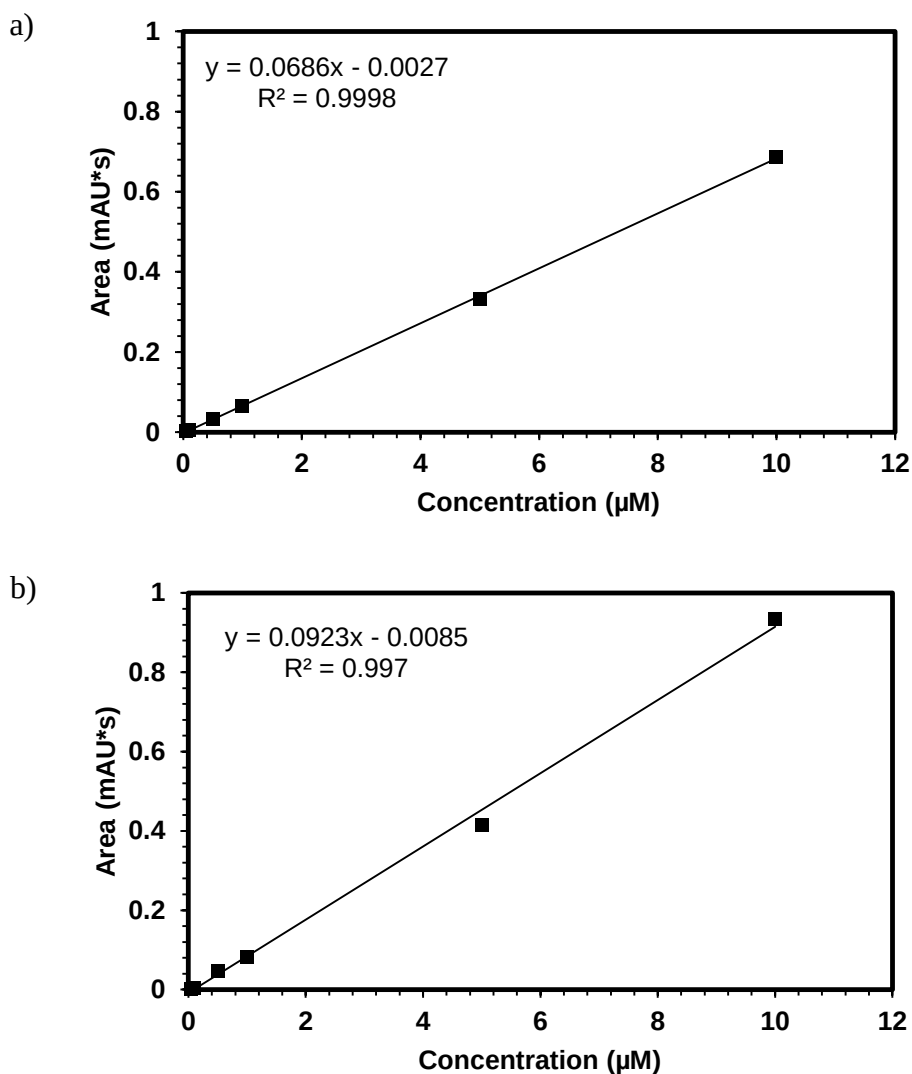


Figure 6.4: Standard curves of a) 76a and b) 5F 203 versus concentration for stability studies in rat and human plasma.

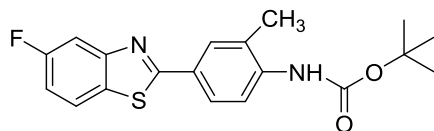
4.2.1.10 Release studies

Gels of **76a** were prepared according to the standard solvent-switch gelation method: in a 20 mL scintillation vial, a hot solution (60 °C, 400 µL) of the compound (0.5% w/v) in binary mixtures of DMSO and ultra-purified water was pipetted into transwell polyethylene terephthalate (PET) membrane inserts (pore size 1.0 µm) and allowed to cool down and gel for approximately 12 h. The transwells were then inserted into 24 well plates containing 1.2 mL of PBS (pH 7.4) and incubated in a Thermo Scientific MaxQ 4000 Shaker at 37 °C at 15 rpm. At each time point a sample (150 µL) was removed from each solution and replaced with the same amount of fresh PBS buffer.

Analyses were performed as described in Chapter 3, section 3.2.1.12.

4.2.2 Experimental data

Tert-Butyl (4-(5-fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)carbamate (74)



5F 203 (0.085 g, 0.33 mmol) was dissolved in dry THF (8 mL) and di-tert-butyl dicarbonate (0.09 g, 0.42 mmol) was

added at 0 °C. After 30 min at 0 °C, the reaction was stirred at r.t. for 3 days. The solution was then poured into water (10 mL) and EtOAc (20 mL) was added. The organic phase was washed saturated NaHCO₃ and water (20 mL) and dried over Na₂SO₄. The crude compound was purified silica gel flash chromatography (gradient elution 10-25% EtOAc in petroleum ether 40-60 °C) to afford a yellow solid (0.04 g, 0.11 mmol, 34% yield).

¹H NMR (DMSO-*d*₆): δ 8.76 (s, 1H, NH), 8.17 (dd, *J*=5.4, 8.8 Hz, 1H, H-7), 7.91 (d, *J*=2.1 Hz, 1H, H-4), 7.88-7.84 (m, 2H, H-2', H-6'), 7.68 (d, *J*=7.65 Hz, 1H, H-5'), 7.35 (td, *J*=2.6, 9.0 Hz, 1H, H-6), 2.32 (s, 3H, CH₃), 1.49 (s, 9H, *t*-C₄H₉).

¹³C NMR (DMSO-*d*₆): δ 169.90, 162.64, 160.32, 154.53, 153.24, 140.25, 131.04, 130.15, 129.04, 128.01, 125.27, 123.54, 113.72, 113.44, 108.58, 79.41, 28.16, 17.92.

Mp: 152-154 °C.

***m/z*:** HRMS (TOF ES⁺) C₁₉H₂₀FN₂O₂S⁺ [MH]⁺ calculated 359.1224; found 359.1130.

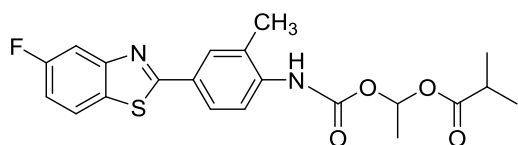
HPLC *t*_R= 24.68 min.

General procedure for the synthesis of 5F 203 carbamate analogues (76):

5F 203 (1 eq) was suspended in dry CH₂Cl₂ at 0 °C and dry pyridine (2 eq) and 1-chloroethyl chloroformate (1.2 eq) were added dropwise and the reaction mixture was stirred at 0 °C for 15 min and then warmed up to r.t. and stirred for 3 h. Water was poured into the reaction mixture and the crude product was extracted 3 times with CH₂Cl₂ and dried over Na₂SO₄. Crude 1-chloroethyl-5F 203 carbamate was used for the following reaction without further purification. 1-chloroethyl-5F 203 carbamate (75, 1 eq) was suspended into

the corresponding acid (35-40 eq) and heated at 70 °C. Ag₂O (1.2 eq), was added and the reaction mixture heated up to 95 °C and stirred for 3 h. Ag₂O was filtered off and the filtrate was collected and concentrated under reduced pressure yielding a yellow oil which was purified by silica gel flash chromatography (gradient elution 10-30% EtOAc in petroleum ether 40-60 °C). Compounds **76a-76d** were obtained from trituration in hexane to remove the residual acids.

1-(((4-(5-Fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)carbamoyl)oxy)ethyl isobutyrate (76a)



Synthesis of **76a** was performed according to the general procedure, using 5F 203 (0.25 g, 0.97 mmol),

pyridine (0.16 mL, 1.94 mmol), 1-chloroethyl chloroformate (0.13 mL, 1.17 mmol), CH₂Cl₂ (6 mL), Ag₂O (0.21 g, 0.90 mmol), isobutyric acid (2.40 mL, 27.00 mmol). A white solid was afforded (0.11 g, 0.26 mmol, 35% yield).

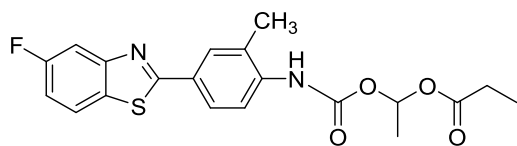
¹H NMR (DMSO-d₆): δ 9.42 (s, 1H, NH), 8.18 (dd, J=5.3, 8.8 Hz, 1H, H-7), 7.94 (d, J=2.3 Hz, 1H, H-4), 7.89 (td, J=2.5, 8.3 Hz, 2H, H-2', H-6'), 7.62 (d, J=8.5 Hz, 1H, H-5'), 7.36 (td, J=2.7, 9.1 Hz, 1H, H-6), 6.79 (q, J=5.4 Hz, 1H, CH-CH₃), 2.55 (sept, J=6.8 Hz, 1H, CH-(CH₃)₂), 2.33 (s, 3H, CH₃-H-3'), 1.50 (d, J=5.8 Hz, 3H, CH-CH₃), 1.10 (d, J=6.9 Hz, 6H, CH-(CH₃)₂).
¹³C NMR (DMSO-d₆): δ 174.41, 169.64, 161.60, 160.19, 154.44, 151.89, 139.13, 129.11, 128.89, 125.36, 123.60, 108.72, 108.48, 89.16, 57.91, 33.08, 19.55, 18.53, 18.39, 17.71, 16.65.

Mp: 107-110°C.

m/z: HRMS (TOF ES⁺) C₂₁H₂₂FN₂O₄S⁺ [MH]⁺ calculated 417.1279; found: 417.1330.

HPLC t_R= 24.03 min.

1-(((4-(5-Fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)carbamoyl)oxy)ethyl propionate (76b)



Synthesis of **76b** was performed according to the general procedure, using 5F 203 (0.25 g, 0.97 mmol),

pyridine (0.16 mL, 1.94 mmol), 1-chloroethyl chloroformate (0.13 mL, 1.17 mmol), CH₂Cl₂ (6 mL), Ag₂O (0.205 g, 0.88 mmol), propionic acid (1.90 mL, 26.00 mmol). A white solid was afforded (0.16 g, 0.40 mmol, 54% yield)

¹H NMR (DMSO-d₆): δ 9.41 (s, 1H, NH), 8.18 (dd, J=5.3, 8.8 Hz, 1H, H-7), 7.93 (d, J=2.3 Hz, 1H, H-4), 7.89 (td, J=2.3, 8.5 Hz, 2H, H-2', H-6'), 7.65 (d, J=8.7 Hz, 1H, H-5'), 7.34 (td, J=2.6, 9.1 Hz, 1H, H-6), 6.81 (q, J=5.4 Hz, 1H, CH-CH₃), 2.39-2.31 (m, 5H, CH₂-CH₃, CH₃-H-3'), 1.50 (d, J=5.4 Hz, 3H, CH-CH₃), 1.04 (t, J=7.5 Hz, 3H, CH₂-CH₃).

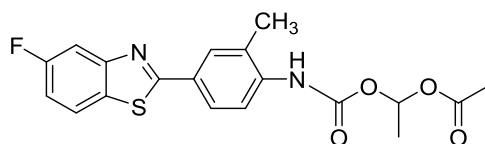
¹³C NMR (DMSO-d₆): δ 172.10, 169.74, 160.27, 154.47, 151.99, 139.27, 131.86, 130.19, 129.14, 128.92, 125.40, 123.80, 114.04, 108.89, 108.56, 89.15, 26.78, 19.74, 17.85, 8.74.

Mp: 138-141 °C.

m/z: HRMS (TOF ES⁺) C₂₀H₂₀FN₂O₄S⁺ [MH]⁺ calculated 403.1123; found: 403.2527.

HPLC t_R= 22.73 min.

1-(((4-(5-Fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)carbamoyl)oxy)ethyl acetate (76c)



Synthesis of **76c** was performed according to the general procedure, using 5F 203 (0.25 g, 0.97 mmol),

pyridine (0.16 mL, 1.94 mmol), 1-chloroethyl chloroformate (0.13 mL, 1.17 mmol), CH₂Cl₂ (6 mL), Ag₂O (0.18 g, 0.78 mmol), acetic acid (1.30 mL, 22.75 mmol). A white solid was afforded (0.08 g, 0.21 mmol, 32% yield)

¹H NMR (DMSO-d₆): δ 9.41 (s, 1H, NH), 8.18 (dd, J=5.5, 8.9 Hz, 1H, H-7), 7.94 (d, J=2.2 Hz, 1H, H-4), 7.89 (td, J=2.0, 9.2 Hz, 2H, H-2', H-6'), 7.66 (d, J=8.1 Hz, 1H, H-5'), 7.36 (td, J=2.4, 9.1 Hz, 1H, H-6), 6.80 (q,

$J=5.3$ Hz, 1H, $\text{CH}-\text{CH}_3$), 2.33 (s, 3H, $\text{CH}_3\text{-H-3'}$), 2.07 (s, 3H, $\text{CO}-\text{CH}_3$), 1.48 (d, $J=5.4$ Hz, 3H, $\text{CH}-\text{CH}_3$).

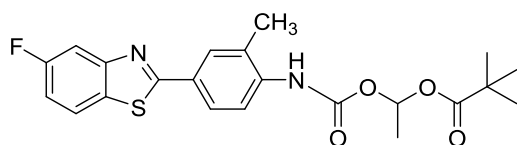
^{13}C NMR (DMSO- d_6): δ 169.65, 168.70, 162.60, 160.21, 154.46, 151.93, 139.17, 131.75, 130.21, 129.10, 128.33, 125.33, 123.58, 113.72, 108.59, 89.08, 20.69, 19.62, 17.70.

Mp: 144-146 °C.

m/z: HRMS (TOF ES^+) $\text{C}_{19}\text{H}_{18}\text{FN}_2\text{O}_4\text{S}^+$ $[\text{MH}]^+$ calculated 389.0966; found 389.2380.

HPLC t_R = 21.99 min

1-(((4-(5-Fluorobenzo[d]thiazol-2-yl)-2-methylphenyl)carbamoyl)oxy)ethyl pivalate (76d)



Synthesis of **76d** was performed according to the general procedure, using 5F 203 (0.15 g, 0.58 mmol),

pyridine (0.09 mL, 1.16 mmol), 1-chloroethyl chloroformate (0.08 mL, 0.07 mmol), CH_2Cl_2 (4.5 mL), Ag_2O (0.13 g, 0.54 mmol), pivalic acid (1.84 g, 18.00 mmol). A white solid was afforded (0.05 g, 0.10 mmol, 23% yield)

^1H NMR (CDCl_3): δ 8.09 (d, $J=7.7$ Hz, 1H, H-6'), 7.94 (d, $J=1.9$ Hz, 1H, H-4), 7.87 (dd, $J=2.6, 8.7$ Hz, 1H, H-5'), 7.81 (dd, $J=4.9, 8.4$ Hz, 1H, H-7), 7.72 (dd, $J=2.3, 9.6$ Hz, 1H, H-2'), 7.14 (td, $J=2.9, 8.8$ Hz, 1H, H-6), 6.93 (q, $J=5.3$ Hz, 1H, $\text{CH}-\text{CH}_3$), 6.60 (s, 1H, NH), 2.36 (s, 3H, $\text{CH}_3\text{-H-3'}$), 1.56 (d, $J=2.7$ Hz, 3H, $\text{CH}-\text{CH}_3$), 1.27 (s, 9H, $\text{C}(\text{CH}_3)_3$).

^{13}C NMR (CDCl_3): δ 176.71, 170.34, 163.38, 160.97, 155.15, 155.10, 151.07, 138.68, 130.45, 129.50, 128.90, 126.93, 122.40, 114.00, 113.77, 109.43, 109.18, 90.04, 38.94, 27.08, 19.84, 17.75.

Mp: 132-135 °C.

m/z: HRMS (TOF ES^+) $\text{C}_{22}\text{H}_{24}\text{FN}_2\text{O}_4\text{S}^+$ $[\text{MH}]^+$ calculated 431.1435; found 431.1465.

HPLC t_R = 25.06 min

4.3 Results and Discussion

4.3.1 Design and synthesis of 5F 203 carbamate prodrugs

Carbamate prodrugs of 5F 203 were designed with the aim of creating potential self-assembling prodrugs of the anticancer drug candidate 5F 203 and improving the rate of conversion into the parent amine compared to the previous series of prodrugs developed (Chapter 3). As guided by the pioneering and successful work of Alexander et al.,⁶⁻⁸ development of esterase-sensitive carbamate prodrugs was undertaken, by synthesising acyloxyalkoxycarbonyl derivatives of 5F 203. Furthermore, a tert-butyl protected (Boc) 5F 203 carbamate conjugate was also synthesised, in order to compare the simple carbamate and the acyloxyalkoxycarbonyl prodrug approaches in terms of in vitro efficacy and self-assembling properties.

5F 203 was synthesised as previously reported in Chapter 3, section 3.3.1.1. Synthesis of the Boc-protected 5F 203 (**74**, Figure 7.4) derivative was carried out following a standard procedure reported for protection of amines,¹² using di-tert-butyl dicarbonate in THF, with a reaction time of 3 days. After an aqueous work up, the crude compound was purified by column chromatography using a mixture of EtOAc and petroleum ether 40-60 °C to afford the compound in 34% yield.

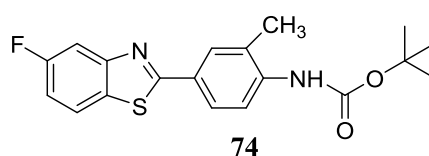
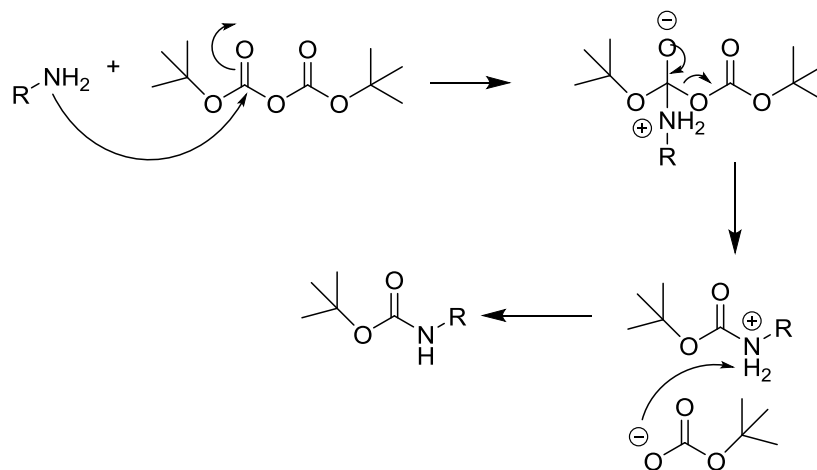


Figure 7.4: Molecular structure of Boc-5F 203 (**74**)

Boc protection occurs via a nucleophilic attack of the amine to a carbonyl group of the di-tert-butyl dicarbonate, followed by leaving of tert-butyl carbonate, formation of the tetrahedral intermediate and final conversion into the Boc-protected compound (Scheme 3.4).



Scheme 3.4: Mechanism of Boc protection of amines with formation of tert-butyl carbamate derivative.

Then, a series of acyloxyalkoxycarbonyl analogues of 5F 203 (**76a-76d**) was synthesised and the effect of substitution at the carbonyl moiety with different alkyl groups was evaluated (Figure 8.4).

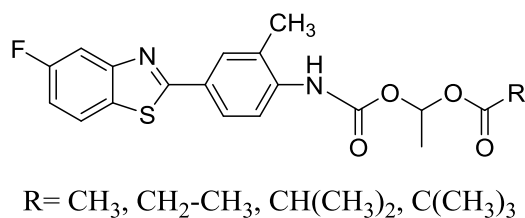


Figure 8.4: Acyloxyalkoxycarbonyl analogues of 5F 203 (**76a-76d**)

This series was synthesised in a two-step reaction: firstly, 5F 203 was acylated with 1-chloroethyl-chloroformate in presence of pyridine and using CH_2Cl_2 as solvent, to afford the intermediate 1-chloroethyl-5F 203 carbamate **75** (Figure 9.4). Then, reaction with the corresponding acids in the presence of silver oxide yielded the desired products **76a-76d**.

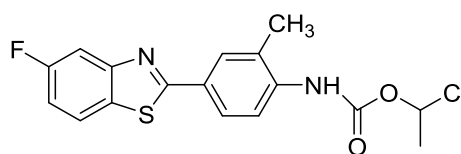
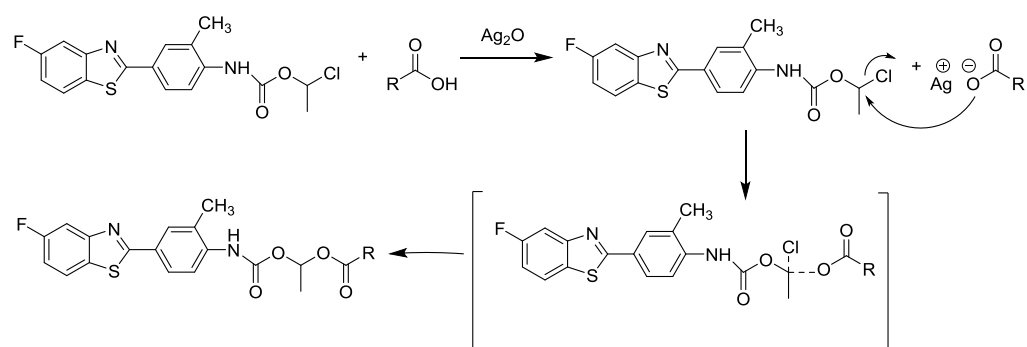


Figure 9.4: Molecular structure of 1-chloroethyl-5F 203 carbamate intermediate **75**.

Reaction of 5F 203 with 1-chloroethyl-chloroformate did not give full conversion into the carbamate intermediate **75**. This was thought to be related to the poor nucleophilicity of the terminal aniline of 5F 203, caused by the electron withdrawing effect of the phenyl-benzothiazole aromatic moiety. Therefore, different bases (i.e. sodium hydride, triethylamine and pyridine) and reaction times were tested for the synthesis of intermediate **75**; pyridine was found to be the most suitable base for this reaction, although full conversion was not obtained.

Analogues **76a-76d** were synthesised by reaction of the carbamate alkyl chloride intermediate **75** with excess of the corresponding acids, using silver(I) oxide (Ag_2O) to generate in situ salts of the carboxylate anions. This strategy is often used for difficult esterifications, in which alkyl halides react with carboxylate anions via silver ion coordination in an $\text{S}_{\text{N}}2$ reaction (Scheme 4.4). Products **76a-76d** were obtained after purification with column chromatography using a mixture of EtOAc and petroleum ether 40-60 °C with yields between 23% and 54%.



Scheme 4.4: Mechanism of $\text{S}_{\text{N}}2$ reaction between carbamate alkyl chloride intermediate **75** and carboxylate anions via silver ion coordination.

4.3.2 Preliminary gelation tests of 5F 203 carbamate prodrugs

Preliminary gelation screening on the synthesised carbamate derivatives of 5F 203 (**74** and **76a-76d**, 0.5% w/v) was carried out to determine their potential self-assembling properties using a solvent and temperature switch method (section 4.2.1.4). Binary mixtures of DMSO and water at different ratios were used as solvents to trigger gelation at solvent volume fractions ϕ_{DMSO} up to 0.4. Vial inversion tests determined formation of gels, metastable gels and precipitates (Table 1.4).

ϕ_{DMSO}	74	76a	76b	76c	76d
0.05	P	MG	G	P	P
0.1	P	MG	G	MG	P
0.2	P	MG	G	G	P
0.3	P	G	G	MG	P
0.4	P	G	G	MG	P

Table 1.4: Data table of vial inversion tests in DMSO/H₂O mixtures with ϕ_{DMSO} from 0.05 to 0.4, where G= gel, MG= metastable gel and P= precipitate.

These preliminary tests showed that three of the acyloxyalkoxycarbonyl derivatives of 5F 203 (**76a-76c**) possessed gelation abilities, whereas the Boc-protected 5F 203 (**74**) samples precipitated in all the different solvent fractions of DMSO/water tested.

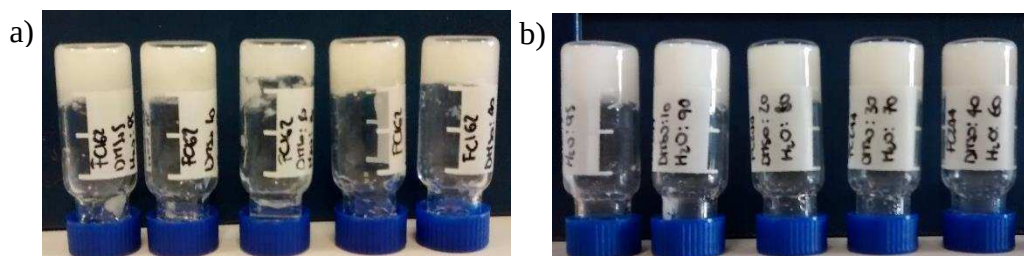


Figure 10.4: Pictures of vial inversion tests of 5F 203 carbamate derivatives **76a** (a) and **76b** (b) using a solvent-switch method (water/DMSO). Vials are presented from low solvent volume fraction ϕ_{DMSO} on the left (0.05) to higher DMSO concentrations ϕ_{DMSO} on the right (0.4).

In particular, the carbamate derivative **76d**, possessing a pivalic group at the carbonyl moiety, did not gelate any of the solvent fractions tested. Compound **76a**, bearing an isobutyryl chain, showed gelation at ϕ_{DMSO} 0.3 and 0.4, whereas it formed metastable gels (which precipitated within the first 2 h) at ϕ_{DMSO} from 0.05 to 0.2 (Figure 10a.4). The analogue **76b**, having an ethyl moiety, was found to be a gelator for each solvent volume fraction (Figure 10b.4). Compound **76c** formed a gel at ϕ_{DMSO} 0.2, whilst it formed metastable gels or precipitates in the other ratios evaluated, with precipitation occurring after 2 h from the gelation tests.

From these preliminary results, a correlation between self-assembly and the predicted 1-octanol/water partition coefficients of compounds (ClogP, calculated with ChemBioDraw Software 2014) was also revealed (Table 2.4).

Compound	ClogP	Gelation
76d	5.21	✗
74	5.14	✗
76a	4.81	✓
76b	4.50	✓
76c	3.97	✓



Table 2.4: ClogP values calculated for 5F 203 carbamate derivatives (**74** and **76a-76d**) and the corresponding gelation results after vial inversion tests. The arrow indicates that gelation occurs at decreasing ClogP values.

In particular, gelation occurred for compounds having ClogP values between 4.81 and 3.97, whereas carbamate derivatives **74** and **76d**, having ClogP values of 5.14 and 5.21 respectively, showed precipitation at all the ratios tested.

$\log P$ (Equation 2.4) measures the distribution of a substance between two partially miscible solvents (1-octanol/water) which are in intimate contact:

$$\log P = \log \left(\frac{[X_{C_8H_{18}O}^{organic}]}{[X_{H_2O}^{aqueous}]} \right)$$

Equation 2.4: Partition coefficient $\log P$

with X being the mole fraction of the substance in each phase.

The role that $\log P$ plays in the self-assembly has been recently studied by Lan and co-workers: they calculated the correlation between solubility parameters and gelation abilities of the known gelator molecule 1,3:2,4-dibenzylidene sorbitol (DBS). Results demonstrated that there is not a clear relationship between gelation and $\log P$ values, as self-assembly of DBS occurred in a wide range of $\log P$ values considered ($-2.5 < \log P < 10$). However, they found that gel opacity was related to $\log P$ values, with formation of clear gels in low-polarity solvents and opaque gels in high-polarity solvents.¹³

In a review article correlating gelation with solvent parameters, the same authors stated that $\log P$ cannot define specific intermolecular interactions, therefore this value cannot be used to predict a priori suitable solvents that will be gelled by specific gelators.¹⁴

Overall, since $\log P$ is related to the lipophilicity of a compound,¹⁴ it was hypothesised that the results showing gelation at decreasing ClogP values (Table 2.4) indicated that highly lipophilic compounds (i.e. **74** and **76d**) are not suitable for self-assembling and that more amphiphilic structures are required for gelation. Therefore, carbamate derivatives **76a-76c** possessed the adequate balance between lipophilicity and hydrophilicity to promote the self-assembly. Moreover, compared to the other carbamate derivatives, Boc-protected 5F 203 **74** does not possess the ester bond that characterises the acyloxyalkoxycarbonyl analogues, therefore this can reduce the number of hydrogen bond acceptor groups necessary for hydrogen bond interactions.

4.3.3 Probing the self-assembling properties of 5F 203 carbamate prodrugs

Carbamate derivatives that formed visually stable gels in the vial inversion tests (**76a**, **76b** and **76c**) were analysed by of rheology, electron microscopy and ^1H -NMR spectroscopy, in order to evaluate their self-assembling properties and confirm their LMWG nature.

4.3.3.1 Dynamic rheological studies

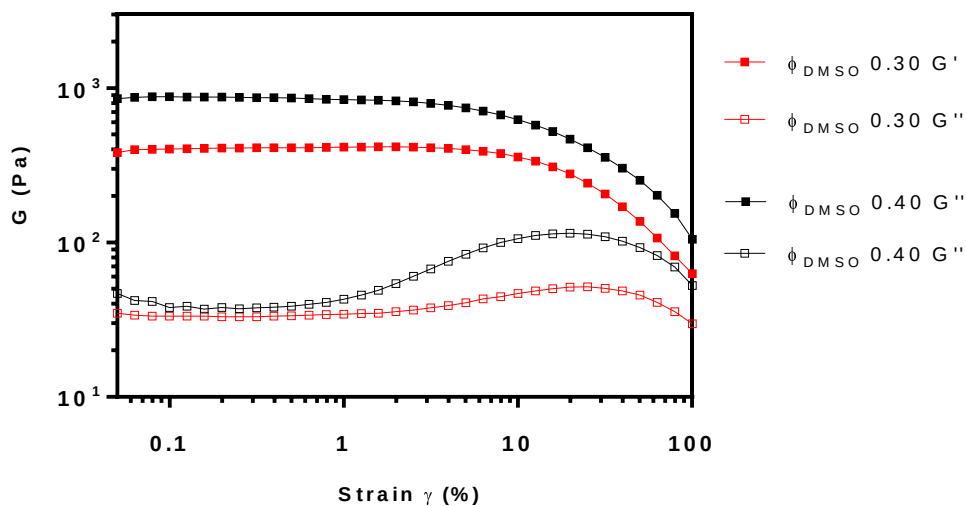
The mechanical properties of compounds **76a**, **76b** and **76c** were studied to confirm the preliminary vial inversion results and to identify, in terms of gelators' mechanical strength, the optimum ratio of DMSO to water (ϕ_{DMSO}) able to produce stable and stiff gels. Indeed, there have been many reports in the literature defining the correlation between ϕ_{DMSO} and the mechanical properties of LMWGs. In particular, Adams and co-workers demonstrated that in the case of Fmoc-Phe-Phe (Fmoc-FF) and Fmoc-Leu-Gly (Fmoc-LG) gels formed via a solvent-switch method, the ϕ_{DMSO} correlated with gels stiffness. For Fmoc-LG, strong gels ($G' \sim 10^4$ Pa) were formed at ϕ_{DMSO} lower than 0.25, whereas between ϕ_{DMSO} of 0.25 and 0.50 G' decreased. Above ϕ_{DMSO} of 0.5 no gels were formed.¹⁵ In the case of Fmoc-FF, an increase of G' was observed with increasing ϕ_{DMSO} (from ϕ_{DMSO} of 0.05 to 0.4).¹⁶ Skilling et al., describing the mechanical properties of a series of cytidine-based LMWGs, reported a similar trend: G' values increased with increasing ϕ_{SOL} from 0.1 to 0.4; above this value, less stiff gels were formed.¹⁷

As described in the reports above, oscillatory rheological studies performed on the gels of compounds **76a** and **76b** confirmed the correlation between the DMSO volume fractions and the mechanical properties.

Analyses of analogue **76a** were carried out on samples (0.5% w/v) with ϕ_{DMSO} of 0.3 and 0.4, solvent fractions that gave the most stable gels in the preliminary vial inversion tests. Initial analyses showed that for both ratios, amplitude and frequency sweeps revealed G' values higher than G'' values of at least one order of magnitude, confirming the LMWG nature of this

analogue (Figure 11.4). Strain amplitude measurements, carried out using variable strain ($\gamma = 0.05\text{--}100\%$) and constant frequency ($\omega = 10\text{ rad/s}$), showed an increase of G'' value at increasing deformation, revealing formation of a broad peak at higher strains, more evident for the gels with ϕ_{DMSO} of 0.4 (Figure 11a.4).

a)



b)

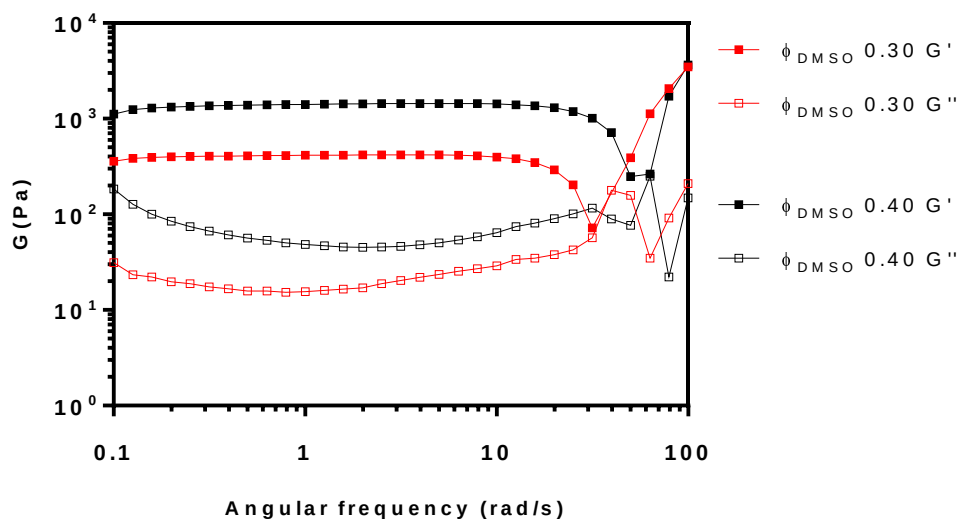


Figure 11.4: a) Oscillatory amplitude sweeps of **76a** with ϕ_{DMSO} of 0.3 and 0.4, $n = 3$, at 37°C , $\gamma = 0.05\text{--}100\%$ and $\omega = 10\text{ rad/s}$. Standard deviations $< 25\%$. b) Frequency sweeps of **76a** with ϕ_{DMSO} of 0.3 and 0.4, $n = 3$ at 37°C , $\gamma = 0.5\%$ and $\omega = 0.1\text{--}100\text{ rad/s}$. Standard deviation $< 18\%$.

This phenomenon, referred as “G” peak”, is related to an increase of deformation energy dissipated, due to an increase motion of the molecules which are not anymore belonging to the gel network, as a result of the higher strains. The presence of a broad G’ peak, along with the tendency of the storage modulus to leave the linear viscoelastic region, explain the formation of an initial cross-linked network, which then resulted into micro-cracks with increasing strains.¹⁸

Frequency sweeps were performed under a strain of 0.5%, and showed a frequency independence of G’ values up to 25.1 rad/s (ϕ_{DMSO} of 0.3) and 39.8 rad/s (ϕ_{DMSO} of 0.4) respectively. G’ values displayed slight frequency dependence in the same range considered. With increasing frequencies, breakdown of the gel network was observed. (Figure 11b.4).¹⁹

In terms of correlation between ϕ_{DMSO} and gels’ stiffness, a comparison of G’ values of **76a** in the LVR at ϕ_{DMSO} of 0.3 and 0.4 showed an increase of this parameter from ~ 400 Pa to ~ 1400 Pa respectively, thus confirming the behaviour previously reported, with increasing G’ at higher solvent fractions of DMSO.

In the case of compound **76b**, which was tested at ϕ_{DMSO} from 0.1 to 0.3, a similar trend was observed (Figure 12.4). Despite the fact that gels with ϕ_{DMSO} of 0.05 and 0.4 displayed stability in vial inversion tests, their preparation in a larger scale for rheology measurements was challenging and resulted in gels’ precipitation. Therefore, only gels with ϕ_{DMSO} of 0.1-0.3 were analysed. When G’ values of **76b** at ϕ_{DMSO} from 0.1 to 0.3 were calculated within the linear viscoelastic region, an increase from ~ 990 Pa to ~ 1360 Pa was observed. Gels with ϕ_{DMSO} of 0.2 and 0.3 exhibited similar values. This trend, although calculated only on the basis of three gels, showed a relationship between gel strength and ratio of DMSO to water.

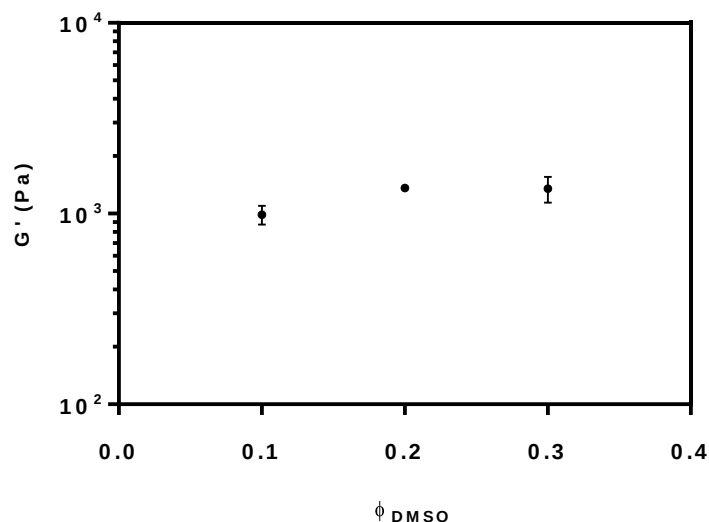


Figure 12.4: G' values of **76b** (0.5% w/v) at different ϕ_{DMSO} at 0.5% strain i.e. in the LVR. All measurements were carried out in triplicate and the mean calculated. Error bars represent standard deviation.

Analyses of oscillatory rheological studies of analogue **76b**, carried out on samples (0.5% w/v) with ϕ_{DMSO} from 0.1 to 0.3 (Figure 13.4), showed that, for all the ratios tested, the elastic behaviour dominated the viscous one in both amplitude and frequency sweeps, i.e. G' values were higher than G'' values of at least one order of magnitude, a characteristic of supramolecular gels.²⁰ However, differences were observed depending on the ϕ_{DMSO} analysed. Amplitude sweep results showed that the sample with ϕ_{DMSO} of 0.1 possessed a longer linear viscoelastic region compared to the other two ratios tested (ϕ_{DMSO} of 0.2 and 0.3), meaning that the transition from gel to sol state (i.e. breakdown of the gel network) was reached at higher strains. Furthermore, an increase of G'' values was observed as a function of applied stress, with formation of G'' curves. Such observations were more evident for samples with ϕ_{DMSO} of 0.2 and 0.3, in which micro-cracks and then macro-cracks occurred.¹⁸ Samples with ϕ_{DMSO} of 0.2 almost reached a crossover point ($G'=G''$) (Figure 13a.4), showing a final large crack at high strains, also visually confirmed.

Frequency sweep studies, carried out at a constant strain of 0.5%, revealed similar behaviour for all the samples tested (Figures 13b.4): G' values showed a linear response by varying the frequency up to 39.8 rad/s; G''

values displayed a slight frequency dependence in all the range of frequencies tested ($\omega = 0.1$ -100 rad/s).

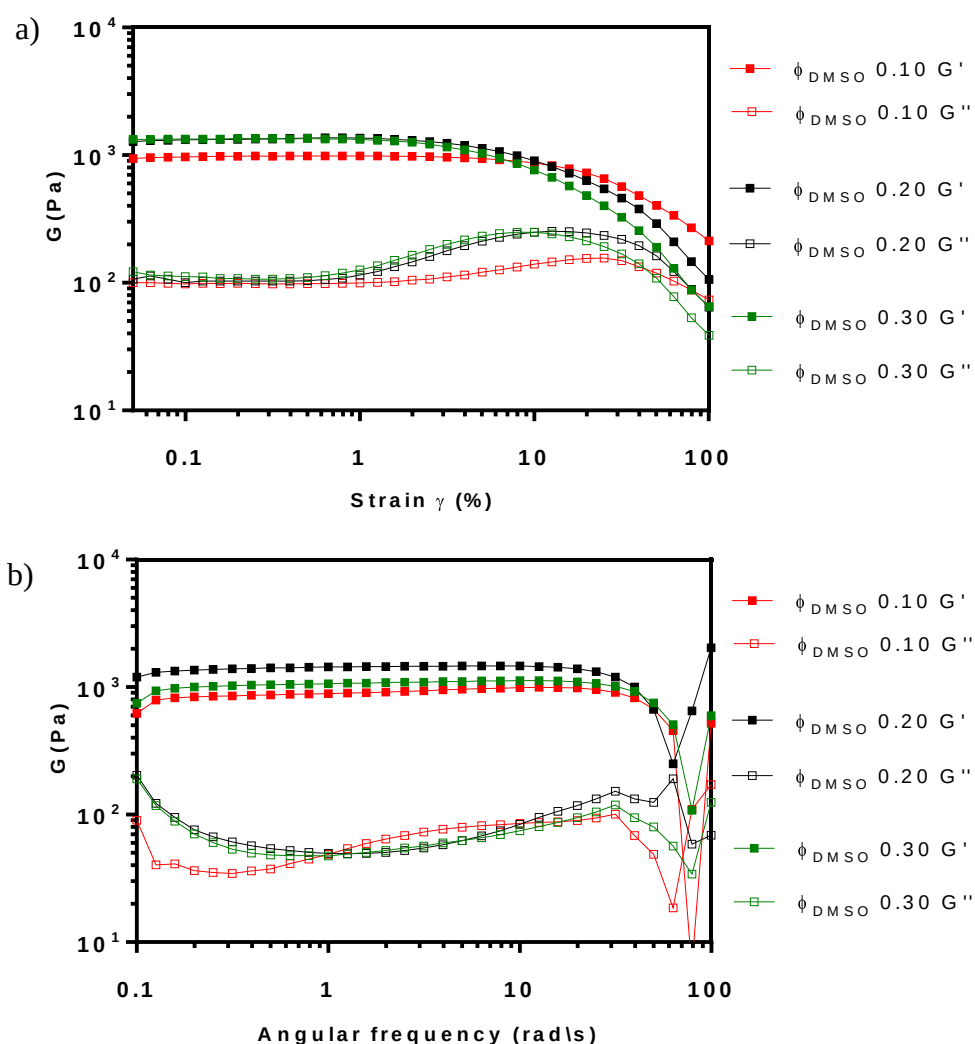


Figure 13.4: a) Oscillatory amplitude sweeps of **76b** with ϕ_{DMSO} of 0.1, 0.2 and 0.3, $n = 3$, at 37 °C, $\gamma = 0.05$ –100 % and $\omega = 10$ rad/s. Standard deviations <16 %. b) Frequency sweeps of **76b** with ϕ_{DMSO} of 0.1, 0.2 and 0.3, $n = 3$ at 37 °C, $\gamma = 0.5$ % and $\omega = 0.1$ -100 rad/s. Standard deviations < 30 %.

Oscillatory rheological measurements performed on the sample of **76c** at ϕ_{DMSO} of 0.2, that showed gelation properties in the preliminary vial inversion tests, displayed G' values higher than G'' values of one order of magnitude, indicating the presence of an elastic rather than a viscous material (Figure 14.4).²¹ Moreover, G' and G'' were found to be frequency independent up to 25.1 rad/s. Compared to the previous analogues analysed, amplitude sweeps did not reveal a G'' curve, and G' values were found to be

the lowest amongst all the series ($G' = 350$ Pa), meaning that gels of **76c** had the weakest gel network.

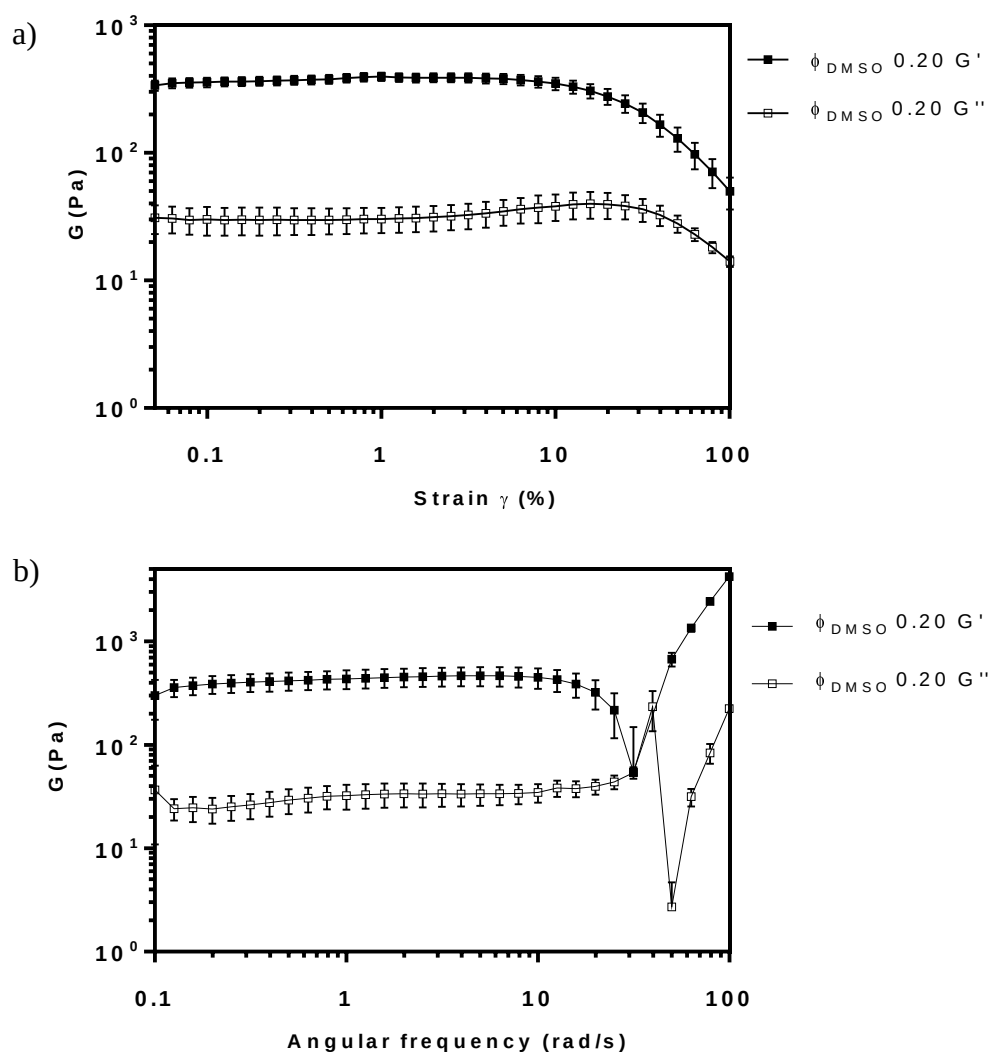


Figure 14.4: a) Oscillatory amplitude of **76c** (0.5% w/v) with ϕ_{DMSO} of 0.2 at 37°C, $n=3$, $\gamma = 0.05$ –100 % and $\omega = 10$ rad/s. b) Frequency sweeps **76c** (0.5% w/v) with ϕ_{DMSO} of 0.2 at 37°C, $n=3$, $\gamma = 0.5$ % and $\omega = 0.1$ –100 rad/s. Error bars represent standard deviation.

Overall, dynamic rheological studies confirmed the gelator nature of the analogues **76a**, **76b** and **76c** and showed that the ϕ_{DMSO} can affect the mechanical strength of the gels.

4.3.3.2 Electron microscopy analyses

In order to acquire more information about self-assembly, the microscopic morphology of analogues **76a**, **76b** and **76c** was analysed by TEM.

TEM micrographs of gels of **76a** at ϕ_{DMSO} of 0.3 and 0.4 showed the presence of an entangled and dense network of fibres, with no differences between the two ratios tested, in terms of fibres' length and width. Specifically, both samples displayed a branched pattern of fibres, with formation of thick aggregates with variable widths between 1100 nm and 400 nm and lengths from 8 μm to 32 μm (Figure 15.4). The different concentrations of DMSO of the samples did not affect the morphology of the fibres and the dimensions of the aggregates.

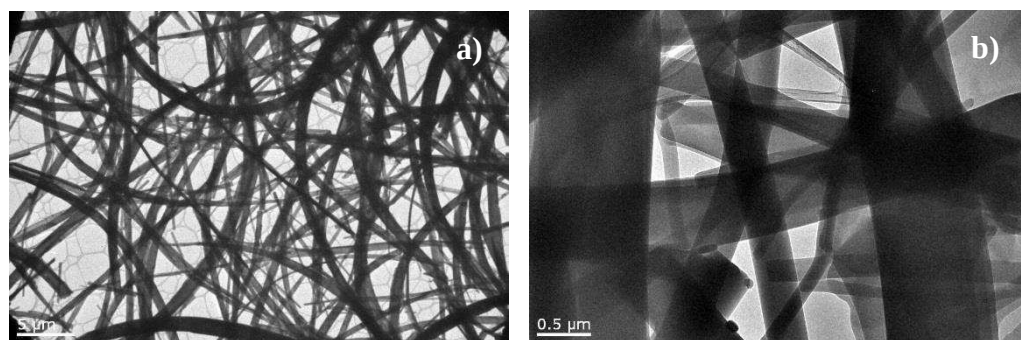


Figure 15.4: TEM micrographs of gels of **76a** (0.5% w/v) at a) ϕ_{DMSO} of 0.3 (scale bar 5 μm) and b) ϕ_{DMSO} of 0.4 (scale bar 0.5 μm).

TEM micrographs of gels of **76b** at ϕ_{DMSO} between 0.1 and 0.3 again reveal formation of networks of fibres, but less dense and thick compared to the previous analogue examined. The sample with ϕ_{DMSO} of 0.3 demonstrated the most similar morphology compared to compound **76a**, with lengths between 5 μm and 10 μm and widths of 280-670 nm (Figure 16a.4). When the concentration of DMSO was decreased to ϕ_{DMSO} of 0.2 and 0.1, the shape of the fibres slightly changed, with formation of inner cavities particularly for the gel with least amount of organic solvent. Aggregates lengths and widths reduced, with fibres 45-200 nm wide and 2-5 μm long (Figure 16b.4). The presence of thinner and dense fibres was also in agreement with rheological analysis, according to which gels with less volume fraction of DMSO had G' values lower than the ones with ϕ_{DMSO} of 0.2 and 0.3,

confirming the contribution that the organic solvent gives to gelation in terms of gel strength.²² Furthermore, **76a** possesses one more methyl group in the alkyl chain of the carbonyl moiety compared to the analogue **76b**; this minor change in the molecular structure could confer to gels of **76a** a higher tendency to aggregate into thicker fibres, due to increased van der Waals interactions, promoted by the additional methyl group. Indeed, a correlation between fibres' morphology and chain length of carbamate gelators has already been reported.¹

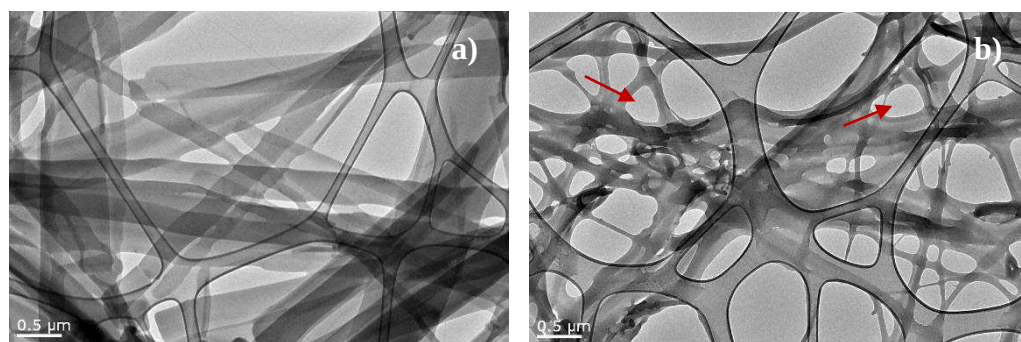


Figure 16.4: TEM micrographs of gels of **76b** (0.5% w/v) at a) ϕ_{DMSO} of 0.3 and b) ϕ_{DMSO} of 0.1. Red arrows indicate formation of inner cavities.

Gels of **76c** at ϕ_{DMSO} of 0.2 showed the presence of an entangled network, formed by shorter and thinner aggregates compared to the previous analogues analysed. Observations also suggested that the gel matrix contained microscopic inner cavities (Figure 17a.4), as already found for gel of **76b** at ϕ_{DMSO} of 0.1. A slight change in the morphology of the aggregates was also observed, with formation of ribbon-like aggregates 40 to 80 nm in width and 0.7 to 1.7 μm in length (Figure 17b.4). As previously hypothesised, the change in fibre morphology and network density could be attributed to the lack of a methyl group in the molecular structure of **76c** compared to analogues **76a** and **76b**; moreover, formation of cavities could be attributed to low ϕ_{DMSO} , as observed for both gels of analogues **76b** and **76c** with ϕ_{DMSO} of 0.1 and 0.2 respectively.

The effect that ϕ_{SOL} has on the morphologies of aggregates has been already described by Cui and his group: they demonstrated the different self-assembling behaviour of folic acid molecules into binary mixtures of methanol and water at different ratios, showing formation of filamentous

nanostructures at high solvent concentrations and lozenge-shaped platelets at low solvent fractions.²³

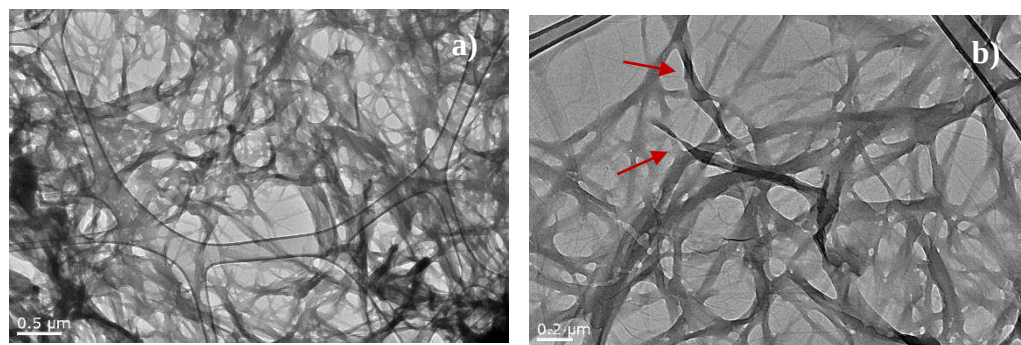


Figure 17.4: TEM micrographs of gels of **76c** (0.5% w/v) at ϕ_{DMSO} of 0.2 indicating a) formation of inner cavities and b) ribbon-like shape of the aggregates (red arrows).

4.3.3.3 Temperature-dependent ^1H -NMR spectroscopy studies

^1H -NMR spectroscopy was used to understand the role of 5F 203 carbamate analogues' functional groups involved in the gelation. As shown from ^1H -NMR and fluorescence spectroscopy studies described in Chapter 3 on 5F 203 succinic acid conjugate (**68a**), the π - π stacking of the phenyl-benzothiazole ring represents the main source of non-covalent interactions that promotes gelation. The acyloxyalkoxycarbonyl derivatives of 5F 203 possess two additional functional groups, i.e. a carbamate and an ester group, both of them able to act as hydrogen-bond donors and acceptors. In addition, as suggested by preliminary gelation tests and electron microscopy studies, the substitution of the carbonyl group with different alkyl moieties could affect the self-assembly, due to the presence of van der Waals interactions.

Temperature-dependent ^1H -NMR studies represent a valid approach for the determination of the gel-sol transition state for gels obtained via a temperature-switch method. Indeed, in the gel state at low temperatures, the functional groups that participate in gelation are immobilised within the solid-like gel network because of the strong intermolecular interactions, with consequent broadening or disappearing of the NMR signal. On the contrary, groups that are not involved in the self-assembly give sharp peaks, as a consequence of their mobility in solution. An increase in temperature results in gel-sol transition, due to an

enhancement of molecules' motion, and subsequent appearance of signals from the groups involved in the self-assembly.²⁰ Several studies reported variation temperature ¹H-NMR analyses to confirm the gelator nature of some compounds and prove the gel-sol transition. For example, Song and co-workers performed temperature-dependent ¹H-NMR measurements on an alkyloxylobenzoyl hydrazine derivative in toluene-d₈ and benzene-d₆ to investigate the role of non-covalent interactions in self-assembly. They found that the downfield shift of NH of the amide bond at high temperatures clearly indicates formation of an intermolecular hydrogen bonding between amide groups.²⁴

Temperature-dependent ¹H-NMR studies were performed on gels (0.5% w/v) of analogues **76a**, **76b** and **76c**, in particular on the samples with volume fractions $\phi_{\text{DMSO-d}_6}$ of 0.3 for **76a** and **76b** and 0.2 for **76c**. It was decided to select these ratios of DMSO-d₆ in D₂O to compare the effect of the same solvent concentration on the gels of **76a** and **76b**. In the case of **76c**, $\phi_{\text{DMSO-d}_6}$ of 0.2 was the only volume fraction in which the analogue displayed gelation properties. Samples of the gels were previously prepared and cooled down in suitable NMR tubes. Then, ¹H-NMR studies were carried out on the gel samples at 25 °C and the temperature was slowly increased at specific points (i.e. 45 °C, 65 °C and 75 °C). Results provided qualitative analyses, giving information on the functional groups involved in the gelation as a function of the temperature.

¹H-NMR spectra of gels of **76a** showed that at 25 °C the signals from the protons of the aliphatic region were sharper compared to the ones of the aromatic region, belonging to phenyl-benzothiazole ring (Figure 18.4). The sharp signals of protons H_a, H_b H_d and H_e in the aliphatic region revealed that these groups were not immobilised in the gel matrix; therefore, their contribution to gelation was negligible. On the contrary, the broadening of the signals of the aromatic protons confirmed the role of π - π stacking in the self-assembling process.

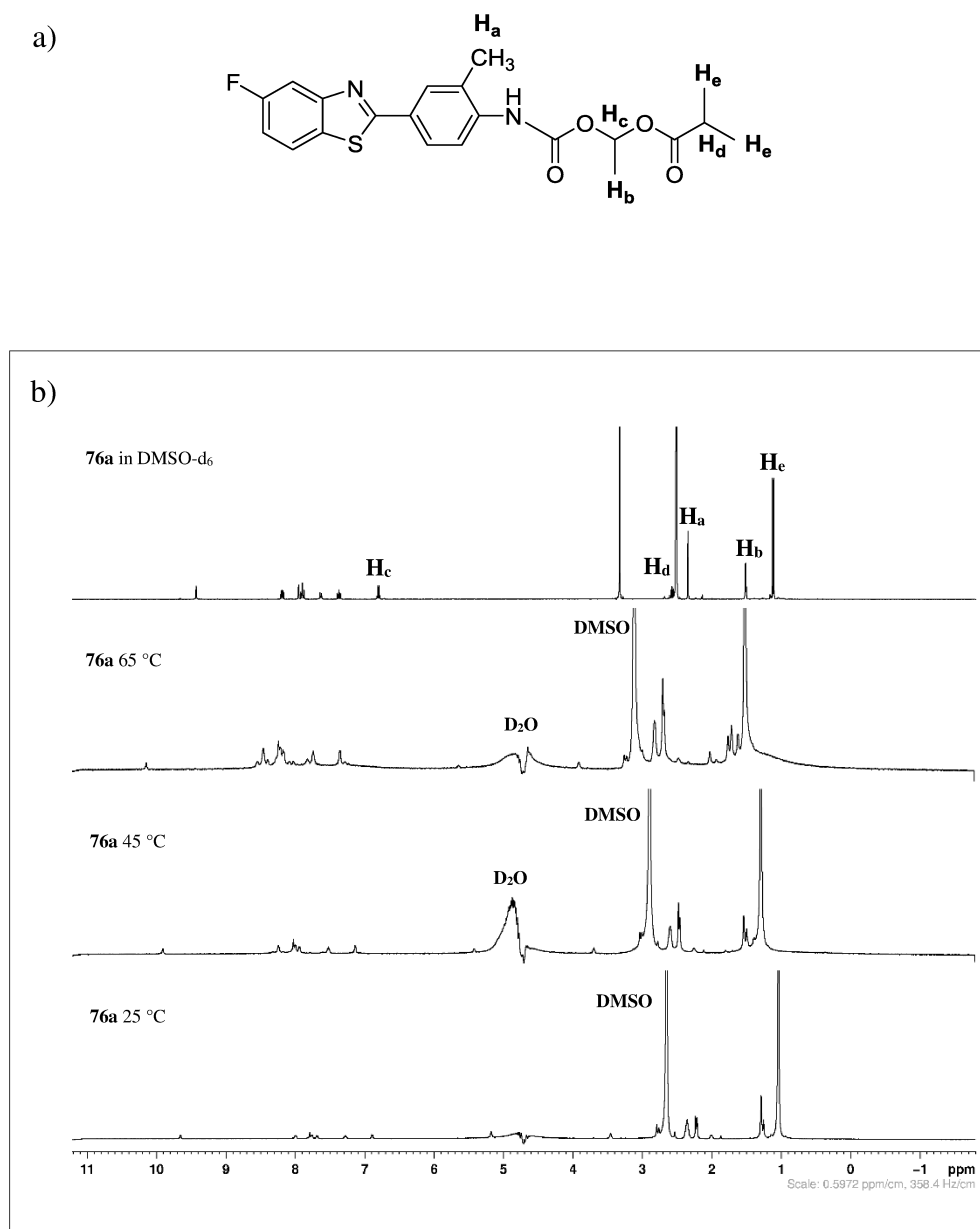


Figure 18.4: a) Molecular structure of **76a**. b) ^1H -NMR spectra of gel of **76a** (0.5% w/v, $\phi_{\text{DMSO-d}_6}$ of 0.3) recorded at different temperatures. The spectrum on the top was used as reference and represents the compound in DMSO-d_6 .

At increasing temperatures, due to increased solubility and breaking of the gel network, the intensities of signals from the aromatic region increased, together with the signal from the NH of the carbamate bond, confirming the role of hydrogen bonds in the self-assembly. The signal of proton H_c did not appear even at high temperatures. Experiments were carried out up to 65 °C, as at higher temperatures the gel collapsed forming non-uniform aggregates which hampered the final analyses.

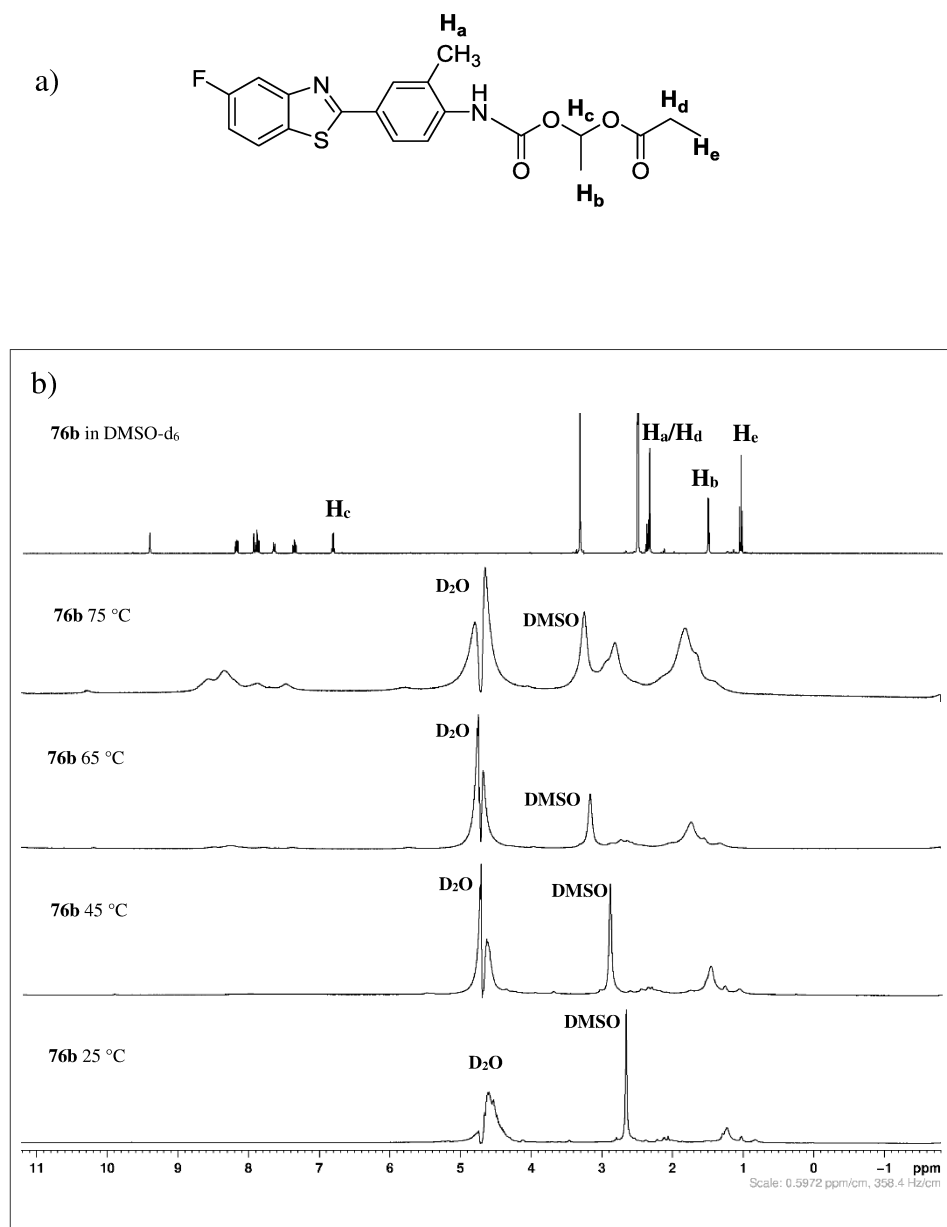


Figure 19.4: a) Molecular structure of **76b**. b) ^1H -NMR spectra of gel of **76b** (0.5% w/v, $\phi_{\text{DMSO-d}_6}$ of 0.3) recorded at different temperatures. The spectrum on the top was used as reference and represents the compound in DMSO-d_6 .

^1H -NMR spectra of gels of **76b** at 25 °C showed broad signals of the protons of the aliphatic region, whereas those of the aromatic region did not produce any signal, as a result of the aggregated state of the gel (Figure 19.4). By increasing the temperature, the signals of aromatic protons and of the NH of the carbamate bond appeared, although they were not sharp. Overall, compared to the previous analyses of gels of **76a**, signals were much broader and this phenomenon could be attributed to a greater stiffness of these gels. Again, the aliphatic protons

showed to belong to the liquid part of the gel, with signals of aliphatic protons H_e and H_b being more visible at 25 °C, whilst protons H_a and H_d appeared at higher temperatures, suggesting a reduced motility and a minor contribution to the gel network.

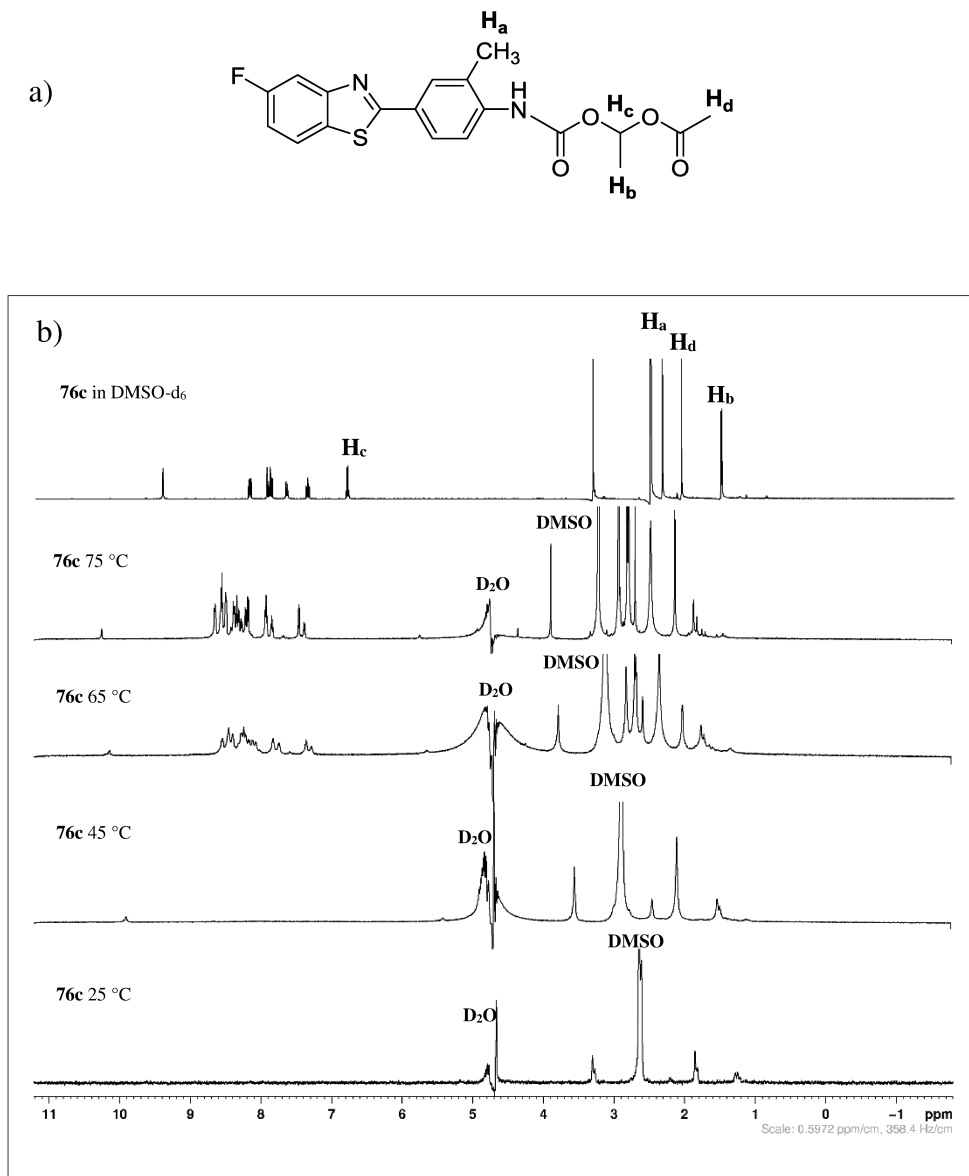


Figure 20.4: a) Molecular structure of **76c**. b) ^1H -NMR spectra of gel of **76c** (0.5% w/v, $\phi_{\text{DMSO-d}_6}$ of 0.2) recorded at different temperatures. The spectrum on the top was used as reference and represents the compound in DMSO-d_6 .

^1H -NMR spectra of gels of **76c** at 25 °C displayed a pattern similar to analogue **76b**, with no detection of protons in the aromatic region and the presence of protons H_a , H_b and H_d in the aliphatic region (Figure 20.4). At 45 °C, the signal of the NH of the carbamate bond became evident. Protons in the aromatic region appeared at 65 °C; at 75 °C the gel network was shown to be completely broken,

showing all the signals corresponding to the original compound in DMSO-d₆. However, detection of new peaks not attributable to **76c** was also unveiled. In particular, due to the presence of these signals in both aliphatic and aromatic regions, it was hypothesised that a degradation product, derivative of 5F 203 carbamates, was formed at high temperatures. Subsequent ¹H-NMR analyses were carried out to elucidate the nature of these side products, although without any success.

Overall, ¹H-NMR studies on the analogues **76a**, **76b** and **76c** confirmed the findings disclosed in Chapter 3, revealing the contribution that the phenyl-benzothiazole ring conferred to gelation via non-covalent π - π stacking interactions. These analyses also proved the role of the carbamate bond in the self-assembling process via formation of hydrogen bonds. Substitution of the carbonyl group with different alkyl chains did not give evidence to any difference, in terms of self-assembling, between the three analogues: in all the spectra examined, the aliphatic protons displayed sharp peaks at all the temperatures tested (25 °C, 45 °C, 65 °C and 75 °C), meaning that these groups belong to the ‘liquid-like part’ of the gel matrix.

4.3.4 In vitro growth inhibitory activity of 5F 203 carbamate prodrugs

The in vitro activity of carbamate prodrugs **74** and **76a-76d** against human cancer cell lines was assessed, to determine whether the newly synthesised derivatives were able to maintain the growth inhibitory activity of the active drug 5F 203. As already described in Chapter 3, prodrugs were tested against MCF-7 breast and IGROV-1 and OVCAR-4 ovarian carcinoma cell lines. Moreover, to confirm the selectivity of these prodrugs against cancer cell lines, in vitro assays against MRC-5 normal human embryonic lung fibroblast cell line were also carried out.

4.3.4.1 Growth inhibitory effects on MCF-7 breast carcinoma cell line

5F 203 carbamate prodrugs retained activity against MCF-7 cancer cells at the concentrations tested (1 nM to 10 μ M) (Table 3.4). As already specified in the previous chapter, the GI₅₀ values of 5F 203 carbamate prodrugs reported in the following tables refer to the growth inhibitory effect of 5F 203 on cells, deriving from the hydrolysis of 5F 203 derivatives into the native drug.

Compound	GI ₅₀ mean $\pm$ SD (nM) in MCF-7
5F 203	38.82 $\pm$ 7.23
74	6111.93 $\pm$ 2038.13
76a	11.63 $\pm$ 5.22
76b	22.57 $\pm$ 13.56
76c	39.88 $\pm$ 9.04
76d	81.22 $\pm$ 64.90

Table 3.4: Growth inhibitory activity of 5F 203 and carbamate prodrugs in MCF-7 carcinoma cells. Cells were exposed to experimental agents for 72 h before viability was assessed by MTT assay (n = 4 per experiment; ≥ 3 independent trials were performed).

However, whereas the acyloxyalkoxycarbonyl analogues of 5F 203 **76a-76d** displayed growth inhibitory activity at nanomolar concentrations, with GI₅₀ values similar to those of the parent amine 5F 203 (GI₅₀ < 82 nM), the Boc-protected 5F 203 showed GI₅₀ values of 6.11 μ M. This difference in activity could be associated to a slower hydrolysis of 5F 203 from **74** compared to the acyloxyalkoxycarbonyl analogues. Indeed, the carbamate bond is more stable than the ester functionality and less susceptible to hydrolysis compared to the

acyloxyalkoxycarbonyl derivatives of amines, which have an enzymatically hydrolysable ester function that releases the parent amine in the presence of an esterase.²⁵ Therefore, these results proved the successful approach of acyloxyalkoxycarbonyl conjugates as prodrugs of primary amines. Indeed, in order to promote cell death, the prodrugs need to be hydrolysed into the parent amine to allow for formation of nitrenium ion intermediates and consequent DNA adducts.²⁶

The lower potency of the simple carbamate **74** could be a result of less intracellular 5F 203 availability, due to a slow hydrolysis of the parent drug from the prodrug.

A comparison of GI₅₀ values between those reported from 5F 203 amide prodrugs in Chapter 3, section 3.3.4.2, and those of the acyloxyalkoxycarbonyl derivatives **76a-76d** revealed a higher potency of the carbamate prodrugs, due to the faster hydrolysis of the ester bond compared to the amide group. Moreover, compounds **76a-76d** showed a dose-dependent growth inhibitory relationship in cancer cells (Figure 21.4).

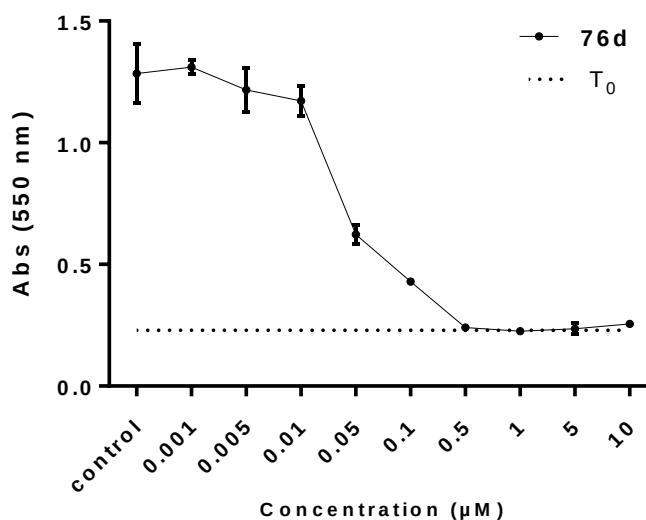


Figure 21.4: Example of dose-response profile of **76d** in the MCF-7 cell line at different concentrations (1 nM to 10 μM, n=4). T₀ represents the absorbance of non-treated cells at the time of drug addition.

4.3.4.2 Growth inhibitory effects on IGROV-1 and OVCAR-4 ovarian carcinoma cell lines

When IGROV-1 and OVCAR-4 cell lines were exposed to prodrugs **76a-76d**, a similar pattern to the previously reported amide prodrugs was observed, with growth inhibitory activity against IGROV-1 cells, whilst negligible activity was observed against OVCAR-4 cells. Results of MTT assays against IGROV-1 cells revealed GI_{50} values similar to those observed for MCF-7 (Table 4.4).

Compound	GI_{50} mean $\pm$ SD (nM) in IGROV-1
5F 203	138.24 $\pm$ 87.99
74	> 10000
76a	43.81 $\pm$ 8.07
76b	157.35 $\pm$ 137.31
76c	4261.08 $\pm$ 2445.07
76d	83.10 $\pm$ 18.97

Table 4.4: Growth inhibitory activity of 5F 203 and carbamate prodrugs in IGROV-1 carcinoma cells. Cells were exposed to experimental agents for 72 h before viability was assessed by MTT assay (n = 4 per experiment; ≥ 3 independent trials were performed).

Acyloxyalkoxycarbonyl derivatives **76a**, **76b** and **76d** displayed GI_{50} values <200 nM, similar to those of the parent amine 5F 203 (138.24 nM), confirming the faster rate of hydrolysis of these group of prodrugs compared to 5F 203 amide conjugates. Compound **76c** exhibited higher GI_{50} values in the micromolar range, i.e. 4.26 μ M. Boc-protected 5F 203 (**74**) did not show any growth inhibitory activity against this cell line at the maximum concentration tested (10 μ M). Again, this phenomenon could be related to slow hydrolysis of the carbamate bond. Also in this cell line as for MCF-7, dose-dependent inhibition of cancer cell growth was observed (Figure 22.4).

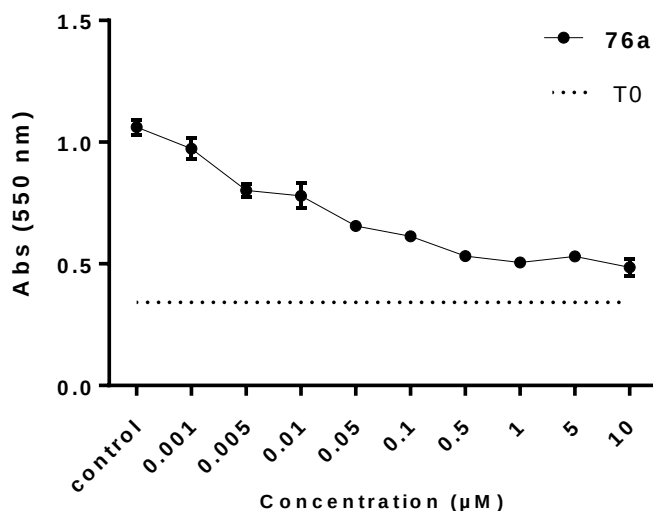


Figure 22.4: Example of dose-response profile of **76a** in the IGROV-1 cell line at different concentrations (1 nM to 10 μM, n=4). T₀ represents the absorbance of non-treated cells at the time of drug addition.

When the compounds were tested against the ovarian cell line OVCAR-4, none of them displayed growth inhibitory activity at the maximum concentrations tested (10 μM) (Table 5.4). As hypothesised in Chapter 3, section 3.3.4.3, the inactivity of carbamate prodrugs against OVCAR-4 may be attributed to absence of CYP1A1 inducibility. Also for these experiments, the same batch of cells was used and, as previously reported, minimal cytosolic AhR might be displayed, with consequent absence of signal transduction and lack of growth inhibitory properties. Another reason could be the lack of specific membrane transporters for these compounds. Indeed, amino acid prodrugs displayed growth inhibitory activity against OVCAR-4 due to the presence of amino acid membrane transporters which may have facilitated a more rapid intracellular uptake of these prodrugs.

4.3.4.3 Effects of prodrugs on MRC-5 normal lung fibroblast cell growth

MTT assays were performed against MRC-5 embryonic lung fibroblasts to assess the selectivity of 5F 203 carbamate prodrugs against cancer cell lines. None of derivatives **74** and **76a-76d** showed any growth inhibitory activity at

the maximum concentration tested (10 μ M), thus revealing selectivity against malignant cell lines (Table 5.4).

Compound	GI ₅₀ mean $\pm$ SD (nM) in OVCAR-4	GI ₅₀ mean $\pm$ SD (nM) in MRC-5
5F 203	>10000	>10000
74	>10000	>10000
76a	>10000	>10000
76b	>10000	>10000
76c	>10000	>10000
76d	>10000	>10000

Table 5.4: Growth inhibitory activity of 5F 203 and carbamate prodrugs in OVCAR-4 ovarian carcinoma cells lines and MRC-5 fibroblast normal cell lines. Cells were exposed to experimental agents for 72 h before viability was assessed by MTT assay (n = 4 per experiment; ≥ 3 independent trials were performed).

4.3.5 In vitro stability assays

After having demonstrated the self-assembling properties of acyloxyalkoxycarbonyl derivatives of 5F 203 and their notable and selective growth inhibitory properties against MCF-7 breast and IGROV-1 ovarian cancer cell lines, the next step towards the development of LMWG-based prodrugs for localised drug delivery consisted in the assessment of their in vitro stability properties in pH 7.4 phosphate buffer. Moreover, the susceptibility of prodrugs to convert into the parent drug by enzymatic cleavage was also evaluated in rat and human plasma.²⁷ Amongst all the acyloxyalkoxycarbonyl analogues of 5F 203, **76a** was selected as the candidate for these studies because of its remarkable in vitro activity displayed against cancer cell lines compared to the other derivatives examined.

Firstly, stability of **76a** in PBS at pH=7.4 was evaluated. A stock solution of **76a** was diluted in PBS and its degradation into the parent amine 5F 203 was analysed. Although initially soluble in the aqueous medium, the compound showed only sparing solubility over time, with a tendency to form aggregates visually detected. Stability tests, performed over a 24 h period, revealed degradation of **76a** into the active free amine, with a decrease of the initial concentration of **76a** (10 μ M) to 0.5 μ M and formation of 5F 203 (up to 0.8 μ M)

(Figure 23.4). At this pH no other side products were observed in the incubation buffer. Therefore, **76a** displayed degradation to the parent amine within the period tested, confirming the prodrug approach of acyloxyalkoxycarbonyl analogues of 5F 203. However, the final concentration of 5F 203 detected was low, and this could be explained by limited solubility of **76a** in the buffer and a consequent hydrolysis only of a small fraction of the prodrug.

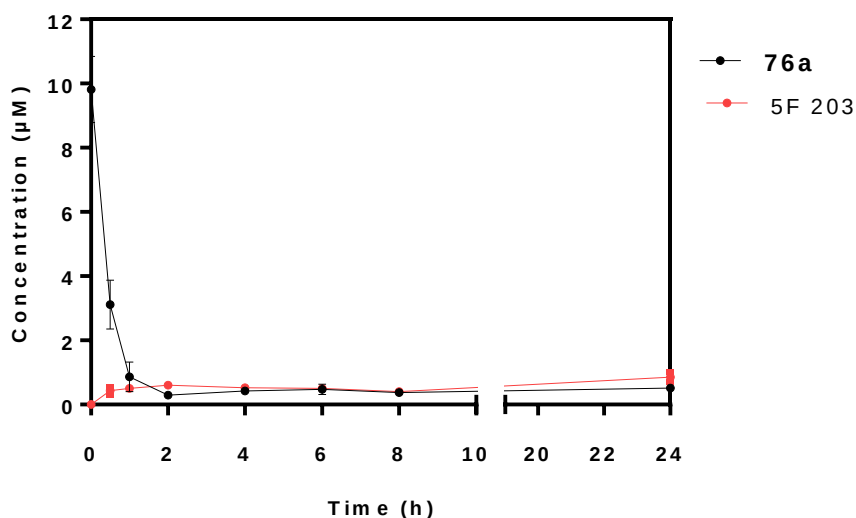


Figure 23.4: Concentrations of **76a** and 5F 203 versus time following incubation with PBS at pH 7.4 at 37 °C. All measurements were carried out in triplicate. Error bars represent standard deviation.

Prodrug **76a** was then incubated in rat plasma, to assess its susceptibility to conversion into the parent drug via enzymatic hydrolysis. Results showed a rapid and complete conversion of **76a** into 5F 203 within 3 h (Figure 24.4). Calculated half-life ($t_{1/2}$) of **76a** was 14.26 ± 0.09 min. Less than 10% of prodrug was found in the samples at 60 min; at 180 min concentration of **76a** was below the lower limit of quantification (50 nM). As the concentration of **76a** decreased, concentration of its converted active drug, 5F203, increased. No other metabolites of **76a** other than 5F 203 were detected. It is noteworthy that the conversion occurred immediately when **76a** came into contact with the rat plasma, as revealed by the samples at time 0 min that contained a small amount of 5F 203 (0.18 µM). However, when **76a** was incubated with denatured rat plasma containing denatured proteins, 5F 203 was not detected, meaning that the enzymes within plasma were involved in the hydrolysis of the prodrug into the parent amine.

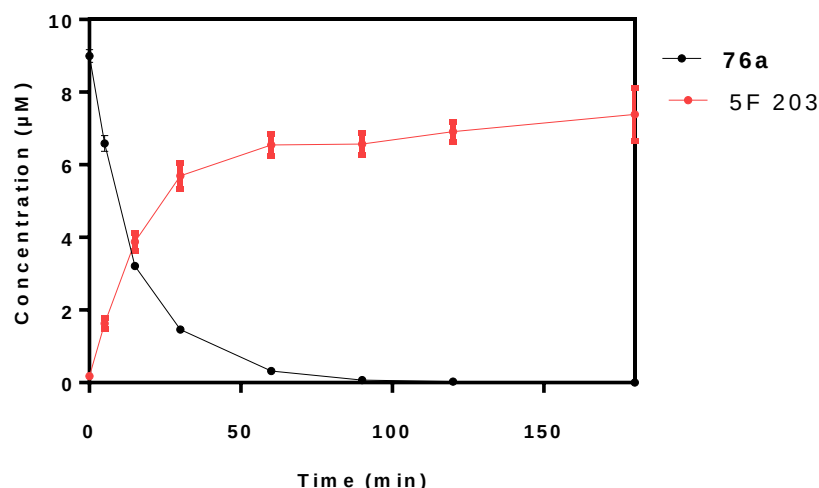


Figure 24.4: Concentrations of **76a** and 5F 203 versus time following incubation with rat plasma at 37 °C. All measurements were carried out in triplicate. Error bars represent standard deviation.

Prodrug **76a** was also incubated with human plasma to assess any difference with rat plasma, in terms of rate of conversion of **76a** into the active drug. As already displayed in rat plasma, **76a** showed complete conversion into 5F 203 also in human plasma. However, the conversion rate was slower and lower in human plasma and the half-life was 96.67 ± 6.86 min (Figure 25.4). Furthermore, disproportionate metabolites (i.e. metabolites identified only in human plasma or metabolites present at disproportionately higher levels in humans than in any other animal species) were not found after incubation with human plasma and 5F 203 was found to be the only metabolite, like in rat plasma.

The differences in conversion rate between rat and human plasma (Figure 26.4) could be ascribed to different hydrolase activities of the two incubation media, as described by Bahar and co-workers. In their study, they compared the hydrolase activity of some model drugs (i.e. Irinotecan, Oseltamivir, and Temocapril) in human and animal plasma finding that, overall, rat and mouse plasma possessed a higher hydrolase activity.²⁸ Therefore, this could explain the slower rate of conversion of **76a** into 5F 203 in human plasma.

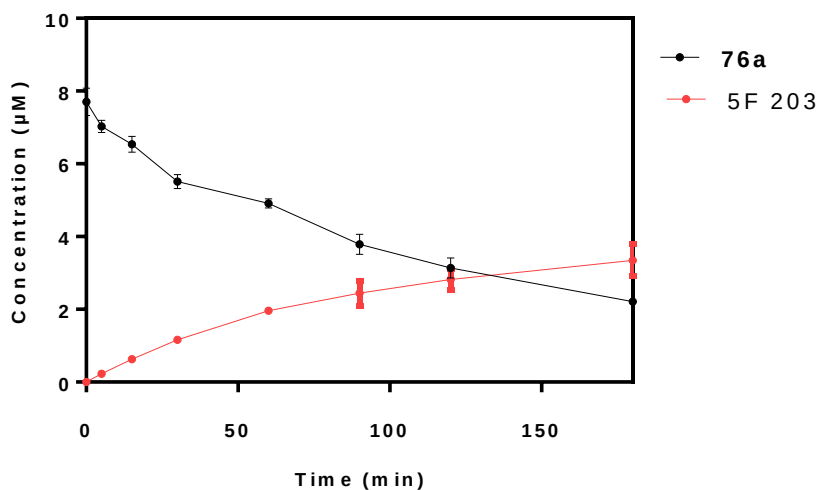


Figure 25.4: Concentrations of **76a** and 5F 203 versus time following incubation with human plasma at 37 °C. All measurements were carried out in triplicate. Error bars represent standard deviation.

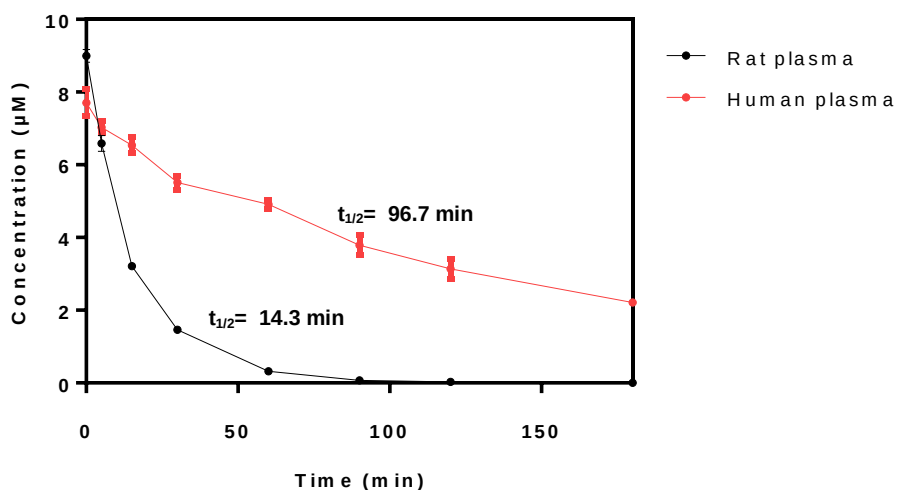


Figure 26.4: Concentrations of **76a** versus time and $t_{1/2}$ following incubation with rat (black line) and human plasma (red line) at 37 °C.

4.3.6 In vitro release studies

The next step towards the development of LMWGs prodrugs for localised drug delivery involved the evaluation of release of 5F 203 from gels of **76a** at ϕ_{DMSO} of 0.3 (the lowest solvent fraction able to form a gel), after having confirmed the self-assembling properties of the acyloxyalkoxycarbonyl derivatives of 5F 203 and the prodrug nature of **76a** by stability tests. Studies were performed to verify the ability of **76a** gels to act as a depot for the sustained release of the therapeutic agent for localised drug delivery. Experiments were carried out with a similar method used for the release studies of 5F 203-succinic acid prodrug hydrogel (Chapter 3, section 3.3.5), at physiological conditions at 37°C. Samples of the gels were previously prepared, cooled on transwell inserts at a concentration of 0.5% w/v and then incubated in 24 well plates containing PBS at pH 7.4. Samples of the wells were taken at specific time points and analysed by HPLC. Results showed no release of prodrug **76a** within the period considered (72 h). However, detection of the active drug 5F 203 was revealed 0.5 h after the onset of the experiment until the end of the time-course (72 h) (Figure 27.4). As previously hypothesised, the presence of 5F 203 in PBS buffer could be a result of the rapid hydrolysis of **76a** in solution, as demonstrated in the previous section. Indeed, low amounts of **76a** could be released from the gel matrix but, as a consequence of its sparing solubility, **76a** in solution was not detected. However, the more soluble active drug 5F 203, rapidly hydrolysed from the prodrug, was detected within the period considered.

Moreover, the limited amount of **76a** released from the gel depot could be a consequence of high rigidity of the gel matrix. This was also visually confirmed, as after 3 days the gel was still intact on the transwell insert; LC-MS analyses of the gel content after the release studies detected only **76a** in the gel matrix, thus proving the hydrolysis of the prodrug into the active drug only after its dissolution in PBS buffer.

Lastly, due to the high, not biocompatible, amount of DMSO contained in the gel of **76a** (ϕ_{DMSO} of 0.3), it was not possible to perform the same release experiment in plasma.

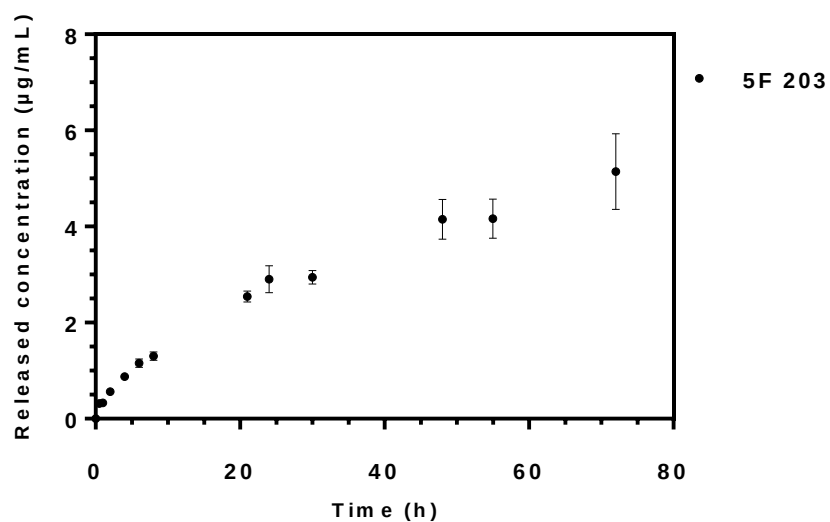


Figure 27.4: Accumulative release profile of 5F 203 from gel of **76a** (ϕ_{DMSO} of 0.3, 0.5% w/v) in PBS (pH 7.4, 37°C). All measurements were carried out in triplicate. Error bars represent standard deviation.

4.4 Conclusions

In this chapter, a new series of carbamate prodrugs of 5F 203 has been synthesised and the self-assembling properties of the compounds have been evaluated, in conjunction with their growth inhibitory activity *in vitro* against cancer cell lines.

Acyloxyalkoxycarbonyl derivatives of 5F 203 (**76a-76d**) have been developed with the aim of synthesising prodrugs susceptible to rapid ester hydrolysis. A simple Boc-protected 5F 203 conjugate (**74**) has also been developed to validate the “prodrug approach” of the acyloxyalkoxycarbonyl derivatives, as demonstrated by Alexander et al.⁶

Preliminary gelation tests, carried out with a solvent-switch method, displayed self-assembling properties for most of the acyloxyalkoxycarbonyl derivatives of 5F 203 (**76a**, **76b** and **76c**) and a correlation between ClogP and gelation was determined, the more lipophilic compounds were less prone to self-assembly. Furthermore, dynamic oscillatory rheological studies were performed to assess the LMWG nature of the new entities, finding at least one order of magnitude difference between the storage modulus and the loss modulus, confirming LMWGs characteristics. Moreover, a correlation between the DMSO volume fraction (ϕ_{DMSO}) and the stiffness of the gels was determined, with higher ϕ_{DMSO} corresponding to higher G' values. TEM analyses revealed formation of nanofibrillar structures of different shapes and dimensions depending on the analogues and the ϕ_{DMSO} analysed. Temperature-dependent $^1\text{H-NMR}$ spectroscopy studies gave information about the functional groups involved in gelation: they confirmed the pivotal role of π - π stacking interactions, derived from the phenyl-benzothiazole ring, in gel formation, as already described in Chapter 3. They also proved the contribution to gelation of hydrogen bonds of carbamate groups.

MTT assays, carried out against breast and ovarian cancer cell lines, showed a high inhibitory activity of all the acyloxyalkoxycarbonyl derivatives of 5F 203 against MCF-7 and IGROV-1, whereas they did not inhibit OVCAR-4 cancer cell growth. Thus, selectivity between cancer cell lines was observed.

Moreover, compounds **76a-76d** were highly cancer cell-selective as they did not inhibit the proliferation of normal fibroblast cells MRC-5 at the concentrations tested. Conversely, Boc-protected 5F 203 (**74**) displayed low potency. In vitro stability of **76a**, the most active prodrug of the series, was then evaluated in PBS and plasma. Analyses demonstrated hydrolysis of **76a** into the parent 5F 203 in buffer at pH 7.4. When incubated with rat plasma, a complete and rapid conversion of **76a** into the active drug occurred within 3 h; the same behaviour was observed when the prodrug was incubated with human plasma, though a lower rate of conversion was detected. Finally, release studies from the gel matrix of **76a** at ϕ_{DMSO} of 0.3 displayed release of 5F 203 in PBS buffer, as a result of hydrolysis of **76a** in solution.

Therefore, the novel acyloxyalkoxycarbonyl prodrugs of 5F 203 developed showed promising capabilities in terms of both self-assembling properties and in vitro anticancer activity.

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Chapter 5: Conclusions and future work

5.1 Conclusions

Use of LMWGs for localised cancer therapy has recently gained increasing popularity¹: one of the approaches, comprising synthesis of gelator-based prodrugs of chemotherapeutic agents, has several advantages compared to the traditional polymeric-based formulations, such as high drug loading, absence of excipients in the gel matrix and increased in situ drug concentration due to the localised delivery. However, one of the main problems of LMWGs-based prodrugs is represented by the limited number of anticancer drugs that have been discovered to have gelation properties: functionalised LMWGs-based prodrugs of the antitumour drug paclitaxel have been the only ones reported up to date.²⁻⁴

In this context, the present work has aimed to find new anticancer agents that display gelation properties by modification of the parent drug with known self-assembling groups, such as amino acids, peptides and alkyl chains of different lengths. In particular, two anticancer agents were evaluated: the clinical antimetabolite MTX and the experimental benzothiazole derivative 5F 203, both antineoplastic agents characterised by significant activity but, at the same time, by systemic toxicity.

Therefore, the conjugation of self-assembling functional groups to MTX and 5F 203 was considered a useful strategy to prepare LMWGs-based prodrugs for localised cancer therapy, as a valid alternative to overcome the systemic toxicity of these antitumour drugs. In addition, because of the completely different chemical structures of MTX and 5F 203, different synthetic approaches were investigated for both anticancer agents and the most successful derivatives were analysed to confirm the LMWG nature of the new materials, their in vitro activity against human-derived cancer cell lines and their release.

5.1.1 Methotrexate peptide and acyl modified derivatives

Different approaches for the synthesis of potential self-assembling MTX peptide and acyl modified conjugates were investigated.

A liquid phase strategy for the synthesis of compound **36**, consisting of MTX conjugated at the γ moiety to Fmoc-Phe-Phe, was initially evaluated. In spite of several attempts carried out to scale up **36** for gelation tests, low yields and failure of alternative synthetic routes hampered the feasibility of preliminary self-assembling tests.

A solid phase synthesis was then evaluated for the design of MTX derivative **52**. However, occurrence of an aminolysis side reaction, between the precursor of MTX and the self-assembling group Phe-Phe was revealed; therefore, also this synthetic strategy was not suitable for the preparation of MTX conjugates.

MTX derivatives bearing alkyl chains and aromatic groups at the α or both carboxylic acid moieties of MTX via an ester bond were then synthesised. The monoester **42**, possessing a dodecyl alkyl chain at the α moiety of the glutamic acid of MTX, and diesters **43a** to **43d**, displaying alkyl chains with different lengths and a benzyl group at the α and γ moieties of MTX, were synthesised and fully characterised.

Unfortunately, preliminary gelation tests performed on MTX acyl derivatives **42** and **43a-43d**, via a solvent-switch method, revealed lack of self-assembling properties and only formation of viscous solutions of **43b-43d** at high ϕ_{DMSO} (from 0.7 to 0.9). Therefore, the MTX conjugates developed in the present study were not suitable for localised drug delivery applications and confirmed the previous reported lack of gelation behaviour of MTX.⁵

5.1.2 Self-assembling 5F 203 amide prodrugs

Due to the absence of self-assembling properties of MTX conjugates, the potential gelation behaviour of novel derivatives of another anticancer candidate, the benzothiazole agent 5F 203, was considered.

Two new amide prodrugs series of 5F 203 were investigated: the first one represented by amino acids conjugates of 5F 203 (compounds **67a-67g**); the second one by 5F 203 derivatives bearing alkyl chains of different length (**68a-68b**). Preliminary gelation tests of the first series, carried out with a solvent-switch method, formed metastable organogels, in particular the parent drug 5F 203 and compound **67f**. The second series of prodrugs were tested with a pH-triggered method, and compound **68a** revealed formation of a hydrogel at physiological pH.

The gelation abilities of **68a** were confirmed by dynamic oscillatory rheological studies, which showed formation of a weak cross-linked gel network, also shown by SEM imaging. The hydrogelation process, as a function of the pH, was studied by TEM imaging and fluorescence spectroscopy. These last analyses also demonstrated the key role of π - π stacking interactions in gel formation. Time-dependent ^1H -NMR spectroscopy studies revealed the additional contribution of the alkyl chain in the self-assembly.

Results of MTT assays showed that conjugates of the first series inhibited proliferation of MCF-7 and IGROV-1 carcinoma cells at nanomolar concentrations, whereas OVCAR-4 growth was inhibited at micromolar concentrations. Conjugates of the second series displayed GI_{50} values in the micromolar range against MCF-7 and IGROV-1 but they were not active against OVCAR-4 at the concentrations tested. When amide prodrugs were tested against MRC-5 non-malignant fibroblast cells, resistance to these compounds was observed, thus providing evidence of the selectivity for tumour over 'normal' cells. Finally, release studies from the gel matrix of compound **68a** revealed a release of prodrug **68a** over three days and of the active drug 5F 203 after 48 h, thus confirming the potential application of **68a** hydrogel as a depot formulation for the localised delivery of 5F 203 at tumour resection sites.

5.1.3 Self-assembling 5F 203 carbamate prodrugs

A new series of prodrugs of 5F 203, the acyloxyalkoxycarbonyl derivatives **76a-76d** and a Boc-protected conjugate **74**, was investigated in terms of self-assembling and in vitro properties, in order to improve the rate of conversion of

prodrugs into the parent amine 5F 203, compared to the previous series of derivatives developed (Chapter 3).

Results of the preliminary gelation tests, carried out with a solvent-switch method, displayed gelation behaviour for derivatives **76a-76c** and also showed a correlation between octanol-water partition coefficient and self-assembly. Rheological studies confirmed the LMWG nature of the new derivatives and determined a correlation between ϕ_{DMSO} and gels' stiffness. TEM imaging revealed formation of nanofibrillar entangled networks, with dimensions of the aggregates depending on the analogue analysed and the ϕ_{DMSO} considered. Temperature-dependent ^1H -NMR spectroscopy analyses proved the crucial role of the π - π stacking interactions, in gel formation, as described in the previous chapter; the hydrogen bonds of the carbamate group also participated in the self-assembly.

Compounds **76a-76d** displayed GI_{50} values in the nanomolar range similar to those of the parent drug 5F 203, when tested in vitro against MCF-7 and IGROV-1 cell lines, whereas they did not showed any inhibition against OVCAR-4 cells. Compound **74** revealed GI_{50} values in the micromolar range when tested against MCF-7, whereas it did not show any inhibitory activity against ovarian cells lines IGROV-1 and OVCAR-4. Moreover, normal fibroblast cells MRC-5 showed resistance to compounds **74** and **76a-76d**, thus providing evidence for the selectivity of the new derivatives of 5F 203 against cancer cells only. In vitro stability of **76a** was then evaluated in PBS and plasma, showing hydrolysis of **76a** into the active drug 5F 203 in buffer at pH 7.4, thus confirming the prodrug nature of **76a**; in rat and human plasma a complete and rapid conversion of **76a** into the active drug was displayed within 3 h. Lastly, release studies from the gel matrix of **76a** (ϕ_{DMSO} of 0.3) showed release of 5F 203 in PBS buffer within 72 h, as a results of the quick hydrolysis of **76a** in solution.

Overall, the potential application of such gels as self-assembling prodrug gelators for the localised delivery of the anticancer agent 5F 203 was fully demonstrated. However, the high concentrations of DMSO in the gel formulations developed (>10 %) are a hindrance, as they are not

biocompatible for drug delivery applications. Therefore, the development of gelators with a minimum amount of organic solvent ($< 5\%$) or with no solvent (i.e. hydrogelators) would be a way to increase the biocompatibility.

5.2 Future work

The work carried out to date on 5F 203 prodrugs has provided the basis for future optimisation and development of LMWG derivatives of 5F 203, as depot formulations for localised delivery of this anticancer agent.

5.2.1 Further development of 5F 203 amide prodrugs

Due to the potential self-assembly and remarkable in vitro properties of the first series of 5F 203 amide prodrugs, the development of a larger library of amino acid-based prodrugs should be considered, using also different methods for triggering gelation (i.e. pH switch). Moreover, there is much scope for investigating 5F 203 peptide-based derivatives, because of the well-established self-assembling properties of peptide gelators.⁶

The second series, consisting of compounds **68a** and **68b**, should be further expanded, using alkyl chains of different lengths and aromatic groups possessing terminal carboxylic acid units, with the aim of developing gelators at physiological pH.

Moreover, in vitro stability studies of derivative **68a** should be carried out in PBS and in plasma, to further prove, as already demonstrated by release studies in PBS, the hydrolysis of **68a** to the parent amine and determine the rate of conversion into 5F 203 by enzymatic hydrolysis. Additionally, evaluation of prodrug uptake and metabolism in sensitive cells (i.e. MCF-7, IGROV-1) could be carried out to study the conversion of **68a** into the parent amine.

The effect of hydrogel of **68a** on cell proliferation in human derived cancer and normal cells should also be evaluated. This experiment would assess the biocompatibility and efficiency of compound **68a** formulated as hydrogel depot. Colorimetric assays, such as the previously reported MTT and a methylene blue staining assay could be used to measure cell proliferation and cell viability at specific time points after treatment with the hydrogel.

5.2.2 Further development of 5F 203 carbamate prodrugs

The carbamate-based prodrug series needs to be further developed with the synthesis of other analogues with alkyl chains of different lengths and aromatic moieties, in order to evaluate the effect of substitution on the gelation properties and the in vitro inhibitory activity compared to the prodrugs already developed **74** and **76a-76d**. The main aim would be to produce gelators using the minimum concentration of organic solvent (or only water), finding the adequate balance between hydrophilicity and hydrophobicity to promote the self-assembly in predominant water conditions. This would guarantee a higher biocompatibility of 5F 203 prodrugs for localised drug delivery.

Moreover, additional characterisation of the developed carbamate prodrug gelators (**76a-76c**) should be carried out, such as determination of the T_{gel} and identification of the side products of **76c** formed during the temperature-dependent ^1H -NMR spectroscopy studies. In addition, use of an internal standard (i.e. 4,4-dimethyl-4-silapentane-1-sulfonic acid, DSS) in the ^1H -NMR studies of the gelators would be useful to quantitatively determine the chemical shift variation, intensity of resonance signals and, overall, evaluate the thermodynamics of gelation process.

Lastly, the remarkable and selective in vitro inhibitory activity of prodrugs **76a-76d** against breast and ovarian cancer cell lines lead to quest for the mechanism of action of these derivatives and assess whether they induce expression of CYP1A1 enzyme, as already reported for 5F 203 and Phortress.^{7,8} Western blots were used to detect expression of CYP1A1 protein; up to date, only preliminary data against MCF-7 cancer cell lines have been produced (see Appendix) and need to be confirmed. To probe this further, also the other cell lines tested in the MTT assays (IGROV-1, OVCAR-4 and MRC-5) should be evaluated, to gain a more comprehensive understanding of prodrugs' mechanism of action.

5.3 References

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Appendix

This appendix briefly summarises the preliminary data obtained from Western blot experiments carried out to elucidate the mechanism of action of 5F 203 prodrugs developed in Chapters 2 and 3.

In particular, the parent drug 5F 203 and the prodrugs **67b**, **68a** and **76a** were tested. 5F 203-norleucine (**67b**) was chosen because it displayed the highest activity in inhibiting cancer cell proliferation amongst all the amino acid prodrugs of 5F 203. Prodrug **76a** was chosen because of its highest potency amongst 5F 203 carbamate prodrugs. **68a** was tested because it formed the most promising hydrogel, in terms of potential application for localised drug delivery. In order to evaluate the mechanism of action of 5F 203 prodrugs, expression of CYP1A1, the protein induced by active drug 5F 203, was investigated in all the cell lines previously tested for MTT assays. However, due to time constraints and problems encountered in protein detection, only preliminary data from MCF-7 cell cultures are reported.

a. Western blot method

a.1 Reagents and protocols used for Western blots

Reagent	Protocol
Nonidet-P40 (NP-40) lysis buffer	150 mM NaCl, 1% NP-40, 50 mM Tris (Trizma base, pH 8.0), protease inhibitor and phosphatase inhibitor
10% APS	1g of ammonium persulfate (APS) was dissolved in 10 mL of distilled H ₂ O
1 x Resolving buffer	139.5 g of Trizma Base and 4g of SDS were dissolved in 1 L of distilled H ₂ O and the pH was adjusted to 8.9 with 1M NaOH
1 x Stacking Gel buffer	45.36 g of Trizma Base and 4g of SDS were dissolved in 1 L of distilled H ₂ O and the pH was adjusted to 6.8 with 1M HCl
10 x Running buffer	15.0 g of Trizma Base, 72.06 g of glycine and 5g of SDS were dissolved in 500 mL of distilled H ₂ O
10 x Transfer buffer	12.11 g Trizma Base, 56.3.g of glycine were dissolved in 500 mL of distilled H ₂ O
1 x Transfer buffer	200 mL of methanol were added to 100 mL of 10 x transfer buffer in 700 mL of distilled H ₂ O
TBS-T (Tris Buffer Saline-Tween)	8g of NaCl, 2.42g of Trizma Base was dissolved in 1 L of distilled H ₂ O and adjusted to pH 7.6 with 1M HCl. 0.05% Tween 20 was added.

Table 1.a: Reagents and protocols used to prepare buffers for Western blots

a.2 Preparation of protein lysates

Cells were seeded at a density of 10^6 in a Petri dish and allowed to adhere for 24 h before test agent was introduced (final concentrations $1\mu\text{M}$ for compounds 5F 203, **67b** and **76a** and $10\mu\text{M}$ for compound **68a**). After 24 h of treatment, cells were lysed with NP-40 lysis buffer (Table a.1). The plates with cells were kept on ice and the medium was transferred into a centrifuge tube. Cells were manually detached using a cold plastic cell scraper and added to the medium previously removed. The samples containing the cells were centrifuged at 1500 rpm for 5 min at 4°C , the medium was aspirated and the cells were washed twice with cold PBS. PBS was disposed and cold NP-40 lysis buffer (0.3 mL) was added to the cell pellet and the sample was left on ice for 30 min. Tubes were

then centrifuged at 13300 rpm for 10 min at 4 °C. The supernatants were collected and frozen at -20 °C until required.

a.3 Determination of protein concentration

Six standard solutions of bovine serum albumin (BSA) 1, 2, 4, 6, 8, 10 mg/mL were prepared using distilled H₂O. These stock solutions were diluted 10 x with distilled H₂O. 10 µL of BSA standards were pipetted and added in triplicate into a microtiter plate; then 150 µL of the Bradford reagent was added into all wells. The same procedure was repeated using 5 µL of protein lysate from each cell line, previously thawed on ice: the protein lysates were diluted 10 x with distilled H₂O and 150 µL of the Bradford reagent was added into all wells.

The plate was shaken for 2 min using the plate shaker to ensure all reagents were mixed well. Afterwards, the plate was incubated for 5 min at r.t. and the absorbance was measured at 595 nm using a microtiter plate reader (PerkinElmer). A standard curve was plotted for the BSA standards with their average absorbance values versus their concentrations in mg/mL. The standard curve was then used to determine the protein concentrations in the volumes of the protein lysates used for the assay according to the principle of Beer-Lambert law. Protein concentration for each cell lysate was calculated and 50 µg protein separated by gel electrophoresis.

a.4 SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis)

SDS-PAGE was used to separate proteins for Western blotting. Firstly, a 10% resolving gel was prepared by mixing the following: 4.16 mL of distilled H₂O, 3.3 mL of acrylamide/bis acrylamide stock solution (Sigma-Aldrich), 2.51 mL of resolving buffer (Table a.1), 0.09 mL of 10% APS (Table a.1) and 0.020 mL of *N, N, N', N'*-Tetramethylethylenediamine (TEMED, Sigma-Aldrich). The gel was left to polymerise for 45 min. Then, a 5% stacking gel was prepared by mixing the following: 3.70 mL of distilled H₂O, 0.67 mL of acrylamide/bis acrylamide stock solution, 0.63 mL of stacking buffer (Table a.1), 0.03 mL of

10% APS (Table a.1) and 0.015 mL of TEMED. The gel was left to polymerise for 45 min. Subsequently, protein lysates were thawed on ice and sample containing 50 µg of protein were prepared. 0.285 mL of loading buffer (Invitrogen) and 0.015 mL of 2-mercaptoethanol were added to the protein lysates and the samples were heated for 5 min at 95 °C. The samples were then centrifuged and vortexed for 1 min before being loaded into sample wells of the stacking gel. Molecular marker (6 µL, Thermo scientific) was also loaded into one of the peripheral lanes of the gel. The proteins were separated by SDS-PAGE in 1 x running buffer (Table a.1) at 150V for ~2 h using an electrophoresis apparatus (Invitrogen).

a.5 Western blotting

Following gel electrophoresis, the separated proteins were transferred to a membrane by semi dry transfer. The gel was soaked in 1 x transfer buffer (Table a.1) for 2 min. In the meantime, nitrocellulose membrane and filter papers were cut to the dimensions of the gel and soaked in 100 mL of 1 x transfer buffer (Table a.1) for 2 min. A Trans-Blot Turbo Transfer System (BioRad) was used to transfer the separated proteins from the gel to the membrane with a “transfer sandwich” by removing all trapped air bubbles and protein transfer was conducted at 25V for 30 min. Afterwards, to block non-specific protein binding, the membrane was incubated in 10% milk (5.0 g of non-fat dried milk in 50 mL TBS-T, Table a.1) for 1 h at r.t. with gentle agitation. Then the membrane was incubated with C1P1A1 monoclonal mouse antibody (6G5, ThermoFisher, 58 kDa) in 5% milk with TBS-T overnight at 4 °C with gentle agitation (1:1000). The day after the membrane was washed 3 x 10 min in TBS-T and was incubated with a goat anti-mouse horseradish peroxidase (HRP, 1:2000) secondary antibody in 5% milk with TBS-T for a further 1 h at r.t. with gentle agitation. This was followed by washing the membrane for 3 x 10 min in TBS-T to remove excess unbound secondary antibody. Monoclonal anti-β actin antibody was used to verify equal protein transfer.

a.6 Detection by enhanced chemiluminescence

The membrane was incubated with 1 mL of enhanced chemiluminescence substrate mixture (WesternSure, Luminol Enhancer solution and stable peroxide solution, mixed to a ratio of 1:1) and placed in a C-Digit Blot Scanner (Li-Cor). Then, the membrane was scanned and analysed by Image Studio Software.

b. Preliminary results

5F 203 has been reported to induce expression of protein CYP1A1, validating the role of this enzyme in 5F 203 mechanism of action (see Chapter 1). Consequently, prodrugs of 5F 203 were tested to determine whether induction of CYP1A1 was evident after incubation of the compounds with sensitive cells. Treatment of MCF-7 cells with 5F 203, **76a** (1 μ M, 24h) and **68a** (10 μ M, 24h) caused induction of CYP1A1 protein (Figure 1.a.a).

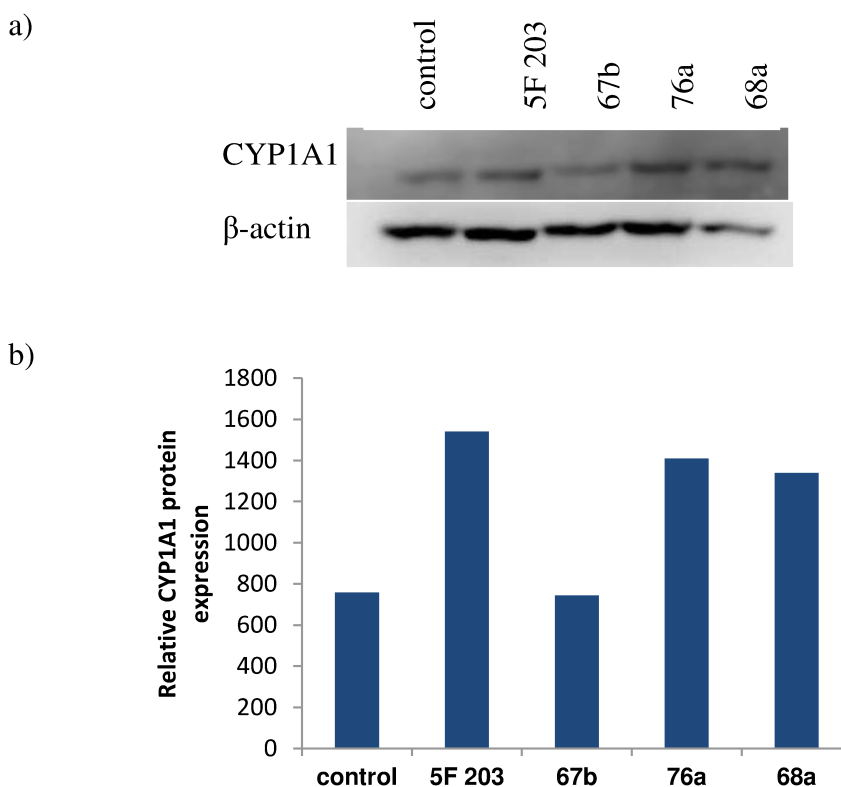


Figure 1.a: a) Detection of CYP1A1 protein induced in lysates of MCF-7 cells exposed to compounds 5F 203, **67b**, **76a** (1 μ M, 24h) and **68a** (10 μ M, 24 h). b) Densitometry quantification of blots.

Densitometry analyses of Western blots (Figure 1.a.b) detected an increased expression of CYP1A1 in cell lysates treated with 5F 203, **76a** and **68a**, whereas 5F 203-norleucine (**67b**) displayed a protein expression similar to the non-treated control. However, **67b** is an amino acid prodrug structurally similar to Phortress, whose ability to induce expression of CYP1A1 has already been demonstrated (see Chapter 1). Therefore, these preliminary experiments need to be repeated to deeply investigate the role of **67b** in inducing expression of CYP1A1. However, this initial data clearly confirm that prodrugs **76a** and **68a**

are able to induce expression of CYP1A1 and, consequently, could have the same mechanism of action of 5F 203.