Synthesis and Evaluation of
G-quadruplex DNA Targeting Agents

A thesis submitted for the degree of
Doctor of Philosophy of the University of London

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This thesis describes research conducted at the School of Pharmacy, University of London between January 2005 and January 2008 under the supervision of Prof. Stephen Neidle. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature

Date 15-8-2008
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I also want to thank my family for their support, especially Marfa who, being closer, had to endure my crises whilst I was writing.

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ABSTRACT

The DNA at the telomeres and promoter regions of eukaryotic genes is G-rich and may form special DNA structures termed G-quadruplexes. These structures have high stability and there is evidence of their existence in vivo. G-quadruplexes in telomeric DNA can inhibit telomerase and potentially affect the architecture of telomeres, whereas their presence in promoter regions can inhibit gene expression.

Telomere maintenance and oncogenes such as c-kit are recognised targets in anti-cancer therapy. G-quadruplex stabilisation by small molecules can selectively affect cancer cell viability by affecting telomere maintenance and the expression of c-kit and other oncogenes.

The work in this thesis describes the design and synthesis of two families of novel G-quadruplex ligands and their evaluation as G-quadruplex stabilising agents and telomerase inhibitors and their preliminary biological evaluation as anti-cancer agents.

The syntheses of two libraries, one composed by eighteen novel 4,5-disubstituted acridones and the other by twenty-two novel tri- and tetra-substituted naphthalene diimides, is discussed together with SAR analyses.

The assessment of the DNA binding ability of the ligands was conducted using fluorescence resonance energy transfer (FRET) DNA melting experiments and surface plasmon resonance (SPR) binding studies. The evaluation of their telomerase inhibitory ability was performed using a modified telomerase repeat amplification protocol (TRAP) assay.

The preliminary biological evaluation of the compounds consisted of cell culture studies using cancer and normal cell lines to assess their cell growth inhibitory ability and to give an insight into their mechanism of action.
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<td>AEBSF</td>
<td>4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride</td>
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<td>ALT</td>
<td>Alternative lengthening of telomeres</td>
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<td>Pyrrolo[2,1-c][1,4]benzodiazepine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pd</td>
<td>Population doublings</td>
</tr>
<tr>
<td>PNA</td>
<td>Peptide nucleic acid</td>
</tr>
<tr>
<td>POT1</td>
<td>Protection of telomeres 1 protein</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PyBOP</td>
<td>Benzotriazol-1-yl-oxytripyrroldinophosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>RAP1</td>
<td>Repressor activator protein 1</td>
</tr>
<tr>
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<td>Ribonucleic acid</td>
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<td>Thymine</td>
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<tr>
<td>TAMRA</td>
<td>6-carboxytetramethrhodamine</td>
</tr>
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<td>Telomere binding protein</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<tr>
<td>TEMPO</td>
<td>2,2,6,6-Tetramethylpiperidine-1-oxyl</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TIN2</td>
<td>TRF1-interacting protein 2</td>
</tr>
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<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
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<tr>
<td>TPP1</td>
<td>Tripeptidyl peptidase 1 protein</td>
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<tr>
<td>TRAP</td>
<td>Telomerase repeat amplification protocol</td>
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<tr>
<td>TRF1/2</td>
<td>Telomeric-repeat binding factor 1/2</td>
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CHAPTER 1

INTRODUCTION
1 INTRODUCTION

1.1 CANCER

1.1.1 General

More than 1/3 of the population of developed countries such as the UK are expected to develop a variety of cancer in their lifetime (Cancer Research UK web site). Almost 300,000 new cases of cancer were diagnosed in the UK in 2004 and 153,491 people died as a consequence of one of the varieties of the disease in 2005 (Cancer Research UK web site). It is estimated that approximately 7.6 million people died worldwide in 2007 as a consequence of cancer (American Association of Cancer Research web site).

The term cancer comprises a group of diseases commonly characterised by uncontrolled cell proliferation, which can eventually spread throughout the body and cause organ malfunction and often death. Cell proliferation in normal human tissues is tightly controlled by an array of proteins that regulate the proliferative and inhibitory stimuli. All types of cancer invariably start when the finely balanced regulation of cell proliferation is lost for a single cell and this replicate uncontrolled. This is a rare event in the human body and a succession of genomic aberrations must accumulate for one cell to transform. These aberrations may have hereditary origin or be caused by mistakes in the DNA replication or environmental factors. The consequences of these genomic alterations can be loss or gain of gene function. The aberrant regulation of the expression of tumour suppressor genes and proto-oncogenes is one of the hallmarks of cancer.
1.1.2 Cancer chemotherapy

The first chemical ever to be used as an anti-cancer therapy was a nitrogen mustard, at the time used as a chemical weapon (Thurston, 2006). This fact is very illustrative and representative of the majority of cancer treatments in the XX century. The use of cytotoxic agents was, and still is for many types of cancer, the only option for chemical treatment of cancer. The selectivity of these treatments is thought to exclusively rely on the faster cell replication that cancer cells present with respect to normal cells, which makes them more susceptible to the toxicity of these agents. The optimisation of these early molecules together with screening programs and serendipity has resulted in the discovery of several compounds with therapeutic activity against cancer. The main disadvantage of these treatments is that they affect most cells in the body, particularly rapidly dividing cells, causing a number of undesirable side effects such as bone marrow suppression, gastrointestinal tract lesions, hair loss and nausea, which generally limit the dosage of the drug that can be safely administered (Thurston, 2006).

1.1.3 New targets in cancer therapy

The progressive understanding of the biochemical pathways implicated in cancer is enabling the development of new types of anti-cancer treatments that exploit the differences between cancer and normal cells. The study of patterns of gene and protein expression enables the identification of enzymes, receptors and other potential molecular targets that are exclusively expressed or overexpressed in cancer cells and not in normal cells. New anti-cancer agents should rely on these differences to achieve maximum potency and, importantly, selectivity.

1.1.3.1 Telomerase as a potential target in cancer therapy

The telomerase enzyme is expressed in approximately 90% of cancer cells, whereas is not expressed at all or only at low levels in normal somatic human cells (Dunham et al., 2000). Telomerase maintains the integrity of telomeres, offsetting the naturally occurring erosion caused by cell replication (Olovnikov, 1973). Normal cells have a limited lifespan before they undergo senescence, and this is regulated by the length of
their telomeres (Lundblad & Szostak, 1989). Cancer cells can divide indefinitely by bypassing the barrier of senescence by maintenance of telomere integrity via activation of telomerase (Bodnar et al., 1998). Inhibition of telomerase can abrogate the inappropriate maintenance of telomeres and limit the replicative potential of cancer cells (Hahn et al., 1999a). The importance of telomerase in oncogenesis was confirmed in an experiment that showed that expression of hTERT (one of the components of telomerase) together with expression of a mutant form of the \textit{k-ras} oncogene and inactivation of the tumor suppressor proteins p53 and pRb achieved the oncogenic transformation of human cells (Hahn et al., 1999b). These data, and other that are discussed in this introduction, show the pivotal role of telomerase in oncogenesis and cancer cell viability and support the notion that regulation of telomerase activity can be an important therapeutic target against cancer.

A more detailed introduction to telomerase and telomeres is given in section 1.2.

\textbf{1.1.3.2 \textit{c-kit} as a potential target in cancer therapy}

The tyrosine kinase protein family is involved in cellular growth signalling cascades and their deregulation is typical in cancer. The search for protein kinase inhibitors is an active field of research in anti-cancer therapy as kinases represent an attractive cancer-specific target (Thurston, 2006). An example of a kinase targeting drug, imatinib (Gleevec), is successfully used in the clinic to treat chronic myelogenous leukemia and gastrointestinal stromal tumors (GIST).

c-kit is a receptor tyrosine kinase and is subject to aberrant hyperactivity in GIST (Sakurai et al., 1999). Imatinib, although initially developed to target a different kinase, is effective at inhibiting the c-kit protein and is usually employed to treat c-kit positive GIST. These tumors, however, quickly develop resistance to imatinib. The inhibition of c-kit may have applications in a variety of cancers, such as GIST, and is an attractive target for drug development.
1.2 TELOMERES

1.2.1 General

Telomeres are highly specialised DNA/protein structures that constitute the end of the chromosomes. Telomeres are needed by the cell to avoid unwanted responses to the presence of free DNA ends. These include: 1- the DNA degradation by nucleases; 2- the aberrant processing to form end-to-end chromosomal fusions; 3- homologous recombination; 4- the triggering of DNA damage checkpoint mechanisms that can cause the onset of senescence or apoptosis (Smogorzevska & de Lange, 2004; Ferreira et al., 2004). The correct functioning of telomeres is an essential requirement for the normal proliferation of cells (Blackburn, 2000a).

1.2.2 Telomere structure

Telomeric DNA is composed in most eukaryotic organisms of series of repeats of particular DNA sequences. Human telomeric DNA is formed by repeats of the sequence d(TTAGGG) in its 5’→3’ strand (Moyzis et al., 1988). Telomeric DNA is mainly double-stranded except for a 100-200 base long 3’ single-stranded overhang (Wright et al., 1997). An accepted model for the arrangement of the human telomeric DNA is the so-called t-loop model (Figure 1.1; Griffith et al., 1999). According to this model, the single-stranded overhang associates with a complementary sequence of the double-stranded telomeric DNA by displacing the second strand and forming two structures called t-loop and D-loop. This peculiar organisation avoids the exposure of the free ends to the DNA repair mechanisms of the cell.

![Figure 1.1](Taken from de Lange, 2005).
Telomeric DNA associates in a complex fashion with a number of proteins. Their individual function is not totally determined for many of them but they confer structural stability by binding specifically to different parts of the telomere and also exert sensing, recruiting and assisting roles for the correct functioning of telomeres.

Telomeres have been proposed to possess at least three different possible configurations: 'capped', 'open' and 'uncapped' (Blackburn, 2000b; Dubrana et al., 2001; de Cian et al., 2008). These terms relate to the capping function performed by telomeric proteins, which protect telomeres against degradation, DNA damage checkpoints and inappropriate processing. The 'capped' status corresponds to a fully protected configuration which avoids the extension of telomeric DNA by telomerase. The 'open' status corresponds to a configuration where the protection is maintained but telomerase and other proteins implicated in the DNA replication can freely operate. The 'uncapped' status is consequence of short telomeres or the displacement of telomeric proteins and in this state telomeres are dysfunctional and cannot exert their protective function. The specific structures of each possible configuration are unknown.

1.2.3 Telomere components

Amongst the proteins that associate with telomeres, six proteins compose the so-called shelterin complex (de Lange, 2005). These are POT1, TRF1, TRF2, TIN2, Rap1 and TPP1. POT1, TRF1 and TRF2 contain DNA-binding domains and associate directly to telomeric DNA. POT1 binds to the single-stranded telomeric overhang, although it can also bind duplex telomeric DNA, whereas TRF1 and TRF2 bind specifically to duplex telomeric DNA. TIN2, Rap1 and TPP1 associate with the DNA-binding proteins and to each other, giving shape to the shelterin complex (Figure 1.2).

The shelterin complex plays an important role in the architecture of telomeres. It has been suggested that the DNA remodelling abilities of TRF1 and TRF2 may direct the formation of the t-loop and D-loop (de Lange, 2005). TRF2 or POT1 inactivation causes uncapped telomere status which results in a number of responses, including the onset of senescence or apoptosis, formation of end-to-end fusions, recruitment of DNA damage repair proteins and attrition of the 3' overhang (de Lange et al., 2002; Van Steensel et al., 1998; Takai et al., 2003). Cells with a mutant form of POT1 unable to
bind to the 3’ overhang show uncontrolled lengthening of their telomeres (Loayza & de Lange, 2003) whereas overexpression of POT1 inhibits the extension of telomeres by telomerase (Kelleher et al., 2005; Lei et al., 2005). Overexpression of TRF1 causes telomere attrition in cells containing telomerase (Li et al, 2000). TRF2 was also found to be a negative regulator of telomere length (Smogorzweska et al., 2000). These data suggests that not telomere length per se but amount of telomere-associated protein, may be important in the regulation of telomerase (Ancelin et al., 2002; Karlseder et al., 2002).

![Diagram of the shelterin complex](image)

**Figure 1.2** The shelterin complex. (Taken from de Lange, 2005).

### 1.2.4 Telomeres and cancer

The end-replication problem of DNA polymerase in the duplication of the 5’→3’ DNA strand (Olovnikov, 1973) coupled to the generation of the 3’ overhang (Wellinger et al., 1996; Dionne & Wellinger, 1996) causes a loss of DNA at telomeres, between 50 and 150 base pairs (bp) in each replication cycle (Harley et al., 1990). Telomere shortening acts as a biological countdown for the lifespan of the cell and when telomeres become critically short, cells stop proliferating. Once telomeres reach this length, known as the Hayflick limit (Hayflick, 1965), the cell enters the state of replicative senescence, which is a metabolically active but non-replicative phenotype (Shay et al., 1991). The number of rounds of replication that a cell can undergo before becoming senescent depends, amongst other factors, on the overall length of telomeric DNA (Hahn et al., 1999a). Telomere length varies amongst different types of cells. For normal somatic cells it is approximately 10 kilo base pairs (kbp), it is approximately 12 kbp for stem cells and is 15-20 kbp for germ cells (McElligott & Wellinger, 1997).
When telomeres become too short they can affect chromosomal integrity, possibly due to inefficient capping (Blackburn, 2001). The onset of senescence can be seen as a tumor suppressor mechanism by which the proliferation of older cells, which possess inherent higher risk of becoming cancerous, is halted (Campisi, 2001). The senescence response is controlled by a series of tumour suppressor proteins, including p53 and pRB (Shay et al., 1991). The oncogenic transformation of a cell requires it to bypass the barrier of senescence. In fact, loss of p53 and pRB function are commonly occurring events in oncogenesis (Beausejour et al., 2003). A model to explain the oncogenic transformation of a cell was proposed by Wright and Shay (Figure 1.3; Wright & Shay, 1992).

\[\text{Telomerase Bypasses M1 Senescence and M2 Crisis Leading to Cell Immortality}\]

\[\begin{array}{c}
\text{Express hTERT} \\
\text{Express hTERT} \\
\text{Express hTERT} \\
\text{Senescence} \\
\text{Crisis} \\
\text{Immortality} \\
\text{Telomere Shortening} \\
\text{Telomere Maintenance} \\
\end{array}\]

\text{Figure 1.3 Telomerase mediated immortalisation based on the M1 and M2 stages model. (Taken from Shay & Wright, 2005).}

According to this model, after deactivation of the tumour suppressor proteins the cells can bypass the stage of senescence, or M1 stage, and carry on replicating until telomeres reach a critically short length and enter a crisis stage, M2. At this stage telomeres are too short to be able to protect the chromosomes and these suffer a high number of chromosomal aberrations commonly causing cell death. Some cells, however, recover from the crisis stage by stabilisation of their shortened telomeres through activation of telomerase by expression of hTERT.

Telomere maintenance is required to keep the replicative potential of cancer cells and those that do not express telomerase utilise an alternative telomere maintenance
mechanism known as ALT, which involves recombination processes between telomeres (Bryan et al., 1995).

1.2.5 Telomerase

Human telomerase is composed of two main sub-units, a protein component of 127 kDa termed human telomerase reverse transcriptase (hTERT) and a RNA component of 451 nucleotides termed human telomerase RNA (hTR) (Feng et al., 1995). The protein sub-unit contains the catalytic centre and the RNA component possesses a complementary sequence that binds to telomeric DNA and at the same time acts as a template for the synthesis of the extended strand (Morin, 1989). These two components are sufficient for telomerase to be active although the telomerase holoenzyme complex associates with other proteins (Weinrich et al., 1997).

Telomerase is upregulated in around 90% of cancer cells, whereas is not expressed in most normal cells (Dunham et al., 2000). Although hTERT expression has been typically regarded as the regulatory event for telomerase activity (Feng et al., 1995), there is increasing evidence supporting a tighter regulation of hTR than was initially supposed (Cairney & Keith, 2008). Some somatic cells also express low levels of telomerase during S phase (Masutomi et al., 2003). The exact role of telomerase in this case is uncertain but it could have a role in the stabilisation of the telomeric complex during DNA synthesis. The important role of telomerase for telomere maintenance and extension of the replicative capacity of cells has been highlighted (Bodnar et al., 1998; Vaziri & Benchimol, 1998; Counter et al., 1998). Although activation of telomerase was found to produce lengthening of telomeres in some cases (Bodnar et al., 1998; Counter et al., 1998), other studies showed that telomerase activation did not produce noticeable lengthening but instead achieved stabilisation of short telomeres (Zhu et al., 1999; Ducray et al., 1999). This supports the proposed structural role of telomerase in the architecture of telomeres (Masutomi et al., 2003).

Inactivation of telomerase by expression of a dominant-negative mutant of hTERT produced cell growth arrest with a delay that depended on telomere length (Hahn et al., 1999a). Inhibition of telomerase is an accepted target for anticancer intervention and several strategies can, in principle, be pursued to achieve it. These include the direct
inhibition of the enzyme and blockage of telomerase expression and immunotherapy (reviewed in Harley, 2008). A small molecule, BIBR1532, a potent direct inhibitor of telomerase in pre-clinical development (Damm et al., 2001; Pascolo et al., 2002), and the hTR template antagonist GRN163 (Akiyama et al., 2003) showed cell growth arrest in cell cultures with a delayed response in concordance with the previously observed time lag (Hahn et al., 1999a). These results are consistent with inhibition of telomerase and cell growth arrest caused by replication-related telomere shortening.

A different approach to inhibit telomerase is to sequester its substrate, telomeric DNA, forming G-quadruplex DNA structures. In vitro stabilisation of G-quadruplexes with high concentrations of K⁺ was shown to inhibit the extension of the DNA by telomerase (Zahler et al., 1991). This approach was pursued and small molecules that could stabilise the G-quadruplexes were developed. They were shown to inhibit telomerase in vitro (Sun et al., 1997). The activity of these agents has been extensively studied in vivo and they represent a new way of targeting telomere maintenance and consequently cancer.

More detailed introductions to G-quadruplex and G-quadruplex ligands are given in sections 1.3 and 1.4 respectively.
1.3 G-QUADRUPLEX DNA

1.3.1 General

The first structural description of the guanine arrangement called G-quartet, composed of guanines associated to guanines by Hoogsteen type hydrogen bonding (Figure 1.4), was given by Gellert and coworkers working with guanine monophosphate (Gellert et al., 1962). Henderson and coworkers detected the first evidence of G-quartet formation in a biologically relevant substrate working with telomeric DNA sequences from different species (Henderson et al., 1987). The particular nature of these structures was revealed by Sen and Gilbert (Sen & Gilbert, 1988). They discovered that G-rich DNA sequences can self-recognise to form structures based on G-quartets. The structures resulting from the stacking of several G-quartets are known as G-quadruplexes.

![Figure 1.4 A G-quartet stabilised by Hoogsteen hydrogen bonding.](image)

The first detailed description of a G-quadruplex structure was reported for the telomeric DNA of *Tetrahymena* (Sundquist & Klug, 1989), resolved using alkylation and electrophoretic mobility experiments. Since then, a large number of G-quadruplex structures have been reported, often in great detail, generally obtained by crystallographic or NMR experiments.

1.3.2 Structure and polymorphism

G-quadruplexes are constituted of two or more G-quartets groups stacked by π-π interactions and further stabilised by cations located in the electronegative central
channel of the structure. A common feature of G-quadruplex forming sequences is the presence of runs of consecutive guanines, the G-tracts. G-quadruplexes can adopt a large number of topologies and several factors influence the structure or structures that a particular G-rich DNA sequence may form. These include the number of DNA molecules involved, which will give rise to intra- or intermolecular G-quadruplexes, sequence and length of the connections between the different G-tracts, which will define the structure of the loops, and the nature of the cations, which may favour the formation of one topology over another.

The different G-quadruplex topologies can be qualitatively classified by describing the sequence, stoichiometry, polarity of each strand, how the loops connect the different strands and conformation of the guanine glycosidic angles. According to the stoichiometry, G-quadruplexes can be classified as unimolecular, bimolecular or tetramolecular (Figure 1.5).

![Figure 1.5 Stoichiometric polymorphism of G-Quadruplexes. A: Unimolecular. B: Bimolecular. C: Tetramolecular. (Taken from Simonsson, 2001).](image)

According to the polarity of the strands, all parallel or different arrangements of antiparallel strands may be encountered (Figure 1.6).

![Figure 1.6 Strand polarity polymorphism of G-Quadruplexes. A: All strands parallel. B: Three parallel strands and one strand antiparallel. C: Two pairs of adjacent parallel strands. D: Alternating antiparallel strands. Arrows indicate 5'→3' polarity. (Taken from Simonsson, 2001).](image)

The loops can be described as propeller type, which connect adjacent strands running in parallel, lateral, which connect adjacent strands running in antiparallel, diagonal, which
connect opposite strands running in antiparallel, and V-shaped, which connect a strand with a guanine that is part of a G-quartet but is not connected to the guanine immediately above or underneath it in the G-quadruplex structure (Figure 1.7).

![Figure 1.7 Loop types in G-Quadruplexes. A: Lateral. B: Diagonal. C: Propeller type. D: V-shaped. (Taken from Patel et al., 2007).]

The conformation of the glycosidic angles of the guanines will depend on the overall topology. All parallel G-quadruplexes contain only guanines in anti conformation while G-quadruplexes with mixed polarities display guanines with both, sin and anti, glycosidic angles (Figure 1.8).

![Figure 1.8 The conformation of the glycosidic torsion angle for a guanine. A: Anti. B: Syn. (Taken from Patel et al., 2007).]

The stability of G-quadruplex structures require the presence of monovalent cations, which neutralise the electronegative potential created in the centre of the structure by the carbonyl groups of the guanines. The cations coordinate in different ways with the G-quartets. In potassium solutions, K\(^+\) resides equidistant to two G-quartets in the central channel, while in sodium, Na\(^+\) can adopt several positions, including insertion in the centre of the G-quartet planes. G-quadruplexes structures are very stable and have been suggested to maintain their structure even in the gas phase (Rueda et al., 2006). G-quadruplex structures form readily in single stranded DNA but also in duplex DNA where their formation is a competitive event against the association of both complementary sequences (Risitano & Fox, 2003).

Although some of the structural characteristics of G-quadruplex DNA are similar to other conformations of DNA, the G-quadruplex structure displays several distinctive features. G-quadruplex DNA possesses four grooves and up to three loops. Furthermore, the G-quartets present a planar surface larger than any other DNA
structure. These features can and should be exploited in the development of selective G-quadruplex targeting agents.

1.3.3 G-quadruplexes in the telomeres

The DNA at telomeres is G-rich and in most eukaryotes contains a single stranded 3' overhang. The telomeric overhang is particularly prone for the formation of G-quadruplexes since there is no complementary sequence which binding could potentially compete with for G-quadruplex formation. Consequently, it is not surprising that many G-quadruplex structures reported to date have been found in sequences from telomeric regions of several species.

Human telomeric DNA, including its single strand overhang, is formed by repeats of the sequence 5'-TTAGGG-3'. The G-quadruplex structures that can be formed in the telomeres have been subject of intense debate in recent years. The structure for a 22-mer sequence containing four human telomeric repeats was resolved by NMR in solution and in presence of sodium (Figure 1.9-A; Wang & Patel, 1993). This structure displayed antiparallel configuration with two lateral and one diagonal loops. More recently, the same sequence was crystallised in potassium solution (Figure 1.9-B; Parkinson et al., 2002). In this structure the four strands run in a parallel arrangement and the loops are propeller-like. The potassium cations were found to symmetrically coordinate the O6 carbonyl groups of the guanines of two consecutives quartets.

It is well known that intramolecular G-quadruplexes can adopt different conformations in the presence of different cations (Ren et al., 2002; Risitano & Fox, 2005).
Furthermore, many studies with telomeric DNA have shown that at least two species, including the parallel and antiparallel conformers, may exist in solution in both sodium and potassium (Ying et al., 2003; Phan & Patel, 2003; Lee et al., 2005). This can explain the apparent discrepancy in the telomeric G-quadruplex structures. The question of which structure is more biologically relevant remains. Recent studies in potassium solution employing molecular crowded conditions to mimic the intracellular environment showed the structure to be all parallel as the described by the crystal structure (Xue et al., 2007). Furthermore, all the crystal structures of telomeric G-quadruplexes bound to ligands have consistently shown the same DNA arrangement (Parkinson et al., 2007; Parkinson et al., 2008; Campbell et al., 2008) and they have revealed the ability of these G-quadruplexes to stack to each other, providing an efficient packing mechanism that can be potentially relevant in the intracellular domain (Parkinson et al., 2002). For these reasons, the use of the all parallel crystal structure as a template for drug design seems more suitable than any of the other alternatives.

The human telomeric G-quadruplex as described by the crystal structure is a 41 Å wide and 6.3 Å high disc-like structure and possesses several distinctive features when compared to other structures (Figure 1.10; Parkinson et al., 2002).

![Figure 1.10](Taken from Parkinson et al., 2002)

The particular disposition of the propeller type loops on the sides of the structure clears both ends of the structure offering the two planar faces for π-π interactions without the need of rearranging the loops. The two faces are significantly different and while the 5’ face is hydrophobic the 3’ face is more hydrophilic. The structure presents four very
similar grooves, between 9.0 and 10.3 Å wide, with environments highly influenced by the loops in close proximity.

1.3.4 G-quadruplexes in promoter regions of oncogenes

Recent advances in the understanding of G-quadruplex formation requirements and bioinformatics applied to the vast amount of information provided by the Human Genome Project have enabled studies that survey the entire human genome for putative G-quadruplex forming sequences (Todd et al., 2005; Huppert & Balasubramanian, 2005). A total of 375,157 non-overlapping sequences of duplex DNA with G-quadruplex forming potential were identified. Putative G-quadruplex forming sequences were found to occur more frequently in the promoter regions of genes and to present a bias towards predicted structures with particularly high stability (Huppert & Balasubramanian, 2007). The occurrence of G-quadruplexes in promoter regions was found to associate with sites with nuclease hypersensitivity (Huppert & Balasubramanian, 2007). Furthermore, in yeast, putative G-quadruplex forming sequences were shown to possess lower histone content, suggesting a role for G-quadruplexes in the displacement of nucleosomes (Hershman et al., 2008). This evidence supports the hypothesised role of G-quadruplexes in the regulation of gene expression.

Some genes important in cancer, such as c-myc, c-kit, bcl-2, c-met and k-ras, contain G-quadruplex forming sequences. In the presence of a complementary sequence the G-quadruplex conformation has to compete with the highly stable duplex DNA, but formation of G-quadruplex is not necessarily restricted to single stranded DNA (Risitano & Fox, 2005) and several studies have shown that under molecular crowded conditions that mimic the intracellular environment, G-quadruplex formation occurs in presence of its complementary sequence (Kan et al., 2006 and 2007).

Structural data for G-quadruplex forming sequences in promoter regions of several genes have been reported, including for c-myc (Phan et al., 2004; Ambrus et al., 2005), c-kit (Rankin et al., 2005; Fernando et al., 2006) and bcl-2 (Dai et al., 2006), although the latter contains a base modification. Of particular interest for this work are the...
structures formed by the two G-quadruplex forming sequences in the promoter of the c-
kit oncogene, ckit1 (Rankin et al., 2005) and ckit2 (Fernando et al., 2006).

The structure for the ckitl sequence was recently resolved using NMR (Phan et al.,
2007). This G-quadruplex presented a unique topology (Figure 1.11). The most striking
novelty was the participation in the formation of a G-quartet of a guanine not coming
from a G-tract but from a guanine initially supposed to be part of a loop (G10).
Furthermore, the structure presented a remarkably distinct pocket that, in principle,
could be selectively targeted.

![Figure 1.11 Schematic and detailed representations of NMR structure for the ckitl sequence G-
quadruplex. Cyan: guanine bases involved in G-quartet formation; Green: other bases; Grey: phosphate
sugar backbone (Taken from Phan et al., 2007).]

The structure of the native ckit2 sequence has not been resolved yet, however NMR
studies suggested that the sequence can adopt multiple topologies (Fernando et al.,
2006). Sequences including single mutations showed a preference for adopting the all
parallel conformation.

1.3.5 Biological relevance of G-quadruplexes

G-quadruplexes have been studied throughout in vitro and there is growing evidence of
G-quadruplex formation in vivo (Oganesian & Bryan, 2007). Indirect evidence comes
from the increasing number of proteins found to specifically interact with G-
quadruplexes. These include helicases that unwind G-quadruplexes, such as the Bloom
and Werner syndrome proteins (Sun et al., 1998; Mohaghegh et al., 2001) and nucleases specific for G-quadruplexes, such as GQN1 (Sun et al., 2001).

Direct detection of G-quadruplexes in vivo was achieved in experiments using antibodies generated to specifically bind antiparallel G-quadruplexes formed by the telomeric sequence of Stylonichia lemnæ. These were detected to accumulate in the macronuclei but not micronuclei (Schaffitzel et al., 2001). Further studies in Stylonichia showed that interference with the expression of the telomere binding proteins βTBP, phosphorylation of which is required for the unfolding of G-quadruplex during DNA synthesis, or αTBP resulted in the abrogation of the macronuclei detection of the antibody, consistent with a role of both proteins in the in vivo formation of the G-quadruplexes (Paeschke et al., 2005). Moreover, a tritiated analogue of the G-quadruplex ligand 360A was found to preferentially bind to the telomere regions of chromosomes (Granotier et al., 2005).
1.4 G-QUADRUPLEX TARGETING AGENTS

1.4.1 General

The potential therapeutic benefits of G-quadruplex stabilisation, particularly in cancer, have made the development of ligands targeting these structures a very active area of research in recent years. G-quadruplex formation in telomeric DNA in vitro was found to inhibit the binding of telomerase and hence its function (Zahler et al., 1991). Furthermore, G-quadruplex stabilisation in promoter regions of genes potentially represents a new method of affecting the expression of proteins important for cancer cell proliferation (Grand et al., 2002; Bejugam et al., 2007).

G-quadruplex DNA has a distinctive group of features that make it selectively targetable with small molecules. However, these molecules must be carefully designed to avoid interference with duplex DNA, which inevitably shares some structural characteristics with G-quadruplex DNA. The structural analysis of G-quadruplexes has been of key importance for the rational design of these ligands and the crystal structure of the human telomeric G-quadruplex (Parkinson et al., 2002) has been the basis for the design of most ligands. The most distinctive feature of G-quadruplex DNA is the large planar surfaces formed by the four interconnected guanines of the G-quartets. This structure is approximately twice larger than the equivalent structure in duplex DNA, with a pair of bases. This difference is of primary importance in terms of ligand discernment between duplex and G-quadruplex DNA. It is not surprising that the most common approach for G-quadruplex ligand design is the use of relatively large aromatic groups to interact with the G-quartets through π–π interactions. The aromatic groups by themselves are generally insufficient for tight binding and often the hydrophobicity of these groups is a drawback in terms of drug-like properties. These problems are generally resolved by the introduction of cationic groups, which can aid in solubilisation but also improve the interactions with the overall electronegative G-quadruplex DNA structure.

The G-quadruplex structures are also characterised by the structure of their loops and grooves. These structures are particular for each G-quadruplex and, in principle,
represent targetable features for tighter interactions and improved discriminatory ability between several DNA structures. G-quadruplex ligands often include side chains with cationic groups that aim to extend the interactions with loops, grooves and phosphorilated backbones. These side chains have been shown to be of critical importance for tight binding and discriminatory ability.

1.4.2 Existing ligands

The first small molecule shown to bind to G-quadruplex DNA was the anthraquinone derivative 1a (Figure 1.12; Sun et al., 1997). This molecule, originally studied as a duplex and triplex DNA interacting agent, was shown to inhibit telomerase activity (EC_{50} = 23 \mu M) through a G-quadruplex mediated mechanism. Several series of analogues were evaluated and higher telomerase potency was achieved (EC_{50} \sim 1 \mu M) (Perry et al., 1998a; Perry et al., 1998b). However, further study of these compounds as G-quadruplex ligands was not pursued due to their affinity towards duplex DNA and their subsequent toxicity.

![Figure 1.12](image)

Figure 1.12 The structure of some G-quadruplex DNA interacting compounds. 1a: Anthraquinone derivative; 1b: Fluorenone derivative; 1c: 3,6 disubstituted acridine; 1d: BRACO-19; 1e: BSG-17.

Following the approach taken with the anthraquinones, other series of molecules containing disubstituted tricyclic cores, fluorenones such as 1b (Figure 1.12; Perry et al., 1998b), and acridines such as 1c (Figure 1.12; Harrison et al., 1999), were
evaluated. The acridine analogues were shown to be the most potent telomerase inhibitors of the group and presented lower toxicity, hence they were chosen for further derivatisation studies. Structure-based studies of new acridine G-quadruplex ligands suggested that tri-substitution at 3, 6 and 9 positions would yield compounds more potent and selective than the 3,6-disubstituted analogues (Read et al., 2001). The telomerase inhibitory ability of some of these compounds was in the nanomolar range. Molecular computing studies showed that the side chains could interact with three of the grooves of the G-quadruplex structure. The lead compound in this series, BRACO-19 (Compound 1d, Figure 1.12) was extensively evaluated. BRACO-19 was found to inhibit cell growth at sub-cytotoxic concentrations with a number of cancer cell lines. The biological responses to BRACO-19 exposure included decrease of intracellular telomerase activity (Gowan et al., 2002; Burger et al., 2005; Gunaratnam et al., 2007), onset of the senescent phenotype (Gowan et al., 2002; Incles et al., 2004; Burger et al., 2005), telomere shortening (Burger et al., 2005; Gunaratnam et al., 2007), formation of chromosomal end-to-end fusions (Incles et al., 2004) and displacement from the telomeres of the telomere associated protein POT1 (Gunaratnam et al., 2007). In vivo studies showed that BRACO-19 inhibited tumour growth as a single agent in xenograft models of uterus carcinoma UXF1138L (Burger et al., 2005) and also administered post-paclitaxel in xenograft models of vulval carcinoma A431 (Gowan et al., 2002). Loss of cellular telomerase activity and irregular mitotic figures, such as anaphase bridges, were detected in xenograft experiments with UXF1138L (Burger et al., 2005), which supported the in vitro observations and the proposed mechanism of action of BRACO-19 as a G-quadruplex binding agent. Acridines were further derivatised using different substitution patterns and side chains (Harrison et al., 2003; Schultes et al., 2004; Moore et al., 2006; Martins et al., 2007) resulting in a new lead compound, BSG-17 (Compound 1e, Figure 1.12), which entered pre-clinical trials as a telomeric targeting agent anti-cancer drug.

A perylene derivative, PIPER (Compound 1f, Figure 1.13), was designed as a G-quadruplex ligand (Fedoroff et al., 1998). The proposed mode of interaction with G-quadruplex DNA, π-π stacking to the G-quartets, was confirmed by NMR. PIPER was found to act as a molecular chaperone for G-quadruplex formation using single stranded DNA (Han et al., 1999) but also duplex DNA in the c-myc promoter region under physiological conditions (Rangan et al., 2001). Furthermore, PIPER-mediated
stabilisation of a G-quadruplex avoided its unwinding by the G-quadruplex specific helicases Sgs1 (Han et al., 2000) and T-ag (Tuesuwan et al., 2008). Side chain optimisation studies were conducted (Rossetti et al., 2002 and 2005; Samudrala et al., 2007; Sissi et al., 2007; Franceschin et al., 2007) and telomerase inhibitory potency, as measured by TRAP, was improved for some analogues. However, the biological data for these compounds is scanty.

Figure 1.13 The structure of some G-quadruplex DNA interacting compounds. If: PIPER; Ig: TMPyP4; Ih: Se2SAP; Ii: Telomestatin; Ij: RHPS4.

TMPyP4 (Compound Ig, Figure 1.13), a cationic porphyrin, was also evaluated as G-quadruplex ligand (Izbicka et al., 1999a). Computing studies showed a good stacking of the porphyrin core to the G-quartet and the four cationic side chains were shown to interact with each of the four grooves (Izbicka et al., 1999a). Crystallographic studies showed a different binding mode that did not involve G-quartet stacking but stacking to base pairs formed by the loops (Parkinson et al., 2006). This supported previous evidence that indicated a poor selectivity of TMPyP4 for G-quadruplex with respect to duplex DNA (Ren & Chaires, 1999). TMPyP4 and analogues were shown to inhibit telomerase in the low micromolar range (Shi et al., 2001). Similarly to PIPER, porphyrin analogues were shown to inhibit the unwinding of G-quadruplex DNA by Sgs1 (Han et al., 2001). They were tested against several cancer cell lines and showed low toxicity, with IC$_{50}$ values generally over 50 μM (Izbicka et al., 1999a and 1999b). The selectivity of TMPyP4 and another analogues for certain types of G-quadruplexes was studied (Han et al., 2001) and some degree of selectivity towards particular G-
quadruplexes was found for \textit{c-myc} with Se2SAP (Compound \textbf{1h}, Figure 1.13; Seenisamy \textit{et al.}, 2005). Furthermore, TMPyP4 was found to be effective at inhibiting the expression of \textit{c-myc} and \textit{k-ras} genes (Siddiqui-Jain \textit{et al.}, 2002; Cogoi \& Xodo, 2006).

Telomestatin (Compound \textbf{1i}, Figure 1.13), a natural product, was shown to be one of the most potent telomerase inhibitors (Shin-ya \textit{et al.}, 2001). Its aromatic structure interacts selectively with the guanine plane of the telomeric G-quadruplex (Kim \textit{et al.}, 2002). Telomestatin was shown to inhibit cell growth in a number of cancer cell lines (Tauchi \textit{et al.}, 2003; Nakajima \textit{et al.}, 2003; Gomez \textit{et al.}, 2003, 2004, 2006a and 2006b; Shammas \textit{et al.}, 2004; Tahara \textit{et al.}, 2006) through a telomere mediated mechanism related to G-overhang and overall telomere shortening, onset of senescence and apoptosis and displacement of POT1 and TRF2 from telomeres. Although telomestatin has been successfully synthesised in the laboratory (Doi \textit{et al.}, 2006), the process is extremely challenging and a number of groups have studied structural related molecules with the aim of reproducing its activity (Jantos \textit{et al.}, 2006; Minhas \textit{et al.}, 2006; Baker \textit{et al.}, 2006).

Polycyclic acridines have been studied as G-quadruplex ligands. RHPS4 (Compound \textbf{1j}, Figure 1.13) was particularly interesting as it was able to inhibit telomerase \textit{in vitro}, as measured by TRAP, and intracellular telomerase activity at sub-cytotoxic concentrations (Gowan \textit{et al.}, 2001). RHPS4 has been proved to be an effective anti-proliferative agent in a number of melanoma cell lines with a mechanism consistent with telomere targeting (Leonetti \textit{et al.}, 2004). RHPS4 produced interruption of the cell cycle, senescence and apoptosis together with a high number of telomere fusions. Moreover, RHPS4 was able to localise in the nuclei of MCF7 cells after 30 minute exposure (Heald \textit{et al.}, 2002). The efficacy of RHPS4 seemed to depend on the initial length of telomeres in cell culture (Cookson \textit{et al.}, 2005a). \textit{In vivo} studies showed RHPS4 to be effective as a single agent and in combination with Taxol in xenografts of uterus carcinoma UXF1138L with a mechanism related to telomere disfunction (Phatak \textit{et al.}, 2007). Further studies with different xenograft models showed RHPS4 to have an effective anti-tumour effect related to telomere damage and apoptotic cell death, an effect inhibited by overexpression of POT1 or TRF2, confirming the presupposed mechanism of action (Salvati \textit{et al.}, 2007).
Other interesting types of compounds include 2,6-pyridine dicarboxylate derivatives such as 360A (Compound 1k, Figure 1.14; Pennarun et al., 2005), found to bind to telomeres in vivo (Granotier et al., 2005), triazine derivatives such as 12459 (Compound 1l, Figure 1.14; Riou et al., 2002), the phenanthroline analogue EDL35 (Compound 1m, Figure 1.14; de Cian et al., 2007a) and isoalloxazines such as 1n (Figure 1.14; Bejugam et al., 2007), which inhibit the expression of c-kit.

![Figure 1.14 The structure of some G-quadruplex DNA interacting compounds. 1k: 360A; 1l: 12459; 1m: EDL35; 1n: An isoalloxazine analogue.](image)

Some of the more studied G-quadruplex ligands have been described herein. However, the field of G-quadruplex ligand discovery is very active and a large number of ligands have been described. For a more comprehensive insight into G-quadruplex ligands refer to two recently published reviews on the subject (Monchaud & Teulade-Fichou, 2008; de Cian et al., 2008).

The cellular effects caused by G-quadruplex ligands include cancer cell growth arrest at sub-cytotoxic concentrations, inhibition of intracellular telomerase activity, senescent and apoptotic responses, telomere and 3' overhang shortening, chromosomal end-to-end fusions and activation of DNA damage responses. Furthermore, several ligands have shown to have an anti-tumour effect in vivo in a number of cancer xenografts.

### 1.4.3 Mechanisms of action of G-quadruplex ligands and working hypotheses

Although the initial hypothesis to explain the mechanism of action of G-quadruplex ligands was telomerase inhibition, the lack of the expected time lag in the response to
these agents, the absence of telomere shortening in some cases and the evidence of the
displacement of TRF2 and POT1 from telomeres by some of the ligands suggests a
different mechanism of action. The inhibition of telomerase may contribute to the
observed effect in some cases but there is increasing evidence that telomeric binding
and the consequent telomere uncapping are responsible for most of the observed cellular
effects. Telomerase seems to play a role in stabilising telomeres independent of its
telomeric DNA synthesis ability (Masutomi et al., 2003). The differences in telomere
length between normal and cancer cells and the presence of telomerase, which could
play a structural role in the telomeres of cancer cells, may suggest a structural difference
in the telomeres of normal and cancer cells which could account for the observed
selective toxicity of G-quadruplex ligands for cancer cells. This difference can in
principle be targeted to obtain a therapeutic agent to selectively kill cancer cells.

In addition, some G-quadruplex ligands showed the ability to inhibit the expression of
several oncogenes such as \textit{c-kit}. Several studies also found reduced intracellular
telomerase activity upon treatment with G-quadruplex ligands, allegedly resulting from
the inhibition of the expression of \textit{c-myc}, which encodes for the transcription factor that
regulates the expression of \textit{hTERT} amongst others genes (Greenberg et al., 1999) or by
direct inhibition of the expression of \textit{hTERT}, which also contains a G-quadruplex
forming sequence. The potential ability of G-quadruplex ligands to inhibit the
transcription of genes may be exploited in the clinic to target cancer cells that
overexpress certain oncoproteins. The inhibition of \textit{c-kit} using this strategy is an
attractive therapeutic target to treat cancers overexpressing this gene, such as GIST.

There is a potential dual mode of action of G-quadruplex DNA stabilising agents. On
one hand, they may act at telomeres by inhibiting telomerase and/or displacing
telomeric proteins from the telomeres. On the other hand, some of the ligands may also
be capable of inhibiting the transcription of particular genes.

The aim of this study was to develop new series of G-quadruplex targeting agents to test
their binding ability and selectivity for the telomeric and \textit{c-kit} G-quadruplexes and
duplex DNA and to obtain a preliminary insight of their hypothesised mechanisms of
action using cell culture studies.
CHAPTER 2

DESIGN AND SYNTHESIS OF ACRIDONE G-QUADRUPLEX TARGETING AGENTS
2 DESIGN AND SYNTHESIS OF ACRIDONE G-QUADRUPLEX TARGETING AGENTS

2.1 BACKGROUND

2.1.1 Acridone

The DNA intercalating properties of acridines have been known for a long time and have made them an archetype of this type of agent. The tricyclic core of acridine compounds confers them the ability of intercalating into DNA. Acridones, or acridine-9(10H)-ones, are chemically related compounds that can be used as synthetic precursors of their acridine counterparts (Albert, 1966). The acridone core is a planar structure composed of three fused rings with a carbonyl group at the 9 position and a neutral NH group, as opposed to the basic nitrogen of acridine, at the 10 position (Figure 2.1).

![Figure 2.1 The acridone general structure.](image)

2.1.2 Acridone compounds in the literature

The acridone scaffold is present in numerous organisms as a natural alkaloid. For example, extracts of plants of the Rutaceae (Citrus) family have been the source of numerous secondary metabolite acridone derivatives, many of which have shown anticancer effects and biological activity against bacteria, fungi, protozoan parasites and viruses. The naturally occurring acridones citrusinine-I (Compound 2a, figure 2.2;
Yamamoto et al., 1989) and citpressine-I (Wu & Furukawa, 1983) presented anti-viral activity against herpes simplex virus. Another natural product, atalaphillinine (Compound 2b, figure 2.2), presented anti-plasmodial activity in mice models (Fujioka et al., 1989) as well as activity against Pneumocystis carinii (Queener et al., 1991). Some acridone alkaloids and synthetic analogues also showed in vitro anti-cancer activity (Kawai et al., 1999). Several studies suggested that this activity is related to the ability of acridones to inhibit the mammalian topoisomerase II enzyme (Bastow et al., 1994; Vance & Bastow, 1999; Tabarrini et al., 1999; Goodell et al., 2006).

Figure 2.2 The structure of some acridone-based biologically active compounds. 2a: Citrusinine-I; 2b: Atalaphillinine; 2c: 1,4 aminoalkyl substituted acridone; 2d: 1,4 substituted acridone dimers; 2e: Tetracyclic acridone dimers; 2f: Acridone-PBD hybrid; 2g: Acridone analogue of amonafide; 2h: Elacridar.

The potential of acridones as topoisomerase inhibitors has been studied by a number of groups. A series of acridones were designed based on the structure of quinolone bacterial topoisomerase II poisons (Tabarrini et al., 1999). Although they produced reduced cancer cell proliferation, the compounds were poor mammalian topoisomerase
II poisons. In another study, tetracyclic pyrimido-acridones (Compound 2g, figure 2.2) were synthesised in the quest for new topoisomerase II inhibitors based on the structure of amonafide (Kamata et al., 2004). The compounds showed a good degree of cell growth inhibition and DNA-protein cross-linking, which is indicative of the stability of the topoisomerase II complex with DNA.

The ability of acridones to interact with DNA and the implications of this interaction in anti-cancer therapy has been extensively investigated in the search for a clinical candidate. Although the acridone chromophore, or simply substituted acridones, might present DNA interacting properties, carefully positioned side chains are required to positively influence the kinetics of the binding process and to stabilise the DNA/ligand complex (Feigon et al., 1984). Acridones having long substituents were reported with improved binding abilities towards duplex DNA when compared to less functionalised analogues. For example, acridones containing aminoalkyl substitutions at the 1 and 4 positions (Compound 2e, figure 2.2) were found to bind strongly to duplex DNA and inhibit cell proliferation in a number of cancer cell lines (Antonini et al., 1997). Some of these agents were also synthesised as bis-intercalator dimers (Compound 2d, figure 2.2) and also showed good DNA binding abilities and \textit{in vitro} anti-cancer properties (Antonini et al., 2003). Similarly, dimers of tetracyclic acridone derivatives (Compound 2e, figure 2.2) were also synthesised and evaluated as anti-cancer agents (Cholody et al., 1995 and 2005).

Acridones and other intercalating molecules have been used as hybrids with minor groove binders to target duplex DNA with high specificity. Acridone, and also acridine, were hybridised to pyrrolo[2,1-c][1,4]benzodiazepine (PBD), a group that convalently binds to guanine in the minor groove of DNA (Compound 2f, figure 2.2; Kamal et al., 2004). The binding to the duplex DNA in this series was tighter for the acridone-containing hybrids than for the acridine analogues. Likewise, acridones were hybridised to dystamycin type polyamides (David-Cordonnier et al., 2007). Results from biophysical experiments supported the hypothesised hybrid binding mode, consisting of intercalation by the acridone and minor groove binding by the polypyrrole.

Acridone has also been employed to deliver DNA damaging groups taking advantage of its intercalating properties. Acridone was bound to a photosensitiser porphyrin as a
delivery method of the porphyrin to the DNA, potentially providing a photodynamic therapy agent (Viola et al., 1997). Similarly, imidazoacridinone C-1311, an anti-cancer agent evaluated in phase II clinical trials, has a proposed mechanism of action which involves intercalation and posterior alkylation of DNA caused by the metabolised side chain (Konopa, 2001).

Muramyl dipeptide derivatives (MDPs) have shown to exert an effect in immunoprotection against several types of foreign bodies and, to a lesser extent, cancer. To study the potential synergistic anti-cancer effects of MDPs and acridones and acridines, a number of acridone(acridine)/peptide hybrid molecules were synthesised and studied in in vitro and in mouse models where they showed anti-cancer properties (Dzierzbicka et al., 2001; Dzierzbicka & Kolodziejczyk, 2003).

Although acridones have been mainly employed as DNA interacting agents, some analogues are effective against different molecular targets. Elacridar (Compound 2h, figure 2.2) is a 4,5 substituted acridone in clinical studies as an inhibitor of the membrane transporters P-gp and ABCG2, involved in multidrug resistance (Wallstab et al., 1999). Several other series of elacridar analogues were studied in the search for novel multidrug resistance protein inhibitors (Krishnegowda et al., 2002; Hegde et al., 2004; Boumendjel et al., 2007).

2.1.3 Acridone compounds as G-quadruplex ligands

Acridones have been also used to target and stabilise G-quadruplex DNA. Series of 2,6, 2,7 and 3,6 disubstituted acridones (Compounds 2i, 2j and 2k, figure 2.3) were synthesised and evaluated as G-quadruplex binders and telomerase inhibitors (Harrison et al., 2004). Modelling studies and DNA melting experiments showed that acridones had lower binding affinity for the G-quadruplex than the acridine analogues. The in vitro anti-cancer activity of the acridones was also lower. In another study, a series of G-quadruplex targeting 3,6 disubstituted acridones with peptide-containing side chains (Compound 2l, figure 2.3) was synthesised by solid phase methods (Ladame et al., 2004). Surface plasmon resonance (SPR) analysis showed the acridone/peptide conjugates to bind with more affinity to the telomeric G-quadruplex than the peptide free analogue and more importantly with increased specificity when compared to duplex
DNA. In the same study, analogues with acridine instead of acridone were also synthesised and were found to bind more strongly to the G-quadruplex.

Molecular modeling for the acridone and acridine analogues used in these studies showed that the acridones displayed a similar binding mode to the G-quadruplex as their acridine counterparts. However, the binding affinity was lower for the acridones than for the acridines as FRET and SPR studies demonstrated. The charge in the acridine has been postulated to contribute to tight binding by electrostatic interactions with the electronegative carbonyl channel of the G-quadruplex, a hypothesis borne out by FRET and SPR studies.

Figure 2.3 Chemical structure of acridone-based compounds evaluated as G-quadruplex ligands. 2i: 3,6 substituted acridone; 2j: 2,6 substituted acridone; 2k: 2,7 substituted acridone; 2l: Peptide-containing 3,6 substituted acridone.

2.2 LIGAND DESIGN

The study of 4,5-disubstituted acridines or acridones as G-quadruplex binders has been neglected and there are no reported ligands presenting this substitution pattern. Molecular modeling studies suggested that the incorporation at the 4 and 5 positions of aromatic side chains conjugated to the acridone core by amide groups could give molecules with an extended planarity (Stephen Neidle and Michael Moore, personal communication). The planar conformation of this type of molecule would be favoured
by the electronic delocalisation between the aromatic side chains and the acridone core through the amide groups and also by the formation of bifurcated hydrogen bonds between the nitrogen of the acridone and the carbonyl groups of the amides (Figure 2.4).

\[ \text{Figure 2.4 Potential bifurcated hydrogen bond formation in a 4,5-disubstituted acridone.} \]

This conformation results in a planar moiety of approximate size 10 by 10 Å which approximately matches in size the G-quartet plane of a G-quadruplex as demonstrated by manual molecular superposition in figure 2.5. The overlapping of the aromatic systems of the G-quadruplex and the ligand is desirable for the establishment of π–π interactions to stabilise the complex. The larger planar moiety conferred by the aromatic side chains would in principle difficult the intercalation into duplex DNA when compared to simpler acridones.

\[ \text{Figure 2.5 Manual superposition of a 4,5-disubstituted acridone and the 5'} \text{ G-quartet of the crystal structure of the human telomeric G-quadruplex.} \]

As discussed before (page 19), side chains containing ionisable groups are important for G-quadruplex binding. The addition of these appendixes to the phenyl rings in the \textit{para} position with respect to the amide groups could direct the side chains towards the exterior of the G-quadruplex for potential groove binding and interaction with the polyphosphate chain of the DNA backbone.
Compound 2.1 (Figure 2.6) was designed in our group following these precepts and was synthesised and evaluated by FRET (Michael Moore, personal communication). The good binding ability of the ligand confirmed the validity of this approach.

![Chemical structure of compound 2.1.](image)

The large planar moieties and ionisable groups that are hypothetically required for G-quadruplex binding makes the target values proposed by Lipinski in his rule of five (Lipinski et al., 1997) very often unreachable and this is the case for most G-quadruplex binders described in the literature (de Cian et al., 2007a). The solubility of compound 2.1 was good in DMSO, DMF and DMA while it was poor in water and alcoholic solvents. However, the use of small amounts of DMSO and acid (HCl) allowed the preparation of aqueous solutions of compound 2.1 at concentrations of at least 10 mM.

### 2.3 AIM

This work explores in a methodical manner, through the synthesis and evaluation of a diverse library of compounds based on 2.1, the effect that changes in different features of these compounds have on their G-quadruplex binding ability and biological activity. A full understanding of the SAR of these molecules could help in the development of new compounds and ultimately a drug candidate.

### 2.4 LIBRARY DESIGN

Compound 2.1 has amide linked substituted benzene rings at the 4 and 5 positions. The benzene rings have propanamide appendixes in the para position that act as linkers for the pyrrolidine groups at the end of the side chains. The new analogues of compound
that form part of this library are described in table 2.1. Starting from the northern part of the molecule, the features that have been investigated were:

1. Benzene rings. It was postulated that the extended planarity obtained by the use of aromatic groups was a necessary feature for increased interactions with G-quadruplex DNA. This issue was addressed by including in the library compounds 2.17 and 2.18. The side chains for compound 2.17 contain N-methyl pyrrole groups in substitution of the benzene rings. This compound maintains the aromaticity of the side chains but its planarity is compromised by the methyl substitution in the pyrrole groups. The rest of the features of the side chains are equivalent to those of compound 2.1. For compound 2.18, the substituents are aliphatic chains containing dimethylamine as an end group with no aromatic group.

2. Substitution pattern in the benzene rings. It was intended to include analogues of 2.1 with the ortho and meta substitutions in the benzene rings as opposed to the para substitution of compound 2.1. Compound 2.16 is the analogue with meta substituted benzene rings. The ortho substituted analogue was, in our hands, synthetically inaccessible.

3. Carbonyl group in the side chain linkers. The amide groups are potential sites of metabolism and contribute to the rigidity of the molecule. The role of these carbonyl groups were investigated by including compounds 2.9-2.12 in the library. These compounds are analogues of compounds 2.1-2.4, respectively, without the carbonyl groups.

4. Length of the side chain linkers. The influence of the length of the carbon chains linking the benzene rings and the end groups was investigated by introduction to the library of compounds 2.13-2.15. These compounds are analogues of compound 2.1 with linker lengths of one, three or four carbons, compounds 2.13, 2.14 and 2.15 respectively.

5. End groups. The end groups of the side chains play a key role in G-quadruplex recognition. Compounds 2.2-2.8, analogues of compound 2.1 with different end groups, were included to the library. Compounds 2.2-2.5 contain a variety of
tertiary amines. Compound 2.6 contains a secondary amine, cyclohexaneamine. Compound 2.7 contains an aromatic amine, imidazole. Compound 2.8 contains a non-charged pyrrolidine substituted carbonyl group.

Finally, another analogue containing a single side chain, compound 2.19, was included to assess the importance of the disubstitution.

Table 2.1 Compounds of the acridone library. "See figure above for side chain descriptor equivalence. \(^{\text{a}}\)para-phenyl. \(^{\text{b}}\)meta-phenyl. \(^{\text{c}}\)4-amino-1-methyl-2-carboxamide-1H-pyrole. \(^{\text{d}}\)N,N-dimethylpropane-1,3-diamine. \(^{\text{e}}\)Not applicable. \(^{\text{f}}\)Monosubstituted analogue. For the complete structures please refer to the structure sheets in the back pocket of this thesis.
2.5 SYNTHESIS

The strategy for the synthesis of compounds 2.1-2.18 is exemplified in the retrosynthetic analysis for compound 2.1 (Figure 2.7). The synthesis of the acridone core and side chains was conducted independently and converged in the last and key step, which was the amide bond formation between 4,5-dicarboxy acridone and the anilinic side chain. The same strategy can be applied for the synthesis of compound 2.19, the monosubstituted analogue, starting from 4-carboxy acridone.

![Figure 2.7 Retrosynthetic analysis for compound 2.1.](image)

References within the text to spectroscopic methods, related to the characterisation of the products, refer to $^1$H, COSY, $^{13}$C and DEPT NMR, MS or HRMS and IR. Some spectroscopic data representative of the different types of compounds or useful for the discussion have been included within the text. For full spectroscopic data and detailed synthetic procedures please refer to the experimental section (Chapter 9).

2.5.1 Synthesis of the acridone core

4,5-dicarboxyacridone, compound 2.0.1, has been previously synthesised using the Jourdan-Ullmann reaction between ortho-iodoisophthalic and anthranilic acids for the key synthetic step (Rewcastle & Denny, 1985). The synthesis of ortho-iodoisophthalic acid involves a lengthy and complicated sequence of reactions (Wakelin et al., 2003). An alternative was found in the use of ortho-bromoisophthalic acid (2.0.1.1) for the Jourdan-Ullmann reaction. The complete synthetic pathway is depicted in figure 2.8.
Chapter 2 – Design and synthesis acridone of G-quadruplex targeting agents

Figure 2.8 Synthetic pathway for the disubstituted acridone core, compound 2.0.1. Reaction conditions: a) KMnO₄, H₂O, reflux, 16 h, 30%; b) Anthranilic acid, Cu(0), K₂CO₃, DMF, 120°C, 16 h, 75%; c) conc. H₂SO₄, 50°C, 2 h, 73%.

The synthesis of 2.0.1.1 was conducted as previously reported by oxidation of 2-bromo-meta-xylene with KMnO₄ in water upon overnight reflux followed by filtration and acidification of the filtrate to give a white precipitate (Padwa et al., 1992). The reported yield of 68% could not be reproduced and a poor yield of 30% was consistently obtained. To try to improve the yield of this step the Jones reagent, often used for the oxidation of phenylic methyl groups, was tried but no product was detected. Eventually, the KMnO₄ method was followed, albeit low yielding, the reaction presented the advantages of simplicity, scalability (reproducibility with up to 20 g of material) and high purity of the crude product. Compound 2.0.1.1 was subject to the Jourdan-Ullmann reaction conducted as reported (Denny et al., 1987) with anthranilic acid, copper (0) and K₂CO₃ in anhydrous DMF under overnight heating to give 2.0.1.2 in good yield (75%). The melting point and spectroscopic data of 2.0.1.2 were consistent with that reported in the literature. Compound 2.0.1.2 was subsequently cyclised by treatment with concentrated H₂SO₄ at 50°C for 2 hours to afford compound 2.0.1 in good yield (73%). The yellow solid showed low solubility in water, alcoholic or chlorinated solvents but could be successfully dissolved in DMSO, DMF or DMA. The ¹H NMR spectrum presented the characteristic peak of the NH of the acridone at 13.99 ppm and a broad singlet for the acidic hydrogens at 13.83 ppm. The acridone carbonyl was also evident in ¹³C NMR as a peak at 176.20 ppm.

Compound 2.0.2, the starting material for the synthesis of the monosubstituted compound 2.19, has been previously reported (Rewcastle & Denny, 1985). The synthesis was conducted in a similar manner to the synthesis of 2.0.1 as depicted in figure 2.9.
The synthesis started with the Jourdan-Ullmann reaction between 2-bromobenzoic and anthranilic acids to obtain the diacid compound 2.0.2.1 in 91% yield. The next step was the ring closure using concentrated H$_2$SO$_4$ to obtain 2.0.2, in poor yield (34%). The identification of the product was carried out by spectroscopic methods. The distinctive acridone carbonyl displayed a peak at 176.35 in $^{13}$C NMR, and the $^1$H NMR spectrum showed the expected signals for the 8 hydrogens of the acridone.

### 2.5.2 Synthesis of the side chains leading to compounds 2.1-2.7

The synthesis of the side chains for compounds 2.1-2.7 was conducted as depicted in figure 2.10.

![Synthetic pathway for side chains for compounds 2.1-2.7](image_url)

**Figure 2.10** Synthetic pathway for side chains for compounds 2.1-2.7. Reaction conditions: a) 3-chloropropionyl chloride, 50°C, 16 h, 80%; b) Amine R, neat or THF or DMF, RT or 150 °C, 16 h or 5 min, 39-98%; c) Ammonium formate, Pd/C, ethanol, MW 120°C, 3 min, 38-100%.

The first step was the acylation of 4-nitroaniline with 3-chloropropionyl chloride used as solvent at 50°C for 16 hours. Addition of diethyl ether to the reaction mixture caused...
the precipitation of the product. The yield of the reaction was 80% and the product did not require further purification. Compound 2.0.3 was characterised by spectroscopic methods including $^1$H NMR, shown in figure 2.11.

![Figure 2.11 $^1$H NMR spectrum of compound 2.0.3 in DMSO-d$_6$. Aromatic protons Ha and Hb showed a characteristic splicing pattern with two doublets at 8.25 and 7.85 respectively ($J = 9.3$ Hz).](image)

The next step was the substitution of the chlorine by treatment with the different amines. The amines for the synthesis of the side chains of compounds 2.1 (pyrrolidine), 2.2 (morpholine), 2.4 ($N$-methylpiperazine), 2.5 (piperidine) and 2.6 (cyclohexaneamine) were liquids at room temperature and were used as solvents. The reactions proceeded at room temperature over 24 hours with 40-90% conversions. The main impurity in these reactions was the alkene product of elimination, which is favoured by several factors. The acrylate product is thermodynamically stable due to the conjugation of the newly formed double bond with the carbonyl group. Additionally, the basicity and bulkiness of the nucleophilic amines and, likely, the high concentration used, may contribute to the elimination reaction. This issue was addressed by the use of lower reaction temperatures and the use of methanol or water as solvents. However, since the reactions at lower temperatures proceeded slowly and the reactions with water or methanol as solvents presented the same elimination problem, the original method was selected for the syntheses. The isolation of the products from the reaction mixtures was achieved by evaporation of the amines in vacuo and EtOAc/aqueous partition. The products were successfully purified in this manner and no chromatography was necessary. The products were characterised by spectroscopic methods. The $^1$H NMR spectrum for compound 2.0.4 is depicted in figure 2.12.
Figure 2.12 $^1$H NMR spectrum of compound 2.0.4 in CDCl$_3$. The characteristic signals of the hydrogens of the pyrrolidine are two multiplets at 1.95 and 2.75 ppm.

Compound 2.0.6 displayed two multiplets in the $^1$H NMR spectrum at 2.65 and 3.83 ppm, integrating four hydrogens each, correspondent to the morpholine signals. For compound 2.0.10 the $^1$H NMR signals correspondent to the $N$-methylpiperazine showed as a broad multiplet at 2.67 ppm integrating 11 hydrogens. Compound 2.0.12 showed the characteristic signals for piperidine in the $^1$H NMR at 1.58, 1.72 and 2.56 ppm. Compound 2.0.14 displayed the characteristic signal of the CH of the cyclohexaneamine in $^1$H NMR as a triplet of triplets at 2.55 ppm and as a signal at 56.28 ppm in $^{13}$C NMR.

The substitution reaction with dimethylamine, for the synthesis of the side chain for compound 2.3, was conducted using an excess of a 2 M solution of the amine in THF under the same conditions described before to obtain the product 2.0.8 in 98% yield. The identity of the product was confirmed by spectroscopic methods. The signals of the dimethylamine methyl groups were characteristic and appeared as a singlet integrating six hydrogens at 2.42 ppm in $^1$H NMR and as a signal at 44.05 ppm in $^{13}$C NMR. The reaction with imidazole for the synthesis of the side chain leading to compound 2.7 was conducted in DMF using 8 equivalents of base. The reaction did not proceed at room temperature due to the poor nucleophilicity of imidazole but went to completion after heating at 150°C for 5 minutes under microwave radiation. The product 2.0.16 was isolated, by addition of water and filtration of the precipitate, in 83% yield. The three aromatic signals of the imidazole group in $^1$H NMR showed at 6.86, 7.16 and 7.61 ppm.

The nitro compounds were reduced to the correspondent amine compounds by catalytic hydrogenation with ammonium formate and Pd/C in ethanol. The reactions were
conducted at 120°C for 3 minutes under microwave radiation. The compounds were isolated by filtration of the reaction mixture through celite, evaporation \textit{in vacuo} and extraction with CHCl$_3$. The compounds were obtained in 38-100% yields as viscous oils. The $^1$H NMR spectrum of the products displayed a shift to higher fields for the aromatic protons when compared to the nitro compounds and appearance of a broad singlet characteristic of the amine. Figure 2.13 depicts the $^1$H NMR spectrum of 2.0.5.

![Figure 2.13](image)

**Figure 2.13** $^1$H NMR spectrum of compound 2.0.5 in CDCl$_3$. The aromatic signals for $H_b$ and $H_c$ experimented a shift to higher fields with respect to the nitro precursor (Figure 2.12). The amine appeared as a broad singlet at 3.57 ppm.

### 2.5.3 Synthesis of the side chains leading to compounds 2.9-2.12

The syntheses of the side chains leading to compounds 2.9-2.12 were carried out from compound 2.0.3 in a similar fashion to the syntheses of the side chains leading to compounds 2.1-2.7 (Figure 2.14).

![Figure 2.14](image)

**Figure 2.14** Synthetic pathway for side chains for compounds 2.9-2.12. Reaction conditions: a) 3-chloropropionyl chloride, 50°C, 16 h, 80%; b) Me$_2$S-BH$_3$, THF, 60°C, 3 h, 91%; c) Amine $R$, neat or THF, RT, 16 h, 75-99%; d) Ammonium formate, Pd/C, ethanol, MW 120°C, 3 min, 37-89%.
An additional step was necessary for the reduction of the carbonyl group, prior to the reaction with the amines, to obtain 2.0.18. There are a number of available methods that can be used to reduce the amide functionality to an amine, such as LiAlH₄ or catalytic hydrogenation, which can potentially interfere with the nitro group. We chose Me₂S·BH₃ complex in THF for this reaction. The reaction went to completion upon treatment with two equivalents of Me₂S·BH₃ at room temperature for 2 hours and 60°C for one hour. The product was obtained in 91% yield. The transformation was confirmed by the appearance of a new signal in the NMR spectra corresponding to the newly formed CH₂ group. The signal of the hydrogen of the NH was significantly shifted high field compared to the amido NH of compound 2.0.3. The ¹H NMR spectrum of compound 2.0.18 is depicted in figure 2.15.

**Figure 2.15** ¹H NMR spectrum of compound 2.0.18 in CDCl₃. Signals for the three CH₂ groups Hd (distorted triplet), He (quintuplet), and Hf (triplet), confirmed the identity of the product.

Compound 2.0.18 was treated with the correspondent amines under similar conditions to those for the substitution reactions with compound 2.0.3. The reactions proceeded to the products in yields in the range of 75-99%. The yields were in general significantly higher than those of the substitution reactions from compound 2.0.3. A possible reason for this difference is that the elimination reaction of the chlorine is less favourable, which was the main side reaction using 2.0.3. The absence of the carbonyl group makes the carbon β to the chlorine less acidic and the alkene product of elimination not as thermodynamically stable as the conjugated carbonyl-containing counterpart.
The nitro compounds were reduced to the correspondent amines as described previously. The characterisation of the compounds was achieved by spectroscopic methods. The $^1$H NMR spectrum for compound 2.0.24 is depicted in figure 2.16.

**Figure 2.16** $^1$H NMR spectrum of compound 2.0.24 in CDCl$_3$. Signals for the three CH$_2$ groups e, f and g and the singlet for the dimethyl amine methyl groups h confirmed the identity of the targeted compound. Hydrogens Ha and Hd appeared together as a broad singlet.

### 2.5.4 Synthesis of the side chains leading to compounds 2.13-2.15

Compounds 2.13-2.15 contain shortened and lengthened side chains with respect to compound 2.1. The synthetic strategy for these side chains was similar to the synthesis of the side chain for compound 2.1 (Figure 2.17).

**Figure 2.17** Synthetic pathway for side chains for compounds 2.13-2.15. Reaction conditions: a) Chloroacyl chloride, 50°C, 16 h, 45-85%; b) Pyrrolidine, RT, 16 h, 34-57%; c) Ammonium formate, Pd/C, ethanol, MW 120°C, 3 min, 57-89%.
4-nitroaniline was treated with the appropriate chloroacetyl chloride. The acid chlorides used were chloroacetyl chloride for 2.0.27, 4-chlorobutyryl chloride for 2.0.30 and 5-chloropentaryl chloride for 2.0.33. The reaction yields were 85% for the reaction for 2.0.27, 45% for 2.0.30 and 66% for 2.0.33. The characterisation of the compounds was achieved by spectroscopic methods. The aliphatic regions of the $^1$H NMR spectra were characteristic of each compound. Compound 2.0.27 presented a singlet for the only CH$_2$ group at 3.27 ppm. Compounds 2.0.30 and 2.0.33 presented three and four signals respectively correspondent to the CH$_2$ groups.

Compounds 2.0.27, 2.0.30 and 2.0.33 were treated with pyrrolidine as solvent at room temperature for 24 hours and the reactions proceeded with poor yields 34-54%. The nitro compounds were reduced to the correspondent amine compounds as described previously. The $^1$H NMR spectrum of 2.0.35 is shown in figure 2.18.

![Figure 2.18 $^1$H NMR spectrum of compound 2.0.35 in CDCl$_3$. The aliphatic region shows the signals for the carbon chain with 2 triplets e and h and 2 quintuplets f (partially overshadowed by the pyrrolidine signal) and g at higher field.](image)

2.5.5 Synthesis of the side chains leading to compounds 2.8, 2.16 and 2.17

Synthesis of 2.0.38, side chain for 2.8, was conducted as described in figure 2.19.

![Figure 2.19 Synthetic pathway for side chain 2.0.38. Reaction conditions: a) Succinic anhydride, toluene, reflux, 1 h, 59%; b) Pyrrolidine, HOBt, EDCI, DMF, RT, 16 h, 86%; c) Ammonium formate, Pd/C, ethanol, MW 120°C, 3 min, 80%.](image)
The synthesis started with the reaction of 4-nitroaniline with succinic anhydride in toluene under reflux for 1 hour to obtain the carboxylic acid 2.0.36 in 59% yield. The next step was the amide formation with pyrrolidine using HOBT and EDCI in DMF overnight at room temperature, which gave the product 2.0.37 in 86% yield. The nitro compound was reduced to the correspondent amine, compound 2.0.38, as described previously. The characterisation of the intermediates and final compound was achieved by spectroscopic methods. Figure 2.20 shows the $^1$H NMR spectrum for compound 2.0.38.

![Figure 2.20](image.png)

Figure 2.20 $^1$H NMR spectrum of compound 2.0.38 in CDCl$_3$. The hydrogens in the CH$_2$ groups of the pyrrolidine lose their equivalence with the presence of the N-carbonyl resulting in a multiplet $g$ and two quintuplets $h$ and $i$. The quintuplets are result of the coupling of each hydrogen type to the other four non-equivalent protons in their vicinity.

Synthesis of 2.0.41, side chain for compound 2.16, was achieved by treatment of 3-nitroaniline with 3-chloropropionyl chloride to obtain 2.0.39 in 79% yield, followed by treatment with pyrrolidine, in a similar manner as described before, to obtain 2.0.40 in poor yield (13%). The nitro compound was reduced to the correspondent amine compound 2.0.41 as described before (Figure 2.21).

![Figure 2.21](image.png)

Figure 2.21 Synthetic pathway for side chain 2.0.41. Reaction conditions: a) 3-chloropropionyl chloride, 50°C, 16 h, 79%; b) Pyrrolidine, RT, 16 h, 13%; c) Ammonium formate, Pd/C, ethanol, MW 120°C, 3 min, 63%.
The characterisation of the compounds was achieved by spectroscopic methods. The \(^1\)H NMR spectrum of compound **2.0.41** is depicted in figure 2.22.

![Figure 2.22](image)

**Figure 2.22** \(^1\)H NMR spectrum of compound **2.0.41** in CDCl\(_3\). The *meta* substituted benzene ring displays the expected couplings. \(H_b\) and \(H_d\) couple (apart from with \(H_e\)) with \(H_e\) with a small \(J\) (2.0 Hz).

The reaction of 2-nitroaniline with 3-chloropropionyl chloride to obtain the ortho-substituted analogue did not proceed even after treatment at 150°C for 3 hours under microwave radiation.

Synthesis of **2.0.44**, side chain for compound **2.17**, was conducted as described in figure 2.23.

![Figure 2.23](image)

**Figure 2.23** Synthetic pathway for side chain **2.0.44**. Reaction conditions: a) \(N,N\)-pyrrolidinepropyldiamine, HOBt, EDCI, MeCN, RT, 16 h, 73%; b) Ammonium formate, Pd/C, ethanol, MW 120°C, 3 min, 100%.

Compound **2.0.42** (kindly provided by Michael Moore) was treated with \(N,N\)-pyrrolidinepropyldiamine and the coupling reagents HOBt and EDCI in acetonitrile overnight at room temperature to obtain compound **2.0.43** in 73% yield. The nitro compound was reduced to the correspondent amine **2.0.44** as described before. The characterisation of the compounds was achieved by spectroscopic methods. The \(^1\)H NMR spectrum for compound **2.0.44** is depicted in figure 2.24.
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Figure 2.24 $^1$H NMR spectrum of compound 2.0.44 in CDCl$_3$. Hydrogens $H_b$ and $H_c$ in the aromatic region show a coupling constant of $J=2.1$ Hz. The methyl group in the pyrrole gives a singlet at 3.79 ppm. In the aliphatic region, the signal of the hydrogens $H_f$ splits into a quartet due to the coupling to the proton of the amide $H_e$, although the $H_e$ signal was as a broad singlet instead of a triplet.

2.5.6 Coupling reactions between the acridone core and side chains

The first attempt to synthesise 2.1 in our labs involved the chlorination of 4,5-dicarboxyacridone 2.0.1 using thionyl chloride followed by the condensation reaction with the anilinic side chain 2.0.5 (Michael Moore, personal communication). However, the chlorination occurred at both, the carboxylic acid groups and the acridone carbonyl, and treatment with the aniline resulted in a complex mixture of different chlorinated and aniline substituted compounds including 2.1 but also 4,5,9-trisubstituted acridine and 9-chloro-4,5-disubstituted acridine amongst other sub-products. Compound 2.1 was obtained after HPLC purification in 6% yield.

To try to improve the reaction conditions to adequate them to the production of a library, different peptide coupling reagents were investigated for the formation of 2.1 from 2.0.1 and 2.0.5. The acridone starting materials 2.0.1 and 2.0.2 presented low solubility in common solvents other than DMSO, DMF and DMA. For this reason, the different reagents were evaluated in DMF. Some of the most commonly used peptide bond forming reagents, EDCI, DCC, DPPA, CDI, HOBT and PyBOP, were tested with a range of reaction conditions, including room temperature and microwave heating at 80°C, different reagents stoichiometries and different bases (when appropriate). The reactions were monitored by LC/MS and product was only detected in the reactions that employed HOBT or PyBOP. However, the reactions with PyBOP were more efficient and gave less side products.
PyBOP is a modified version of the HOBt (benzotriazole-1-hydroxy) reagent. PyBOP contains a tripyrrolidinophosphonium modification at the hydroxyl group of HOBt. The formation of the active species in the coupling reaction, the benzotriazolyl ester, occurs more readily with the use of PyBOP due to the mediation of the tripyrrolidinophosphonium ester intermediate.

The reactions at room temperature proceeded in 24 hours. Reactions using less than 3 equivalents of the aniline 2.0.5 often gave some monocoupled product. To avoid this and increase the conversion, four equivalents of the side chain with respect to the acridone were preferentially used. The reaction conditions were further optimised by investigating different solvent mixtures. Mixtures of DMF and DMA with DCM and MeCN were tested and mixtures of DMF and MeCN consistently resulted in cleaner reactions. A 3:1 ratio DMF/MeCN was used for the synthesis of the library.

The optimised conditions (acridone/side chain in 1 : 4 ratio, 3 equivalents of PyBOP, in a DMF/MeCN mixture 3 : 1 ratio and room temperature for 24 hours) were used for the synthesis of compounds 2.1-2.19 by reaction of the acridone core 2.0.1 (or 2.0.2 for compound 2.19) with the appropriate side chain (Figure 2.25).

![Figure 2.25 Synthetic pathway for compound 2.1. Reaction conditions: a) PyBOP, DMF/MeCN, RT, 24 h, 85%.](image)

Isolation of the product was accomplished by precipitation from the reaction mixture by addition of EtOAc and collection by filtration. Purification was achieved by repeated precipitation of the product from a DMF solution using EtOAc. Typically, the purity obtained after re-precipitation was over 90% and the yield higher than 50%. Preparative HPLC was used when the purity (as measured by HPLC) was lower than 95%. The characterisation of the compounds was carried out by spectroscopic methods. The \(^1\)H NMR spectrum of compound 2.1 is depicted in figure 2.26.
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Figure 2.26 $^1$H NMR spectrum of compound 2.1 in DMSO-$d_6$. Inset: COSY NMR spectrum detail showing the coupling between protons Hj and Hl with protons Hk and Hl respectively (signals overshadowed by the residual solvent peak).

2.6 CONCLUSIONS

The synthesis of 19 novel acridone compounds was accomplished. A novel approach to obtain a larger planar moiety was employed by introducing conjugated aromatic side chains to the acridone core. This library contains analogues of compound 2.1 with a number of modifications on the side chains potentially useful to obtain structure-activity relationships, following the biophysical and biological evaluation described in this thesis (Chapters 3 and 4).

A new and more efficient methodology for the synthesis of precursor compound 2.0.1 was found in the use of ortho-bromoisophthalic acid for the Jourdan-Ullmann reaction.

The synthetic route for the side chains was shown to be able to accommodate the utilisation of a variety of acid chlorides and amines. The reduction of the nitro groups was successfully conducted using ammonium formate under microwave heating and these conditions represent a fast and high yielding method for the reduction of a variety of aromatic nitro groups.

The coupling reaction between compound 2.0.1 and the anilinic side chains was optimised for the reaction with 2.0.5. The applicability of this reaction with a variety of aromatic and not aromatic side chains was proven and it represents a robust synthetic tool to access compounds of this type.
CHAPTER 3

BIOPHYSICAL EVALUATION OF ACRIDONE G-QUADRUPLEX TARGETING AGENTS
3 BIOPHYSICAL EVALUATION OF G-QUADRUPLEX TARGETING AGENTS

3.1 ASSAYS FOR THE EVALUATION OF G-QUADRUPLEX LIGANDS

3.1.1 Background

The interaction of compounds with DNA has been evaluated in the past by the use of various techniques that take advantage of some physical-chemical properties of the DNA, ligands or DNA/ligand complexes. They include:

1. UV/fluorescence techniques. The UV or fluorescence spectra of molecules often show significant changes when these are in solution or bound to macromolecules such as DNA. These differences can be exploited to measure the binding ability of small molecules to DNA by monitoring the variations in intensity or wavelength of their different absorption and/or emission signals. Experiments using different concentration ratios of the species allow one to study the stoichiometry and binding constants of small molecules with DNA (Cheng et al., 2008).

2. Circular dichroism. Circular dichroism (CD) spectroscopy measures the difference in absorption between left and right handed polarised light by a sample. The different absorption is caused by the different interaction of light with the molecules in the sample, which is consequence of their chirality. This property has been used in structural analysis of proteins and nucleic acids (Fasman, 1996). The effect that altering the DNA environment of a DNA solution (for example with changes in salinity or introduction of ligands) causes on some characteristic peaks of the CD spectrum has been used to detect changes in the secondary conformation of DNA.
3. Isothermal titration calorimetry. The energy in form of heat (enthalpy) released or taken upon the interaction of two species in solution can be measured. The enthalpy can be used to calculate other thermodynamic parameters. Isothermal titration calorimetry has been employed to measure enthalpy and evaluate the binding constants and stoichiometry of small molecules with DNA (Harding & Chowdhry, 2000). It has been successfully used in many studies including the study of acridine-based DNA interacting drugs (Hutchins et al., 2003).

4. Voltammetric methods. The interaction of ligands with DNA has been evaluated in the past exploiting the electrochemical properties of redox active species (Carter & Rodriguez, 1989; Aslanoglu & Oge, 2005). The square wave voltammograms are a useful way of measuring this interaction. The electro-stimulation of a solution of the electroactive species by application of a voltage creates an increase in the electric current, the intensity of which is dependent on the concentration of the species under study. DNA-bound molecules experiment a decreased diffusion rate and do not contribute significantly to the observed current (Welch & Thorp, 1996). Titrations of ligand solutions with DNA can be employed to establish the binding constants of the ligands with the DNA.

5. Equilibrium dialysis. This technique relies on the diffusion of small molecules through a selectively permeable membrane that separates two chambered solutions, one of the DNA and another one of the ligand (Chaires, 2005). The system equilibrates until the concentration of free ligand in both chambers equalises. Measurements of the concentration of free ligand and DNA-bound ligand can be employed to calculate the equilibrium binding constants.

6. Viscosimetry titrations. The binding affinity of duplex DNA intercalators has been traditionally evaluated by viscosimetry titrations (Yen et al., 1982). Duplex DNA intercalators produce a structural distortion of the DNA, necessary to accommodate the ligand between two pairs of bases. This unwinding distortion results in an increase of the length of the DNA helix. The viscosity of DNA solutions is dependent, among other factors, on the concentration and length of the DNA. Ligand titrations of duplex DNA result in a progressive increase in the relative specific viscosity as the molar ratio of the
compound to DNA base pairs is increased. This technique allows one to study the stoichiometry and binding affinity of the ligands.

7. DNA footprinting. In DNase I footprinting assays, a radiolabelled DNA fragment is digested with DNase I enzyme in presence of ligand. The resulting fragments of DNA can be visualised using polyacrylamide gels. The tight binding of a ligand to a specific region of the sequence is detected as an absence of the fragments corresponding to the excision in that region of the DNA. Titration experiments with ligands can be used to study the binding ability of ligands and also importantly their sequence specificity (Elliott et al., 1989; Wang et al., 2000).

8. Melting experiments. The stability of a DNA sequence or a DNA/ligand complex can be evaluated by measuring the temperature at which the DNA loses its secondary conformation. This temperature is known as the melting temperature. The process of melting can be monitored by the use of different techniques, such as UV, CD spectroscopy or fluorescence (Wilson et al., 1997; Rachwal & Fox, 2007). See next section for a further introduction to these techniques.

The surface plasmon resonance (SPR) technique will be presented in chapter 6.

Other techniques, such as electrophoresis and luminescence-based techniques have also been occasionally employed.

3.1.2 Fluorescence resonance energy transfer assay (FRET)

3.1.2.1 Background

A widely used method to measure variations in the melting temperature of DNA, is the so-called FRET assay (Mergny, 1999; Mergny & Maurizot, 2001; Darby et al., 2002). The assay is based on the fluorescence resonance energy transfer (FRET) phenomenon.

The FRET phenomenon occurs between two fluorophores, named donor and acceptor. Upon irradiation of the donor with an appropriate light source, electrons are promoted to high energy states. The energy gained is subsequently released as vibrational energy
first and finally as radiation. The emitted radiation is of lower energy, hence longer wavelength. When a second fluorophore, the acceptor, is appropriately located, the released energy is transferred to it by a non-radiative process and FRET is said to occur. The donor and acceptor must accomplish a series of requirements:

1. Their fluorescent properties must be compatible. The absorption and emission profiles must be so that the wavelength of the radiation emitted by the donor is similar (ideally the same) to the optimum wavelength for the excitation of the acceptor.

2. They must be close in space. The efficiency of the energy transfer is dependent on the distance between donor and acceptor and their relative orientation. The efficiency of the transfer ($E$) can be evaluated by the following equations:

$$E = (1 + R^6/R_0^6)^{-1} \quad (1); \quad R_0^6 = 8.8 \times 10^{-28} \phi_D \kappa^2 n^4 J(v) \quad (2)$$

where $R$ is the distance between both fluorophores and $R_0$ is the Forster radius which defines the distance at which the FRET is 50% efficient. $R_0$ depends on: $\phi_D$, the fluorescence quantum yield for the donor; $\kappa$, a parameter that depends on the orientation of the fluorophores respect to each other; $n$, the refractive index of the medium; and $J(v)$, the integral of the overlap of the emission spectrum of the donor and excitation spectrum of the acceptor.

The FRET phenomenon can occur freely when both fluorophores are independent molecules in solution. The strategic attachment of these fluorophores as tags to biological macromolecules limits the occurrence of FRET to some situations when the donor and acceptor are appropriately situated. This can be of use to study variations in the structure of macromolecules.

### 3.1.2.2 FRET applied to G-quadruplexes

The FRET phenomenon has been exploited to study the stability of nucleic acid structures and, in particular, G-quadruplex DNA (Mergny & Maurizot, 2001). The transition between two conformations of a DNA molecule modified with the
fluorophores can be monitored by measuring the efficiency of FRET throughout the process (Figure 3.1). G-quadruplex forming sequences have been studied with the introduction of fluorophores 6-carboxyfluorescein (FAM), to act as the donor, and 6-carboxytetramethylrhodamine (TAMRA), to act as the acceptor (Darby et al., 2002), attached to the 5’ and 3’ ends respectively via 6-carbon linkers. This pair of fluorophores have a Forster radius $R_o$ of approximately 5 nm and when the DNA forms a G-quadruplex, the fluorophores lay close enough to each other so FRET can efficiently occur. When the DNA is in the unfolded random coil form the fluorophores are separated from each other and the FRET efficiency significantly diminishes.

![Figure 3.1](image)

**Figure 3.1** The FRET experiment applied to the study of G-quadruplex DNA. a) Structure of the donor (FAM) including the 6-carbon linker; b) Structure of the acceptor (TAMRA) including the 6-carbon linker; c) Schematic representation of the melting process of a tagged G-quadruplex: arrows represent the excitation radiation of the donor and the emitted energy by the donor, absorbed by the acceptor when the DNA is folded or detected as radiation when is unfolded.

This property allows one to monitor the melting process of G-quadruplex structures, which will be detected as a decrease in the FRET efficiency and consequent increase in the intensity of the radiation emitted by the donor. The values of fluorescence measurements throughout the melting process plotted against the temperature result in a sigmoidal curve, from which a melting point for the structure can be obtained (corresponding to the point with maximum slope).

It must be noted that the curves obtained for the different experiments are, to various degrees, not pure sigmoidal curves. The melting process is unlikely to occur in one single step, instead, several partially unfolded intermediate structures are likely to occur. The different intermediates may present different FRET efficiencies and this may result in the detection of a multiphasic melting process. Furthermore, G-quadruplexes are highly polymorphic and several conformations can be adopted by a single sequence upon annealing or they can be part of an equilibrium. The different structures may
display different stabilities under the conditions of the experiment and this may contribute to an apparent multiphasic melting process.

The FRET experiment can be conducted in high-throughput fashion using 96-well plates. For this reason, a script was developed in our group to process the large amounts of data resulting from the experiments (Christoph Schultes, PhD thesis, 2004). The program normalises the fluorescence readings, calculates the first derivative and measures the maximum point of the derivative. For a sigmoidal curve, the maximum of the first derivative corresponds to the summit point of the curve and this can be assigned as the melting temperature (Figure 3.2-a). When several transitions occur during the melting process the curve contains “shoulders” and the use of this script would misjudge the melting temperature (Figure 3.2-b). For these cases an average melting temperature can be obtained by manually measuring the temperature at which a 50% increase in the fluorescence is recorded.

![Figure 3.2](image.png)

**Figure 3.2** Representations of normalised fluorescence against temperature in two examples of FRET melting experiments. a) On the left, a single transition melting process results in a sigmoidal curve, which can be processed with a script to obtain the melting temperature \( T_m \) from the first derivative graph; b) On the right, a multi-step melting process yields a curve having several “shoulders” and the first derivative curve contains several maxima. The \( T_m \) can be obtained in this case from the value at which the normalised fluorescence is 0.5.

It is necessary to remember at this point that FRET is a semi-quantitative method and the structural analysis of the different topologies or intermediate structures adopted in the melting process is beyond the scope of the method.

The use of fluorescence for monitoring the melting process of DNA has several advantages over other alternatives, such as CD or UV (Rachwal & Fox, 2007). CD
utilises sophisticated apparatus and requires high concentrations of oligonucleotide, resulting in high costs. UV measurements also require high concentrations of materials. Furthermore, the absorbance changes detected using UV are small in comparison with the larger signal changes obtainable using fluorescence. In addition, UV and CD measurements cannot be conducted in high-throughput fashion.

3.1.2.3 FRET applied to the screening of G-quadruplex ligands

FRET has been extensively used for the evaluation of the binding affinities of G-quadruplex ligands (for example, Guyen et al., 2004). The association of DNA and ligand results in a complex with lower energy. This complex melts, consequently, at a higher temperature. The different affinity of the ligands for DNA can be assessed using FRET by measuring the different stabilisations that they cause. The FRET results have been shown to correlate with results from UV melting profiles (Mergny & Maurizot, 2001). Upon monitoring of the melting process of a DNA sequence in presence of increasing concentrations of ligand, curves representing melting temperatures ($T_m$) against ligand concentration can be obtained (Figure 3.3-a) or, similarly, increment of the melting temperature with respect to the control experiment ($\Delta T_m$) against ligand concentration (Figure 3.3-b). To compare the abilities of different ligands, the $\Delta T_m$ at 1 $\mu$M is generally used.

![Figure 3.3](image)

**Figure 3.3** Two types of representations of FRET melting experiments results for two examples of ligand. a) On the left, melting temperature ($T_m$) is represented against ligand concentration. b) On the right, increment of the melting temperature ($\Delta T_m$) is represented, by normalising with the control experiment, against ligand concentration.

Three G-quadruplex sequences were chosen for FRET evaluation with the ligands. They were the mimic of the human telomeric G-quadruplex sequence, F21T, and two
sequences present in the promoter region of the *c-kit* gene that have been shown to be able to form G-quadruplexes, Ckit-1 and Ckit-2 (Rankin *et al.*, 2005; Fernando *et al.*, 2006). In addition a sequence that forms a duplex DNA structure, Dup, was also used to evaluate the selectivity between G-quadruplex and duplex DNA. The modified sequences are:

- **F21T**: 5'-*FAM*-GGGTTAGGGTTAGGGTTAGGG-3'  
- **Ckit-1**: 5'-*FAM*-AGAGGGAGGGCGCTGGGAGGAGGGGCT-3'  
- **Ckit-2**: 5'-*FAM*-CCCGGGCGGGCGCGAGGGAGGGGAGG-3'  
- **Dup**: 5'-*FAM*-TATAGCTATA-HEG-TATAGCTATA-3'  
  (*HEG* linker: [(-CH₂-CH₂-O-)₆]).

The stability of the DNA structures, hence their melting temperature, depend on a number of factors such as salinity and pH. Critically in the case of the G-quadruplexes, the type of monovalent cation can determine the conformation that is adopted by a particular sequence. This has been previously reported for the human telomeric G-quadruplex. The sequence in Na⁺ adopted an antiparallel conformation (Wang & Patel, 1993), however in K⁺, the adopted structure was all parallel (Parkinson *et al.*, 2002). To obtain a set of conditions analogous to the physiological conditions the FRET experiment was run in a K⁺ based buffer at pH 7.4, from now on referred to as “FRET buffer” (50 mM potassium cacodylate, pH 7.4). Under these conditions, the melting temperatures of the sequences are relatively low enabling the observation of large melting temperature changes (Table 3.1).

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Melting Temperature in FRET buffer (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F21T</td>
<td>58.4 ± 0.9</td>
</tr>
<tr>
<td>Ckit-1</td>
<td>52.5 ± 1.1</td>
</tr>
<tr>
<td>Ckit-2</td>
<td>53.5 ± 0.5</td>
</tr>
<tr>
<td>Dup</td>
<td>54.7 ± 0.5</td>
</tr>
</tbody>
</table>

*Table 3.1 Temperatures of melting of the sequences in FRET buffer.*
3.1.2.4 Competition FRET assay

The amount of G-quadruplexes in the genome is overwhelmed by the abundance of duplex DNA. Ligands targeting G-quadruplexes must present an exceptional discriminatory ability to avoid side effects as a consequence of their interaction with duplex DNA. A modified FRET assay, the competition FRET assay, was developed to evaluate ligand selectivity between two DNA structures, with both sequences present in solution (Mergny & Maurizot, 2001). The assay is performed in an analogous manner to the G-quadruplex experiments described above with the addition of a certain amount of non-labelled duplex DNA, which will "compete" for the ligand with the G-quadruplex DNA. If the ligand shows any affinity for the duplex DNA, the effect on the stabilisation of the G-quadruplex structures will be diminished, on the contrary, if the ligand is completely selective for G-quadruplex DNA, the addition of duplex DNA should not alter the results of the G-quadruplex melting (Figure 3.4).

Figure 3.4 Representation of two cases in the competition FRET experiment. a) The ligand (blue square) shows high selectivity for G-quadruplex DNA resulting in high $\Delta T_m$; b) A ligand with high affinity for duplex DNA does not influence the melting of the G-quadruplex resulting in low $\Delta T_m$.

Different sources of duplex DNA have been used for this type of assay. It was decided to use the unexpensive and commercially available purified DNA of calf thymus, as used previously (For example, Moorhouse et al., 2006).
3.1.3 Telomerase repeat amplification protocol assay (TRAP)

3.1.3.1 Background

The telomeric repeat amplification protocol assay (TRAP) has been widely employed to evaluate the telomerase inhibitory ability of compounds and to detect telomerase activity in cell extracts (Kim et al., 1994; Kim & Mu, 1997; Gomez et al., 2002). G-quadruplex formation in telomeric DNA can avoid telomerase binding and result in the inhibition of telomerase activity. G-quadruplex ligands have been evaluated as telomerase inhibitors using the TRAP assay, schematically represented in figure 3.5 (Gomez et al., 2002).

**Figure 3.5** Schematic representation of the TRAP assay principle. Telomerase extends the DNA primer adding successive copies of the telomeric repeats. Upon addition of four units or more, the DNA can fold into a G-quadruplex, particularly when aided by a ligand. The G-quadruplex inhibits the further extension of the primer by telomerase, resulting in inhibition of the formation of the longer DNA molecules.

The assay has been traditionally conducted in two steps:

1. In the first step a DNA primer and telomerase are incubated under conditions ideal for primer extension by telomerase. The addition of four or more telomeric units to the primer confers it the ability to form G-quadruplexes. When the single stranded DNA is sequestered by a ligand to form G-quadruplex DNA, the sequence is no longer able to act as a substrate for telomerase and no more telomeric units can be added. The analysis
of the different products of this process enables the evaluation of the inhibitory ability of the ligands.

2. The second step in the assay is a PCR to amplify the resulting telomerase products to enable quantification. To validate the TRAP assay, internal standard controls or independent Taq inhibition assays have been used to discard the possibility of PCR inhibition by the ligands. However, the evaluation of G-quadruplex ligands with this assay was recently found to be not adequate since the ligand-stabilised G-quadruplexes were found to efficiently inhibit Taq polymerase (de Cian et al., 2007b).

The main alternative to the TRAP assay is the so-called direct assay, in which a radiolabelled nucleotide is used to detect the extension products by means of radioactivity measurements without the need of a PCR step (de Cian et al., 2007b). This method presents several problems. First, the amount of telomerase needed is very high and is not easily sourced from normal cellular extracts. Secondly, there is an inherent risk in the use of the relatively high amounts of radioactive materials needed for this assay.

3.1.3.2 Modified TRAP assay

A set of modifications to the TRAP assay was envisaged in our laboratories to solve the experimental design problems (Reed et al., 2008). The main issue with the traditional assay is the presence of the ligand in the PCR step. To avoid this interference a new step was introduced to obtain ligand-free DNA prior to the PCR step. This was achieved by using the QIAquick nucleotide purification kit (Qiagen). This kit allows the purification of single and double-stranded oligonucleotides of 17 bases or longer. A positively charged membrane is employed to retain the DNA, which can be washed and subsequently eluted. This assay has been successfully employed with a number of established telomerase inhibitors and similar values to those obtained with the direct assay have been found.
3.2 MATERIALS AND METHODS

3.2.1 Compound preparation

The solids, in free base form, were dissolved in DMSO to obtain 10 mM solutions and were kept as stock solutions at -20°C. The stability of the compounds under these conditions was confirmed by periodical HPLC analysis.

3.2.2 Fluorescence resonance energy transfer assay (FRET)

The appropriate tagged DNA (page 74) (Eurogentec, UK) stock solution in water (20 μM) was diluted to 400 nM using FRET buffer (50 mM potassium cacodylate, pH 7.4) and annealed by heating at 85°C for 5 minutes and cooling down to room temperature over 5 hours. Compound solutions were prepared in concentrations twice the final ones. The first dilution of the 10 mM DMSO stock solutions was done with 1 mM HCl in water to obtain 1 mM solutions. The rest of the dilutions were performed using FRET buffer. 50 μL of annealed DNA and 50 μL of compound solution were put into each well of a 96-well plate (MJ Research, Waltham, MA) and processed in a DNA Engine Opticon (MJ Research). Fluorescence readings were taken at intervals of 0.5°C over the range 30-100°C, with a constant temperature being maintained for 30 seconds prior to each reading. The incident radiation was 450-495 nm and the detection was conducted at 515-545 nm. The raw data were imported into the program Origin (version 7.0, OriginLab Corp.) and the graphs were smoothed using a 10-point running average and subsequently normalised. For the experiments with sequences F21T, Ckit-2 and Dup, the determination of the melting temperatures was performed by obtaining the values at the maxima of the first derivative of the smoothed melting curves using a script. For the experiments with the Ckit-1 sequence, for which pure sigmoidal curves were not obtained, the melting temperatures were obtained manually as the temperature at which a 50% increase in the normalised fluorescence was obtained. The difference between the melting temperature of the experiments with compound and the melting temperature for the blank experiment (ΔTm) was used for comparison.
3.2.3 Competition FRET assay

The competition FRET assay was run using the tagged G-quadruplex sequence F21T. The assay was performed in a similar way to that described above with the following modifications: compound dilutions were prepared in concentrations four times the final ones and added as 25 μL aliquots. The competitor duplex DNA, calf thymus DNA (Sigma-Aldrich, UK), was diluted from the stock solution in FRET buffer to a concentration four times the final one and added as 25 μL aliquots.

The stock calf thymus DNA solution was obtained by dissolving the solid in FRET buffer by gentle shaking over 48 hours and removal of the remaining solid by centrifugation and decantation of the supernatant. The DNA concentration was measured by UV.

To express the relative concentrations of both DNA types it was considered appropriate to calculate them in terms of G-quartets and pairs of bases concentrations for the G-quadruplex and duplex DNA respectively. Experiments at the concentration ratios 1:1, 1:10, 1:100 and 1:300 (G-quartets : pair of bases) were performed.

3.2.4 Telomerase repeat amplification protocol assay (TRAP)

All reagents and solutions were prepared using PCR-grade water. The assay consisted of four independent steps and for consistency they were performed in the same day:

1. Primer elongation step. A mixture containing TS forward primer (0.1 μg; 5'-AAT CCG TCG AGC AGA GTT-3'), bovine serum albumin (0.05 μg), and dNTPs (125 μM each) diluted in TRAP buffer (20 mM Tris-HCl pH 8.3, 68 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 0.05% v/v Tween-20) to make 34 μL was prepared for each experiment. The appropriate amount of protein cell extract containing telomerase, obtained from exponentially growing A2780 human ovarian carcinoma cells, was added at 0°C to TRAP lysis buffer (10 mM Tris-HCl pH 7.4, 0.5% CHAPS, 1 mM MgCl₂, 1 mM EGTA, 5 mM β-mercaptoethanol, 10% glycerol, 0.1 mM AEBSF) to obtain the required protein concentration (500 μg/μL) and the appropriate amount (2 μl) was added
to the experiments and positive controls. TRAP lysis buffer (2 µl) was added to the negative controls instead of protein. Compound solutions were prepared in concentrations 10 times the final one. A first dilution of the 10 mM DMSO stock solutions with 1 mM HCl in water was done to obtain 1 mM solutions. The rest of the dilutions were performed using water. 4 µL of these solutions were added to the reaction mixtures on ice to make a final volume of 40 µL. Water was added instead to the control vehicles. The initial telomere elongation step was carried out for 10 minutes at 30°C, followed by a protein heat inactivation step (4 minutes at 94°C) and final maintenance of the mixture at 20°C.

2. Ligand removal step. The ligands were then separated from the DNA using the QIAquick nucleotide purification kit (Qiagen) following the manufacturers instructions but eluting the DNA with water. In brief, the reaction mixtures resulting from step 1 were diluted in isopropanol-based buffer and the mixture applied to a spin tube and centrifuged. The membrane was then washed twice with ethanol-based buffer by centrifugation. The DNA was then eluted water. This mixture was taken to dryness in vacuo and the DNA was re-dissolved in water (40 µL).

3. PCR amplification step. 10 µl of the PCR reaction mixture were added to each experiment. The mixture contained: ACX reverse primer (1 µM; 5'-GCG CGG [CTTACC]3 CTA ACC-3'), TS forward primer (0.1 µg), BSA (5 µg), 0.5 mM dNTPs and 2U of Taq polymerase (RedHot, ABgene, Surrey, UK) in 5X TRAP buffer. The PCR step consisted of 2 minutes at 92°C and 35 cycles of 94°C for 30 seconds, 61°C for 1 minute and 72°C for 1 minute with final maintenance at 4°C.

4. Visualisation step. The products were separated using a 10% w/w PAGE gel in TBE buffer and stained with SYBR green I (Sigma-Aldrich, UK) for 5 min. The fluorescence was measured in a gel scanner with GeneTools software (Syngene, Cambridge, UK). Measurements were corrected by subtraction of the background using the negative controls and normalisation against the positive control. The values were exported to the program Origin and plotted against compound concentration. A logistic dose-response curve was fitted and the values of concentration for which fluorescence was 50% of the positive control were taken as the telomerase EC<sub>50</sub>. 
3.3 RESULTS OF THE BIOPHYSICAL EVALUATION OF THE ACRIDONE SERIES

3.3.1 Fluorescence resonance energy transfer assay (FRET)

The DNA binding abilities of compounds 2.1-2.19 and reference compound BRACO-19 (BR-19) with the sequences F21T, Ckit-1, Ckit-2 and Dup were assessed using the FRET assay. The values of $\Delta T_m$ at 1 $\mu$M were used for comparison (Table 3.2).

<table>
<thead>
<tr>
<th>Comp. Code</th>
<th>FRET F21T $\Delta T_m$ [c=1 $\mu$M] ($^\circ$C)</th>
<th>FRET Dup $\Delta T_m$ [c=1 $\mu$M] ($^\circ$C)</th>
<th>FRET Ckit-1 $\Delta T_m$ [c=1 $\mu$M] ($^\circ$C)</th>
<th>FRET Ckit-2 $\Delta T_m$ [c=1 $\mu$M] ($^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
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<td>13.3</td>
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</tr>
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<td>2.5</td>
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<td>24</td>
</tr>
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<td>10.7</td>
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<td>3.8</td>
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<td>7</td>
<td>1</td>
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</tr>
<tr>
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<td>n.a.</td>
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</tr>
<tr>
<td>BR-19</td>
<td>32</td>
<td>6</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Table 3.2 FRET data results for the acridone ligands and reference compound BRACO-19. The values were calculated as the mean of two experiments. The uncertainty in the $\Delta T_m$ values is $\pm 0.5^\circ$C.

There is a large difference in the behaviour of the ligands with the G-quadruplex and duplex DNA sequences. The highest stabilisation obtained with the acridones for Dup is $3^\circ$C. The shifts in the $T_m$ were more pronounced for the G-quadruplex sequences and for F21T a maximum shift of $34^\circ$C was obtained while for Ckit-1 and Ckit-2 the values were $13.7^\circ$C and $25.7^\circ$C respectively.
There is a good correlation of binding abilities for the different sequences, especially so for the three G-quadruplex sequences, and good binders for one are generally good binders for the others (Figure 3.6).

This seems to suggest that the ligand modifications affect their binding mode and stabilisation ability in a similar fashion for all three G-quadruplexes, which could mean that all they share a similar binding mode. The duplex data is less conclusive and although the seven most potent binders for duplex are the most potent binders for the G-quadruplex sequences, the rest of the ligands do not seem to follow this trend.
Chapter 3 – Biophysical evaluation of acridone G-quadruplex targeting agents

The stabilisation of the F21T sequence reaches a temperature plateau of around 34°C, probably as a result of the saturation of the binding sites. For the F21T sequence, concentrations of 2.1 slightly higher than 1 µM achieve this saturation (Figure 3.7). None of the ligands of this series achieved the saturation of the Ckit-1, Ckit-2 or Dup sequences, since no plateau in the Tm was achieved at even 10 µM. This suggests a certain degree of discriminatory ability of the ligands between the F21T sequence and the rest.

The differences obtained by side chain modification are discussed next based on the data for the F21T sequence. Due to the good correlation between the different G-quadruplex sequences (Figure 3.6), the conclusions are extrapolatable to the Ckit sequences when not indicated otherwise.

The results supported several assumptions made in the library design (Table 2.1). The importance of the benzene rings in the side chains for G-quadruplex stabilisation was postulated. The relatively low ΔTm obtained for the compound with non-aromatic side chains 2.18 (ΔTm(1 µM) = 7°C) supports this notion. Compound 2.17, the analogue with the N-methylpyrrole substituting the benzene, showed limited stabilisation (ΔTm(1 µM) = 15°C) possibly due to an unfavourable planar conformation, a consequence of the steric clash that the methyl groups would cause.

Compounds 2.1, 2.3, 2.4, 2.5, 2.9, 2.11, 2.12 and 2.16 showed similar and potent stabilisation ability for F21T (ΔTm(1 µM) between 28 and 34°C) and comparable to the 32°C obtained for BRACO-19 (compound 2.12 had a different behaviour with Ckit-1 and its stabilisation ability was lower than expected with this sequence). All these compounds contain basic tertiary amines at the end of the side chains, which are expected to be protonated at the pH of the experiment, 7.4. The comparison with the less basic morpholine analogues 2.2 and 2.10 (ΔTm(1 µM) of 19 and 20°C respectively) and especially with the neutral amide analogue 2.8 (ΔTm(1 µM) = 0°C) highlights the importance of the positively charged groups. Compound 2.6, with the secondary cyclohexaneamine (ΔTm(1 µM) = 24.5°C), and 2.7, with the aromatic imidazole (ΔTm(1 µM) = 15°C), did not perform as well as the compounds with aliphatic tertiary amines.
Interestingly, the length of the side chains is critical for the potency of the ligands. Compounds 2.1, 2.3, 2.4, 2.5, 2.9, 2.11, 2.12 and 2.16 all have the same number of atoms between the acridone core and the tertiary amine end groups. Comparing their ΔT_m values with the obtained for the analogue with shorter side chains, compound 2.13 (ΔT_m(1 μM) = 25°C), and the ones with longer side chains, compounds 2.14 (ΔT_m(1 μM) = 15°C) and 2.15 (ΔT_m(1 μM) = 22°C), shows that the optimum length is the one for 2.1. This observation agrees with preliminary molecular modelling done with compound 2.1 with the human telomeric G-quadruplex crystal structure (Stephen Neidle and Michael Moore, personal communication).

The similarity of the values between 2.1 and 2.9, 2.2 and 2.10, 2.3 and 2.11 and 2.4 and 2.12 shows that the presence of the carbonyl group in the side chains is irrelevant for G-quadruplex stabilisation. The amide bond is a potential site for metabolism and this point is important for further development of these compounds.

As expected the monosubstituted acridone 2.19 showed little stabilisation of the G-quadruplex (ΔT_m(1 μM) = 11°C).

The duplex experiment showed the compounds to be selective towards G-quadruplex DNA. The most potent compounds in the G-quadruplex experiments showed small stabilisation abilities with Dup (ΔT_m(1 μM) between 1.5 and 3°C). Even at 10 μM they showed low interaction (ΔT_m(10 μM) between 2 and 10°C) with the exception of the meta-substituted 2.16 (ΔT_m(10 μM) = 30°C). In general, the effect on the stabilisation of duplex DNA at the range of concentrations where the G-quadruplex DNA showed a strong stabilisation was very low so the selectivity of these compounds at this concentration is considerable. BRACO-19 showed higher affinity towards the duplex DNA (ΔT_m(1 μM) = 6°C). This is in accordance with previous reports of high duplex binding of this molecule (Moorhouse et al., 2006). Competition FRET experiments offer further insight into this issue in the next section.

3.3.2 Competition FRET assay

A selected group of representative acridone ligands, compounds 2.1, 2.3, 2.5, 2.9 and 2.10 were evaluated in the competition FRET experiments.
All compounds showed exceptional selectivity for the G-quadruplex DNA, with no loss of the stabilisation ability even in the 1:300 experiment (Figure 3.8). These results confirm the results obtained in the traditional FRET with the duplex sequence Dup, in which the stabilisation observed was very low.

![Figure 3.8 FRET competition profiles for a selected group of acridone ligands. X axes represent the different competition experiments: A: 1:0 ratio (G-quartets : pairs of bases); B: 1:1 ratio; C: 1:10 ratio; D: 1:100 ratio; E: 1:300 ratio. Y axes represent the percentage of stabilisation ability retained respect to the experiment with no competitor, which is column A in the X axes (retained stabilisation ability of 100%).]

BRACO-19, which showed remarkably higher stabilisation ability for Dup in the traditional FRET, has been previously evaluated in competition experiments in a similar manner (Moorhouse et al., 2006). As expected, BRACO-19 was shown to present a much lower discriminatory ability than the ligands presented herein.

### 3.3.3 Telomerase repeat amplification protocol assay (TRAP)

The TRAP evaluation of Telomestatin was conducted by Dr. Mekala Gunaratnam and Monica Beltran in our laboratories.

A representative group of acridone ligands, compounds 2.1, 2.3, 2.5, 2.9 and 2.10 together with three well established G-quadruplex ligands, BRACO-19, TMPyP4 and telomestatin were selected to be evaluated as telomerase inhibitors with the new modified TRAP assay.

The acridone ligands were tested at concentrations of up to 100 μM (Figure 3.9).
Figure 3.9 TRAP gel for ligand 2.10 and band quantification. Lane description: 1: positive control, no ligand; 2: 1 μM; 3: 5 μM; 4: 20 μM; 5: 100 μM; 6: negative control. The emission intensity of the bands, excepting the shorter one, was quantified and normalised using the controls.

No significant inhibition of the telomerase extension products was observed for any of the acridone ligands at these concentrations. For BRACO-19, TMPyP4 and telomestatin on the contrary, inhibition was observed at lower concentrations. Telomestatin, as reported previously, presented the lower EC$_{50}$ of all.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TRAP EC$_{50}$ (μM)</th>
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<tbody>
<tr>
<td>2.1</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>2.3</td>
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<tr>
<td>2.5</td>
<td>&gt; 100</td>
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<tr>
<td>2.9</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>2.10</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>BRACO19</td>
<td>7.9 ±0.5</td>
</tr>
<tr>
<td>TMPyP4</td>
<td>16.4 ±11.9</td>
</tr>
<tr>
<td>Telomestatin</td>
<td>0.6 ±0.1</td>
</tr>
</tbody>
</table>

Table 3.3 TRAP EC$_{50}$ values for a selected group of acridone ligands and reference ligands. TRAP evaluation was done by duplicate for the acridone ligands and at least three times for the reference compounds.

These results show that G-quadruplex binding ability, as measured by FRET, does not always correlate with telomerase inhibitory potency. For BRACO-19, the ΔT$_{m}$ for F21T obtained by FRET was lower than for compounds 2.1 and 2.3 for example, but in TRAP it performed much better. The recent discovery of the invalidity of the traditional TRAP
assay (de Cian et al., 2007b) limits the conclusions that can be obtained from these data until more ligands are evaluated with the new modified TRAP assay.

3.4 CONCLUSIONS

The acridone ligands in this series showed a wide range of effects in the biophysical assays described in this chapter.

In the FRET assays they showed very strong affinity for G-quadruplex sequences, in particular for the telomeric F21T. The competition FRET experiments uncovered the extraordinary ability of the ligands in discriminating between telomeric G-quadruplex and duplex DNA. Although this selectivity was already shown in the traditional FRET experiments, the confirmation of these results in a competition assay, where the ligands are tested in a more realistic environment, was reassuring. Most of the strong G-quadruplex ligands reported to date, including TMPyP4 and BRACO-19, show a higher degree of duplex interaction. This is a highly undesirable property since it almost certainly represents the cause of many of the adverse effects detected in vivo with this type of ligands.

In contrast, the ability of the acridone ligands for inhibiting telomerase in vitro did not accord with expectations. Several of the ligands showed stronger binding ability for F21T than other well established G-quadruplex ligands, such as TMPyP4 or BRACO-19 for example, but despite of this, their inhibitory ability for telomerase was at least 10 fold lower than for TMPyP4 or BRACO-19. The reasons for the different behaviour in the TRAP assay of different ligands with an apparent similar binding affinity for the G-quadruplex DNA as shown by FRET may be related to a number of reasons, including binding mode and binding kinetics, properties which FRET is oblivious to.
CHAPTER 4

PRELIMINARY BIOLOGICAL EVALUATION OF ACRIDONE G-QUADRUPLEX TARGETING AGENTS
4 PRELIMINARY BIOLOGICAL EVALUATION OF ACRIDONE G-QUADRUPLEX TARGETING AGENTS

4.1 ASSAYS FOR THE BIOLOGICAL EVALUATION OF G-QUADRUPLEX LIGANDS

4.1.1 Background

The biophysical evaluation of G-quadruplex ligands in cell-free assays assesses their potency in G-quadruplex stabilisation and telomerase inhibition, as described for the acridone series in chapter 3. In principle, these biochemical properties can be of utility to achieve a biological response and, ultimately, an anti-cancer effect in patients.

Clinical evaluation is the final and key stage for any drug discovery programme. Prior to the clinical evaluation, a potential drug must be tested in a number of pre-clinical studies, which include in vitro and in vivo experiments. The purpose is to obtain proof of principle for the therapeutic strategy and an initial assessment of potency and potential adverse effects. In anti-cancer drug discovery, the initial biological evaluation of a new agent is generally performed using tumour cell cultures techniques. These experiments allow one to quickly estimate the toxicity of the compounds in different tissues and offer a preliminary insight into the mechanism of action and potential secondary effects.

The cell lines used in the in vitro evaluation of anti-cancer agents are derived from tumour cells from human patients. The process of immortalisation of the cancer cells to allow them to grow in the laboratory necessarily implies the abrogation of some of the native characteristics of the cells. This drawback is compensated by the superior time and cost efficiency of these in vitro models of cancer when compared to the alternative in vivo pre-clinical models, such as xenografts. The disadvantages include the inability
to predict systemic toxicity and to detect potential beneficial or detrimental effects that
the metabolic system of a living animal can exert on the efficiency of the test compound. The in vitro screening is a validated method for pre-clinical testing of anti-
cancer agents and it has been the screening method of choice by the National Cancer
Institute (NCI) of the US for the last 27 years in the search for novel therapeutic agents
(www.cancer.gov).

At present time, G-quadruplex interaction within cells cannot be directly detected or
measured. This experimental limitation can be partially overcome by detecting or
measuring different biochemical outcomes or phenotypes that are, or can be, consequences of G-quadruplex targeting. The ligands can potentially have other unwanted and/or unexpected targets, duplex DNA being the most obvious, and all
conclusions derived from these observations must be drawn carefully as they might not necessarily be consequences of G-quadruplex interaction.

4.1.2 General

The compounds were evaluated against a small panel of cell lines: MCF7, human breast
adenocarcinoma derived cell line; A549, human lung adenocarcinoma derived cell line;
and IMR90, derived from human foetal lung fibroblasts. MCF7 and A549 are models
for two of the most common types of cancer and both express telomerase (Raymond et
al., 1999; Woo & Choi, 2005). IMR90 was used as a model for healthy somatic tissue, necessary to assess the selectivity of the compounds between healthy and cancerous
cells.

4.1.3 Surforhodamine B assay (SRB)

Assays to evaluate the ability of compounds to produce short-term cell growth
inhibition have been adapted to the high-throughput needs of the modern drug discovery
industry. The first method that implemented these needs used a tetrazolium salt to detect
and quantify metabolically active cells (Mosmann, 1983).

The surforhodamine B assay (SRB) was alternatively developed and was quickly
adopted as the routine method by the NCI for their cell line panel screening program
(Skehan et al., 1990). The assay presented improved performance compared to tetrazolium-based assays in terms of linearity, sensitivity, cost and reproducibility (Papazisis et al., 1997).

The SRB assay is performed in 96-well plates and utilises the dye sulforhodamine B, which binds to the basic residues of proteins, to quantify the amount of living cells after short-term exposure to the test compound. The amount of dye per well, which can be quantified spectroscopically as function of the absorbance at 540 nm, is related to the total protein content and consequently to the number of cells. Different modifications to optimise this assay have been implemented since it was first described. The protocol followed herein was adapted from the method of Papazisis and co-workers (Papazisis et al., 1997), and has been extensively used for evaluation of G-quadruplex ligands in our group (for example, Incles et al., 2004).

4.1.4 Long-term studies

The evaluation of toxicity using short-term acute exposure assays such as SRB provides fast and valuable preliminary information about the activity of the compounds in cells. However, the toxic effect measured by SRB is a combination of cytotoxic and cytostatic responses by the cells. Hence, the effect of the ligands in cells can be caused by their interaction with their molecular targets such as telomeres, but potentially also by their interaction with other unforeseen targets that can cause indiscriminate cell damage. Any potential drug must present a therapeutic window of concentrations by which the therapeutic effect is achieved at lower concentrations than the ones that would cause indiscriminate cytotoxic effect.

In order to study the mechanism of action of the G-quadruplex ligands, cells must be exposed to sub-cytotoxic concentrations for longer periods. These cells can be subsequently studied by a number of techniques to investigate the cellular pathways that are responsible for the observed anti-proliferative effect. These techniques include senescence detection, telomere length measurement, chromosome fusion detection and gene expression studies. The particular experiments will be explained in more depth in the following sections or in chapter 7 (gene expression).
4.1.5 Senescence

As discussed in the introduction of this thesis (page 24), dysfunctional telomeres can trigger a DNA damage mechanism that forces the cells to enter the non-replicative state of senescence. To evaluate if the anti-proliferative effects shown by the G-quadruplex ligands reported herein was related with the targeting of telomere integrity, MCF7 and A549 cells exposed to sub-cytotoxic concentrations of ligand were screened for senescence at different time points.

Senescent cells present enlarged morphology and pH-dependent expression of β-galactosidase activity. The β-galactosidase activity has been used as a biomarker for the senescent phenotype (Dimri et al., 1995) and has been exploited in a commercially available senescence detection kit (Cell Signaling Technology, USA). The assay utilises X-gal, a chemically modified galactoside, which produces a blue precipitate upon cleavage caused by β-galactosidase processing. The blue precipitate localises within the cells with β-galactosidase activity and the senescent cells can be visualised and quantified by microscopy.

4.1.6 Telomere length

The telomere length assays described in this thesis were conducted by Ms. Olga Greciano in our laboratories.

As discussed earlier, the telomeres shorten in every round of cell replication and in approximately 90% of cancer cell types the shortening is offset by activation of telomerase. Inhibition of telomerase results in a progressive shortening of the telomeres and eventual onset of senescence. The therapeutic benefit of this strategy has been demonstrated in clinical studies with direct inhibitors of telomerase (Hochreiter et al., 2006). G-quadruplex ligands have also been found to produce shortening of the telomeres (Gunaratnam et al., 2007). Although the mechanisms that explain telomere shortening are not clear for G-quadruplex ligands, undoubtedly the detection of abnormal telomere shortening would prove that this type of agents act at the telomeres.
In order to gain an insight into the mechanism of action of the acridone ligands in cell culture, telomere length in MCF7 and A549 cells undergoing long-term exposure to the ligands was evaluated. For this, an appropriate amount of treated cells was harvested and the cellular DNA extracted. The use of restriction enzymes that do not process telomeric and subtelomeric DNA followed by hybridisation with radioactive nucleotides allows one to detect the length of these DNA fragments.
4.2 MATERIAL AND METHODS

4.2.1 General

The cell lines MCF7 (European Collection of Cell Cultures), A549 (European Collection of Cell Cultures) and IMR90 (American Type Culture Collection) were maintained in monolayer culture at 37°C under a humidified 5% CO\(_2\) atmosphere in 75 cm\(^2\) flasks (TPP, Switzerland). Unless specified otherwise, all incubations in the protocols described herein were done in the incubator under these conditions.

The medium used for the cell lines MCF7, A549 and IMR90 was Dulbecco’s MEM (GIBCO 21969, Invitrogen, UK) supplemented with 2 mM L-glutamine (GIBCO 25030, Invitrogen, UK), 1% essential amino-acids (GIBCO 11140, Invitrogen, UK), 10% foetal calf serum (S1810, Biosera, UK) and 0.5 μg/ml hydrocortisone (Acros Organics, UK).

Cell passages were performed routinely by washing with PBS (GIBCO 14040, Invitrogen, UK), trypsinisation (GIBCO 25300, Invitrogen, UK) and re-seeding into fresh medium adjusting the initial cell density to approximately 1x10\(^4\) cells/ml medium. Cell counting was performed using a Neubauer haemocytometer (Assistant, Germany) by microscopy on a suspension of cells obtained by washing with PBS, trypsinisation, centrifugation at 4°C at 6000 rpm for 1 minute, and re-suspension in fresh medium.

4.2.2 Surforhodamine B assay (SRB)

The experiments were performed in 96-well plates (Nunc, Denmark). Following counting of the cells as described above, 4000 cells per well were seeded with 160 μL of medium and were incubated for 24 hours. The compounds to test were then added dissolved in 40 μL of medium and the cells were incubated for 96 hours. The medium was then flicked off and the cells fixed by incubating them on ice with 10% TCA (Sigma-Aldrich, UK) in water for 30 minutes. The TCA solution was flicked off and the wells washed with water 5 times and dried in an oven at 60°C for one hour. The wells
were treated with 80 μL of 0.4% sulforhodamine B (Acros Organics, UK) in 1% acetic acid and incubated for 15 minutes at room temperature. The SRB solution was then flicked off and the plates washed with 160 μL of 1% acetic acid and dried in an oven at 60°C for one hour. 100 μL of 10 mM Tris-base (Acros Organics, UK) solution was added to each well and the plates were gently shaken for 15 minutes. The absorbance at 540 nm was measured using a plate reader (Anthos 2010, Anthos Labtec, Austria). The data were normalised to the value of 100 for the control experiment (untreated cells) and the IC$_{50}$ values were obtained as the concentration required for a reduction of 50% in the absorbance intensity.

4.2.3 Long-term studies

Following counting of the cells as described above, 1x10$^5$ cells were seeded in 75 cm$^2$ cell culture flasks in 10 ml of medium. The compound to test was added and the cells were incubated. The cells were re-treated with drug once (on day 4), twice (on days 3 and 5) or three times (on days 2, 4 and 6) weekly. For the re-treatment the medium was discarded and the cells washed with PBS. Fresh media and compound solution were then added. The cells were counted on day 7 as described above and 1x10$^5$ of these cells were re-seeded and the process repeated for the whole length of the experiment (4-8 weeks). Population doublings were calculated according to the formula:

\[ N'_f = N_0 \times 2^{pd} \]

\[ pd = \log \left( \frac{N_f}{N_0} \right) / \log 2 \]

where $N_0$ is the number of cells at the time of seeding (1x10$^5$ in this case), $N_f$ is the number of cells at the time of counting and pd is the number of population doublings.

4.2.4 Senescence

Senescence detection and quantification experiments were carried out using a commercially available kit (Senescence β-galactosidase staining kit, Cell Signalling Technology, MA, USA) following the manufacturer's instructions. In brief, following counting of the cells as described above, 1x10$^5$ cells were seeded in a 35 mm well of a 6-well plate (Nunc, Denmark) in 2 ml of medium and compound to test. The cells were
incubated for 24 hours. The medium was then removed and the cells were washed with 2 ml of PBS. The cells were fixed by treating them with 1 ml of fixative solution (2% formaldehyde and 2% glutaraldehyde in PBS) for 15 minutes at room temperature. The fixative solution was then removed and the wells washed with PBS (2 x 2 ml). Freshly prepared staining solution (a mixture of 930 µL of 40 mM citric acid/sodium phosphate (pH 6.0), 0.15 M NaCl and 2 mM MgCl₂, 10 µL of 500 mM potassium ferrocyanide, 10 µL of 500 mM potassium ferricyanide and 50 µL of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in DMF) (1 ml) was added and the cells were incubated overnight. The number of senescent cells, detected by their blue pigmentation, was quantified by microscopy.

4.2.5 Telomere length

Cells undergoing long-term studies were collected and their DNA extracted (Blood and cell culture DNA Mini Kit, QIAGEN, UK). The DNA was quantified by measurement of the absorbance at 260 nm and its purity analysed by measuring the ratio between the 260 nm and 280 nm absorbances using a spectrophotometer (GeneQuant, Biochrom, UK). The DNA was kept at -20°C until required.

The DNA probe 3'-CCCTAACCCCTAACCCCTAACCC-5' (Invitrogen, UK) was labelled with γ³²P-ATP. For this, the DNA (20 pmol) was incubated with γ³²P-ATP (80 µCi), T4PNK enzyme (1 µl) (BioLabs, UK), T4 phosphonucleotide kinase buffer (3 µl) (BioLabs, UK) and TE buffer (16 µl) (10 mM Tris-Cl, 1 mM EDTA, pH 8) for 1 hour at 37°C. The labelled DNA was purified (QIAquick nucleotide removal kit, QIAGEN, UK) and re-suspended in EB buffer (100 µl) (10 mM Tris-Cl, pH 8.5).

The cellular DNA (2 µg) was digested with Hinfl (1.5 µl) and RsaI (1.5 µl) restriction enzymes (Roche, Germany) and hybridised to the ³²P-C-rich probe (3.5 µl) by overnight treatment at 37°C with NEB buffer 2 (3 µl) (50 mM NaCl, 10 mM Tris-Cl, 10 mM MgCl₂, 1 mM DTT) (BioLabs, UK) in a total volume of 30 µl of TE buffer.

Loading buffer (5 µl) was added to each tube and the samples were separated for 2.5 hours at 115V by electrophoresis using 0.7% agarose gels with 0.5 mg/ml of ethidium
bromide in Tris-Borate-EDTA buffer. The gel was subsequently dried for two hours in filter paper and paper towel, followed by 20 minutes in a gel dryer. The gel was then exposed to an X-ray film overnight (Molecular Dynamics). Telomeric smears were visualised using a phosphorimager (Molecular Dynamics).
4.3 RESULTS OF THE BIOLOGICAL EVALUATION OF THE ACRIDONE SERIES

4.3.1 Surforhodamine B assay (SRB)

The short-term cell growth inhibitory ability of the acridone ligands was evaluated using the SRB assay. The results showed most of the compounds to be selectively active against the cancer cell lines A549 and MCF7, when compared to the normal cell line IMR90 (Table 4.1).

<table>
<thead>
<tr>
<th>Compound Code</th>
<th>MCF7 (pM)</th>
<th>A549 (pM)</th>
<th>IMR90 (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>23 ± 7</td>
<td>16 ± 5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.3</td>
<td>55 ± 11</td>
<td>15 ± 7</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.4</td>
<td>99</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.5</td>
<td>62 ± 22</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.6</td>
<td>28 *</td>
<td>31 *</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.7</td>
<td>17 *</td>
<td>19 *</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.8</td>
<td>21 *</td>
<td>27 *</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.9</td>
<td>60 ± 16</td>
<td>66 ± 26</td>
<td>&gt;100</td>
</tr>
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<td>2.10</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.11</td>
<td>53 ± 9</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.12</td>
<td>68 ± 10</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.13</td>
<td>72 ± 27</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.14</td>
<td>15 ± 3</td>
<td>8 ± 3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.15</td>
<td>2 ± 1</td>
<td>43 ± 6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.16</td>
<td>84 ± 9</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.17</td>
<td>19 *</td>
<td>21 *</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.18</td>
<td>40 ± 12</td>
<td>62 ± 13</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2.19</td>
<td>9 ± 3</td>
<td>16 ± 5</td>
<td>7.5 ± 2</td>
</tr>
<tr>
<td>BR-19</td>
<td>2.5 *</td>
<td>2.4 *</td>
<td>26 *</td>
</tr>
</tbody>
</table>

Table 4.1 SRB IC_{50} results for the evaluation of the acridone ligands. The concentration of 100 μM was the highest tested as the majority of compounds presented solubility problems at higher concentrations. * Experiment performed by duplicate.

The cell growth inhibitory potency was, in general, lower than for other similar G-quadruplex ligands, such as acridines e. g. BRACO-19 (Incles et al., 2004). The relative low toxicity found for these compounds has been previously observed for other acridone-based G-quadruplex ligands (Harrison et al., 2004). Remarkably, all acridone compounds except the monosubstituted acridone compound 2.19, which showed high
and unselective toxicity for the three tested cell lines, displayed no acute toxicity at concentrations of up to 100 μM with IMR90 cells. This suggests the existence of a therapeutic window to specifically treat cancer cells over normal cells.

![Graph showing SRB evaluation results](image)

**Figure 4.1** Results for the SRB evaluation of compound 2.3 with cell line MCF7. On the Y axes, the percentage of cellular protein, which is measured by reading the absorbance of the associated SRB dye, normalised with the control experiment. On the X axes, the concentration of the different treatments.

Some of the compounds that showed the strongest binding to G-quadruplex DNA in the FRET assay, such as 2.1, 2.3 and 2.9, showed some of the strongest and most selective effect towards the cancer cell lines in the SRB assay. On the other hand, compounds 2.2 and 2.10, which were poor performers in FRET, showed no cell kill at values as high as 100 μM for any of the tested cell lines. However, several compounds showed high toxicity while being poor G-quadruplex ligands and some others stabilised the G-quadruplex strongly but had high IC₅₀ values. Therefore, no clear correlation between DNA binding abilities and short term toxicity could be established for this series (Figure 4.2).

![Graph showing IC₅₀ values](image)

**Figure 4.2** Plots showing the lack of apparent correlation between the DNA binding abilities and cytotoxicity for the acridone ligands.
4.3.2 Long-term studies

The long-term experiments with compound 2.1 described herein were conducted by Ms. Hanna Seidling in our laboratories.

Compounds 2.1, 2.3 and 2.16 were selected for long-term cell growth inhibition studies. The ligands were evaluated at different sub-cytotoxic concentrations during a period of between three and six weeks against several cancer cell lines.

To discard some initial concerns about the stability of the ligands in the cell culture medium, preliminary long-term studies where fresh compound was added two, three or four times per week were conducted. The results were almost identical, which indicated that the compounds are stable under the cell culture conditions in this time scale. The following experiments were run changing the medium twice per week.

Compound 2.1 was evaluated with the cell lines MCF7 and DU145 (human prostate carcinoma brain metastasis cell line) (Figure 4.3). The IC$_{50}$ for compound 2.1 were 23 μM for MCF7 and >100 μM for DU145 (it could not be determined exactly as solubility problems limited the highest usable concentration).

![Figure 4.3 Long-term cell culture studies for compound 2.1 and cell lines MCF7 (left) and DU145 (right).](image)

Compound 2.1 had a strong cell growth inhibitory effect in both cell lines. In MCF7 cultures, a 25 μM treatment produced a difference of 5 population doublings (pd) after
three weeks with respect to the vehicle control. In DU145 cultures, a 15 μM treatment produced a difference of 10 pd after four weeks with respect to the vehicle control.

Compound 2.3 was evaluated with the cell lines MCF7 and A549 (Figure 4.4). The IC$_{50}$ for compound 2.3 were 55 μM for MCF7 and 15 μM for A549. In the MCF7 experiment, concentrations of 2.3 of 10 and 20 μM showed an intense effect of 5 and 17 pd after six weeks. In the A549 experiment, treatments with 2.3 of 7 μM had a mild effect and cells underwent 22 pd, compared to the 25 pd of the control experiment.

![Figure 4.4](image1.png)

**Figure 4.4** Long-term cell culture studies for compound 2.3 and cell lines MCF7 (left) and A549 (right).

Compound 2.16 was evaluated with the cell lines MCF7 and A549 (Figure 4.5). The IC$_{50}$ for compound 2.16 were 84 μM for MCF7 and > 100 μM for A549 (it could not be determined as solubility problems limited the highest usable concentration).

![Figure 4.5](image2.png)

**Figure 4.5** Long-term cell culture studies for compound 2.16 and cell lines MCF7 (left) and A549 (right).

In MCF7 cultures, compound 2.16 treatments at concentrations of 25 μM or higher had to be discontinued after three weeks or less. The 10 μM treatment showed an effect in
cell proliferation and after five weeks, cells receiving this treatment underwent 17.5 pd while the control cells underwent 22.5 pd. In A549 cultures, compound 2.16 treatment at 50 μM resulted in lack of cells after 2 weeks. Cells under the 35 μM treatment underwent 15.5 pd compared to the 24 pd of the control cells after six weeks.

4.3.3 Senescence

The number of senescent cells at different stages of the long-term experiments was quantified for experiments using compound 2.16 with MCF7 (Table 4.2) and A549 cells (Table 4.3).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% senescent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>week 1</td>
</tr>
<tr>
<td>VC</td>
<td>0</td>
</tr>
<tr>
<td>10 μM</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>25 μM</td>
<td>3.3 ± 2.1</td>
</tr>
<tr>
<td>35 μM</td>
<td>11 ± 4.3</td>
</tr>
<tr>
<td>50 μM</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Table 4.2 Senescence results for compound 2.16 with cell line MCF7. n.a: not available because of lack of cells; disc.: long-term experiment was discontinued earlier because of lack of cells.

MCF7 cultures under treatment with 35 μM of compound 2.16 caused a 11% of senescent cells after one week and treatment at 25 μM caused a 23% of senescent cells after 3 weeks. However, the 10 μM treatment caused no senescence. There seems to be a combined mechanism of cell death, an initial one not senescence related and a final one, following longer exposure to the compound that triggers the final senescence related growth arrest.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% senescent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>week 1</td>
</tr>
<tr>
<td>VC</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>10 μM</td>
<td>0</td>
</tr>
<tr>
<td>25 μM</td>
<td>9 ± 2.0</td>
</tr>
<tr>
<td>35 μM</td>
<td>8 ± 2.2</td>
</tr>
<tr>
<td>50 μM</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Table 4.3 Senescence results for compound 2.16 with cell line A549. n.a: not available because of lack of cells; disc.: long-term experiment was discontinued earlier because of lack of cells.
In A549 cultures, compound 2.16 caused senescence in a concentration dependent manner until week 3, from then onwards no senescence was detected. This seems to correlate with the anti-proliferative effect observed, as a more intense inhibition was observed for the first two weeks of treatment (Figure 4.5-right). This could be caused by development of resistance to compound 2.16 by the cells after week 2.

![Figure 4.6 Picture of A549 cells under treatment with compound 2.16 after staining for senescence. Red arrows indicate cells with the characteristic blue color associated to senescent cells.](image)

### 4.3.4 Telomere length

*The telomere length assays described in this thesis were conducted by Ms. Olga Greciano in our laboratories.*

Telomere length measurements on MCF7 (Figure 4.7) and A549 cells (Figure 4.8) undergoing long-term exposures to compounds 2.3 were conducted. Two time points were chosen for this evaluation, week 1 and week 6. This would allow the detection of both immediate and progressive telomere attrition upon treatment.

![Figure 4.7 Telomere length measurement gel for MCF7 cells. Lane 1 to 6 correspond to: 1: Vehicle control, week 1; 2: 10 μM treatment, week 1; 3: 20 μM treatment, week 1; 4: Vehicle control, week 6; 5: 10 μM treatment, week 6; 6: 20 μM treatment, week 6.](image)
No significant telomere shortening was detected in cells treated for up to six weeks with any of the cell lines tested.

![Figure 4.8 Telomere length measurement gel for A549 cells. Lane 1 to 5 correspond to: 1: Vehicle control, week 1; 2: 3 μM treatment, week 1; 3: 7 μM treatment, week 1; 4: 3 μM treatment, week 6; 5: 7 μM treatment, week 6.](image)

**Figure 4.8** Telomere length measurement gel for A549 cells. Lane 1 to 5 correspond to: 1: Vehicle control, week 1; 2: 3 μM treatment, week 1; 3: 7 μM treatment, week 1; 4: 3 μM treatment, week 6; 5: 7 μM treatment, week 6.

### 4.4 CONCLUSIONS

The activity of the acridone ligands in cell culture studies was modest. All ligands showed IC$_{50}$ values in the micromolar range. This low activity contrasts with the strong G-quadruplex binding ability shown by FRET (Chapter 3). Most compounds were selectively toxic towards the cancer cell lines MCF7 and A549. The toxicity values did not present a clear correlation with the DNA binding affinities, so their mechanism of action is not clear.

In long-term studies, all the tested compounds inhibited cell proliferation at some extent at sub-cytotoxic concentrations. The mechanism of cell death seems to be partially related to the onset of senescence for compound 2.16, although senescence does not seem to be the only mechanism of the observed effect.

Compound 2.3 did not produce significant telomere shortening in MCF7 or A549 cells upon six weeks treatment.
CHAPTER 5

DESIGN AND SYNTHESIS OF NAPHTHALENE DIIMIDE G-QUADRUPLEX TARGETING AGENTS
5 DESIGN AND SYNTHESIS OF NAPHTHALENE DIIMIDE G-QUADRUPLEX TARGETING AGENTS

5.1 BACKGROUND

5.1.1 Naphthalene diimide compounds in the literature

The capacity of naphthalene diimides derivatives (Figure 5.1) to interact with DNA has been demonstrated in several studies. However, the development of this category of compound as DNA targeting agents is still in its infancy. A metasearch in the medical bibliography search engine PubMed (US National Library of Medicine) of “naphthalene diimide DNA” returned 28 hits whereas, for instance, “acridine DNA” returned over 4000 hits and “anthraquinone DNA” over 1000 (as in November 2007). Disubstituted naphthalene diimide derivatives were first identified as DNA interactive agents by Gabbay and coworkers (Gabbay et al., 1973), a work carried on by Wilson’s group that studied in depth their intercalating properties with duplex DNA sequences (Yen et al., 1982; Tanious et al., 1991). They found that disubstituted ligands (Compound 5a, figure 5.2) bound strongly to DNA and showed a 25-fold specificity for GC over AT base pairs. Although strong binders, they found the naphthalene diimides to have slow kinetics of association and disassociation and a proposed threading intercalation mode of binding by which the two cationic side chains reside in different grooves.

Figure 5.1 The naphthalene diimide structure.
Chapter 5 – Design and synthesis of naphthalene diimide G-quadruplex targeting agents

The intercalating properties of naphthalene diimide compounds have been exploited in different ways. Modification of disubstituted naphthalene diimides with different side chains afforded a number of compounds with strong binding affinity towards duplex DNA (Liu et al., 1996; Sato et al., 2000 and 2002), and with preference for G:C base pairs (Liu et al., 1996). The naphthalene diimide core was employed to deliver highly reactive groups that could act as DNA damaging agents, such as alkylating groups (Compound 5b, figure 5.2; Okamoto et al., 2000) and reactive radical species generators (Compound 5c, figure 5.2; Matsugo et al., 1991). Large molecules containing several naphthalene diimide units (Compound 5e, figure 5.2) were investigated as DNA poly-intercalators (Lokey et al., 1997; Guelev et al., 2002; Nojima et al., 2003). Naphthalene diimides have been used as molecular anchors in DNA, PNA and aminoglycoside hybrids to direct the binding of these molecules to single and double-stranded DNA and RNA (Núñez et al., 2000; Mokhir & Kraemer, 2003; Tok &
Fenker, 2001). The application of naphthalene diimides as part of non-nucleoside linkers in a DNA sequence favoured the tethering of the Watson-Crick and the Hoogsteen strands of triplex DNA by π–π interaction of the naphthalene diimide with the terminal base triplet of the hairpin triplex (Nunez et al., 2000; Bevers et al., 2000; Gianolio & McLaughlin, 2001). These studies demonstrated that the binding ability of these molecules is not limited to duplex DNA.

Naphthalene diimide intercalators have been employed to induce photooxidation-mediated cleavage of DNA by light excitation of the DNA bound ligand (Aveline et al., 1997). The photosensitisation can occur through several mechanisms, such as generation of the reactive species hydroxyl radical or singlet molecular oxygen or by electron transfer between DNA and one of the excited states of the ligand. Although several groups have addressed this question, the exact mechanism of action for the DNA photoxidation by naphthalene diimides remains unclear (Aveline et al., 1997; Rogers & Kelly, 1999; Rogers et al., 2000; Abraham et al., 2004). This property makes naphthalene diimides interesting candidates as photodynamic therapy agents, similarly to other DNA binding agents such as porphyrins, acridines and phenazines that are used or are being developed in the clinic for the treatment of cancer amongst others diseases (for example, Kusuzaki et al., 2000; reviewed in Dolmans et al., 2003).

Naphthalimides, a group of molecules closely related to the naphthalene diimides, also have demonstrated duplex DNA binding properties. Monomeric and dimeric analogues have been developed as anti-cancer agents and two lead compounds, amonafide (Compound 5f, figure 5.2) and elinafide (Compound 5g, figure 5.2), were studied in clinical trials, although they did not progress due to toxicity and limited activity (Braña & Ramos, 2001).

5.1.2 Naphthalene diimide compounds as G-quadruplex ligands

To date, only two groups have evaluated naphthalene diimide based compounds as G-quadruplex DNA ligands. The first compound was the ferrocenylnaphthalene diimide derivative 5d, initially developed as a duplex DNA ligand (Sato et al., 2005). The authors were interested in finding a metal containing ligand for its application in a telomerase activity detection device. Circular dicroism studies showed that this
compound exerts a stabilisation effect on a telomeric G-quadruplex with a binding stoichiometry of three molecules per G-quadruplex. In a separate study, six disubstituted naphthalene diimides were synthesised as part of a systematic study of the SAR of molecules structurally related to perylenes (including compound 5a, figure 5.2; Sissi et al., 2007). The compounds were evaluated for telomerase inhibitory activity and cell proliferation with two cancer cell lines but no DNA binding assays were reported. They demonstrated inhibition of Taq polymerase, which invalidated the results of TRAP, and several compounds showed potent toxicity in cancer cell lines, varying between 0.2 and 8 µM.

5.2 LIGAND DESIGN

Recent chemical developments enabled to envisage a synthetic route to tetra-substituted naphthalene diimides (Thalacker et al., 2006), which according to initial molecular modeling (Stephen Neidle, personal communication) would possess the desired characteristics to target G-quadruplex structures (Figure 5.3).

![Tetra-substituted naphthalene diimide](image)

*Figure 5.3 Tetra-substituted naphthalene diimide.*

The planar structure of these molecules could stack onto a G-quartet forming π-π interactions, as shown by manual molecular superposition (Figure 5.4). Furthermore, the relative position of the side chains substituents in the naphthalene diimide core would direct each of them towards one side of the G-quadruplex DNA, which would facilitate the interaction of the side chains with these regions of the structure.
It is proposed that the discriminatory ability between G-quadruplex and duplex DNA would also be improved by the use of four side chains when compared to the existing disubstituted DNA ligands. The increased bulkiness conferred by the four side chains could hinder the threading intercalation mechanism by which these molecules seem to bind duplex DNA (Tanious et al., 1991).

Figure 5.4 Manual superposition of the naphthalene diimide core and the 5’ G-quartet of the crystal structure of the human telomeric G-quadruplex.

The synthetic route described by Thalacker and coworkers enables the introduction to the naphthalene diimide core of up to three different side chains (Figure 5.5). This, in principle, can be exploited to design ligands possessing side chains tailored to target specific features in the topology of particular grooves and/or loops within a G-quadruplex.

Figure 5.5 Three different substitution types for naphthalene diimide ligands synthetically accessible. Type I with the same side chain at the four positions; Type II with two different side chains; Type III with three different side chains.
Chapter 5 – Design and synthesis of naphthalene diimide G-quadruplex targeting agents

This could maximise the affinity towards a particular G-quadruplex and also increase the specificity of a certain ligand between different G-quadruplexes. The use of symmetrical substitution in ligands targeting G-quadruplexes with different loops cannot be justified other than for reasons of ease of synthesis. The telomeric G-quadruplex is an exception due to the repetitiveness of the telomeric DNA sequence but only in the parallel topology described for the crystal structure (Parkinson et al., 2002).

5.3 AIM

The aim of this work was to adapt the existing route for the synthesis of naphthalene diimide dyes for the development of G-quadruplex targeting ligands and to synthesise and evaluate a library of compounds based on the naphthalene diimide core. The synthesis of analogues containing four equal side chains (Type I in Figure 5.5) was of particular interest for an initial assessment of the SAR of these molecules. An initial exploration of the effects of using different side chains was also of interest (Type II in Figure 5.5), however, an in depth investigation of the analogues containing different side chains was beyond the scope of this work.

5.4 LIBRARY DESIGN

The synthetic route to the final compounds consisted of the condensation of a functionalised naphthalene core with the side chains, which were commercially available amines. The amines of interest were primary amines substituted with carbon chains containing heteroatomic end groups. The differences in the amines provided the diversity to the library, namely, linker length and nature of the end group. The length of the carbon linkers was two or three carbons. The end groups were tertiary amines (dimethylamine, diethylamine, pyrrolidine, morpholine or piperidine) or the hydroxyl group. Two analogues containing two different side chains, compounds 5.21 and 5.22, and two previously reported disubstituted compounds, 5.23 and 5.24, were also included in the library (Tanious et al., 1991; Sissi et al., 2007). See table 5.1 for the structures of the different analogues of the library.
As explained below, tri-substituted analogues were consistently obtained as secondary products in the reactions towards the tetra-substituted targets. They were also included as members of the library.

<table>
<thead>
<tr>
<th>Compound code</th>
<th>Core⁸</th>
<th>Side Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>4-ND</td>
<td>3</td>
</tr>
<tr>
<td>5.2</td>
<td>3-ND</td>
<td>3</td>
</tr>
<tr>
<td>5.3</td>
<td>4-ND</td>
<td>2</td>
</tr>
<tr>
<td>5.4</td>
<td>3-ND</td>
<td>2</td>
</tr>
<tr>
<td>5.5</td>
<td>4-ND</td>
<td>3</td>
</tr>
<tr>
<td>5.6</td>
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<tr>
<td>5.7</td>
<td>4-ND</td>
<td>2</td>
</tr>
<tr>
<td>5.8</td>
<td>3-ND</td>
<td>2</td>
</tr>
<tr>
<td>5.9</td>
<td>4-ND</td>
<td>3</td>
</tr>
<tr>
<td>5.10</td>
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<tr>
<td>5.11</td>
<td>4-ND</td>
<td>2</td>
</tr>
<tr>
<td>5.12</td>
<td>3-ND</td>
<td>2</td>
</tr>
<tr>
<td>5.13</td>
<td>4-ND</td>
<td>3</td>
</tr>
<tr>
<td>5.14</td>
<td>3-ND</td>
<td>3</td>
</tr>
<tr>
<td>5.15</td>
<td>4-ND</td>
<td>2</td>
</tr>
<tr>
<td>5.16</td>
<td>3-ND</td>
<td>2</td>
</tr>
<tr>
<td>5.17</td>
<td>4-ND</td>
<td>2</td>
</tr>
<tr>
<td>5.18</td>
<td>3-ND</td>
<td>2</td>
</tr>
<tr>
<td>5.19</td>
<td>4-ND</td>
<td>5</td>
</tr>
<tr>
<td>5.20</td>
<td>3-ND</td>
<td>5</td>
</tr>
<tr>
<td>5.21</td>
<td>(2+2)-ND</td>
<td>R₁: 3 R₂: 2</td>
</tr>
<tr>
<td>5.22</td>
<td>(2+2)-ND</td>
<td>R₁: 3 R₂: 3</td>
</tr>
<tr>
<td>5.23</td>
<td>2-ND</td>
<td>3</td>
</tr>
<tr>
<td>5.24</td>
<td>2-ND</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5.1 Final compounds of the naphthalene diimide library. Molecular descriptors: a) See figures above; b) Number of \(\text{CH}_2\) in the linker; c) End group. DMA: dimethylamine; DEA: diethylamine; Pyrr: pyrrolidine; Mor: morpholine; Pip: piperidine; OH: hydroxyl. ND: Naphthalene diimide.
5.5 SYNTHESIS

The synthetic route to tetra-substituted naphthalene diimide dyes reported by Thalacker and colleagues (Thalacker et al., 2006) was the basis for the synthesis of the ligands of this series. In their work, two new methods to prepare the precursors for the tetra-substituted dyes, compound 5.0.1 (2,6-dichloro-1,4,5,8-naphthalenetetracarboxylic acid) and compound 5.0.2 (2,6-dibromo-1,4,5,8-naphthalenetetracarboxylic acid), were described (Figure 5.6).

They reported a new and improved method for the synthesis of compound 5.0.1 in four steps and a new route to 5.0.2 in one step. In their paper, these precursors were subject to substitution reactions at the anhydride and halogen groups for the introduction of a number of alkyl amines as side chains. Treatment of compound 5.0.1 with alkylamine in acetic acid caused the reaction to occur exclusively at the anhydride groups. Treatment of the resulting compound with a second amine at room temperature followed by treatment with a third amine under heating afforded compounds containing three different side chains. Although it has been claimed that selectivity at the anhydride groups can also be achieved in a similar way using compound 5.0.2 (Chaignon et al., 2007; Jones et al., 2007), in our hands this reaction did not stop at the imides and, to some extent, substitution of the bromines also occurred.

Consequently, the syntheses of compounds 5.21 and 5.22, containing two different side chains, were conducted from compound 5.0.1. The syntheses of the compounds containing four equal side chains were conducted by treatment of compound 5.0.2 with the appropriate amine in one step. Compound 5.0.1 could also be successfully used to synthesise these compounds, however its synthetic route is more demanding and compound 5.0.2 was preferentially used.
The retrosynthetic analysis for the tetra-substituted analogues containing one or two types of side chains and the disubstituted analogues is depicted in figure 5.7.

Figure 5.7 Retrosynthetic analysis for the naphthalene diimide final compounds. Top: retrosynthetic analysis for the tetra-substituted analogues with four equal side chains; Middle: retrosynthetic analysis for the tetra-substituted analogues with two different sets of side chains; Bottom: retrosynthetic analysis for the disubstituted analogues.

References in the text to “spectroscopic methods”, related to the characterisation of the products, refer to $^1$H, COSY, $^{13}$C and DEPT NMR and MS or HRMS. Some spectroscopic data, representative of the different types of compounds or useful for the discussion, have been included within the text. For full spectroscopic data and detailed synthetic procedures please refer to the experimental section.
5.5.1 Synthesis of the naphthalene diimide core

The synthesis of compound **5.0.1** was performed as described in figure 5.8 (Thalacker et al., 2006).

![Synthetic route for compound 5.0.1](image)

**Figure 5.8** Synthetic route for compound 5.0.1. Reaction conditions: a) Cl, I₂ (catalytic), 1,2,4-trichlorobenzene, RT-110°C, 6 h, 20%; b) KOH, ethanol, 80°C, 5 h, 96% (isomeric mixture); c) fuming HNO₃, -5-5°C, 15 min, 19% (for 2 steps); d) fuming HNO₃, conc. H₂SO₄, 100-120°C, 30 min, 35%.

Pyrene was chlorinated by treatment with chlorine gas to obtain **5.0.1.1** in 20% yield. Reportedly, the low solubility of this compound does not allow the recording of NMR spectra. In our hands, however, it was possible to dissolve the product in CDCl₃ or DMSO-d₆ and obtain ¹H and ¹³C NMR spectra in agreement with the structure of **5.0.1.1** (Figure 5.9).

![NMR spectra](image)

**Figure 5.9** ¹H and ¹³C NMR spectra of compound **5.0.1.1** in CDCl₃. Left: ¹H NMR spectrum showing the two signals for Ha and Hb as singlets. Right: ¹³C NMR spectrum shows the 5 expected peaks, 2 of them resolved as CH peaks by DEPT.
The next step was the re-aromatisation of 5.0.1.1 by elimination of hydrochloric acid to obtain the isomeric mixture of 5.0.1.2.a and 5.0.1.2.b in 96% yield (combined). The mixture presented low solubility and was characterised by elemental analysis and MALDI-MS (ES-MS failed to detect the product). No purification was intended at this stage. The mixture was treated with fuming HNO\textsubscript{3} to obtain the oxidation products 5.0.1.3.a and 5.0.1.3.b. Compound 5.0.1.3.a was purified by sublimation at 1-2 mbar and 250°C and was obtained in an overall yield of 19% (for two steps). The product was soluble in CDCl\textsubscript{3} and the use of D\textsubscript{2}SO\textsubscript{4} (solvent used in the literature) for the \textsuperscript{1}H and \textsuperscript{13}C NMR spectra was not necessary. The \textsuperscript{1}H NMR spectrum displayed as expected two singlets of equal integration in the aromatic region. Treatment of a solution of compound 5.0.1.3.a in concentrated H\textsubscript{2}SO\textsubscript{4} with fuming HNO\textsubscript{3} afforded the dichloro compound 5.0.1, which was purified by crystallisation from AcOH, in 35% yield. The product was soluble in DMSO-\textsubscript{d}6 and the use of THF-\textsubscript{d}8 (solvent used in the literature) for the \textsuperscript{1}H and \textsuperscript{13}C NMR spectra was not necessary. The \textsuperscript{1}H NMR presented a single singlet and the two types of carbonyls were evident in \textsuperscript{13}C NMR as two signals at 159.0 and 165.46 ppm.

The synthesis of compound 5.0.2 was performed as described in figure 5.10 (Thalacker \textit{et al.}, 2006).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{5.0.2}
\caption{Synthesis of compound 5.0.2. Reaction conditions: a) DBI, oleum (20% SO\textsubscript{3}), RT, 5 h, 82\%.
}
\end{figure}

Naphthalene dianhydride was treated with freshly prepared DBI (dibromoisocyanuric acid) in oleum at room temperature to obtain compound 5.0.2 in 82% yield. DBI was synthesised as described (Gottardi, 1968) by reaction of cianuric acid with bromine in 60% yield. Compound 5.0.2 was extremely insoluble and could only be characterised by elemental analysis. The elemental analysis was 39.50\% [C] and 0.47\% [H] and was in good agreement with theoretical (39.48\% [C] and 0.47\% [H]). The melting point was higher than 350°C, as reported. No mass spectrum could be recorded after several failures using both EI and MALDI MS.
5.5.2 Synthesis of the tri- and tetra-substituted analogues 5.1-5.20

The tetra-substituted analogues containing four equal side chains were synthesised from either 5.0.1 or 5.0.2 using neat amine as solvent at 150°C for 10 minutes under microwave radiation. The more economical dibromo compound 5.0.2 was preferentially used for these reactions. The work-up of the reactions consisted of evaporation of the amine \textit{in vacuo} followed by purification with HPLC. The amine employed for the synthesis of compound 5.19 was not volatile and required a different work-up which consisted of addition of water and collection of the precipitate by centrifugation.

It was found that the tri-substituted (and occasionally disubstituted) analogues were regularly obtained as subproducts in the reactions using 5.0.2 but not using 5.0.1 (Figure 5.11).

\[ \text{O}^\text{O}^\text{O} \]
\[ \text{N} \text{H} \text{c} \]
\[ \text{O'' N}^\text{O} \text{O'' N}^\text{O} \]

\[ \text{Figure 5.11 Synthesis of compounds 5.1 and 5.2. Compound 5.2 was obtained as product of a side reaction. Reaction conditions: a) Amine used as solvent, MW 150°C, 10 min, 15.5\% (5.1), 10\% (5.2) after HPLC purification.} \]

The formation of the tri-substituted by-products could be due to the presence of impurities in the starting material compound 5.0.2, in that it could have contained monobrominated or unbrominated materials, precursors for the di- and tri-substituted side products, if the bromination reaction with DBI had not gone to completion. However, the tri-substituted compounds were often obtained in similar stoichiometry as the tetra-substituted analogues and from the evidence of the elemental analysis data of compound 5.0.2 (which was the only characterisation possible for this material) this hypothesis was discarded.
Furthermore, a number of other unidentified subproducts were also obtained in trace amounts. These compounds presented high colour intensity, which suggested that they contained the naphthalene diimide chromophore (Figure 5.12).

![Figure 5.12 Fractions from preparative HPLC for compound 5.1. The subproducts were present in trace amounts but showed intense colours.](image)

It was hypothesised that the occurrence of the tri- and disubstituted subproducts was due to radical debromination of the starting materials caused by traces of DBI used in the previous synthetic step. The other trace subproducts could also be the result of other radical reactions. DBI (dibromoisocyanuric acid) could be a source of diatomic bromine by acting in a similar manner to N-bromosuccinimide (Figure 5.13; Clayden et al., 2005).

![Figure 5.13 Bromine radical generation by traces of DBI. The DBI can release Br₂ in an analogous manner to N-bromosuccinimide. Br₂ can form bromine radicals by the action of light.](image)
The bromine anion generated in the condensation reaction between 5.0.2 and the amine can attack the bromine atom in the succinimide to form diatomic bromine. This reaction is favoured by the high stability of the resulting anion. Diatomic bromine can be disassociated into bromine radical by the action of light.

Bromine radicals can then promote the debromination of the intermediates in the reactions towards the tetra-substituted analogues, as exemplified in figure 5.14.

![Figure 5.14 Proposed mechanism for the radical debromination in reactions using the starting material 5.0.2. In the first step the bromine radical debrominates the naphthalene core creating a radical in the carbon, which can be neutralised by, for example, the amine used as solvent, which can propagate the radical reaction to new molecules.]

To prove this hypothesis, amination reactions were conducted in the presence of compounds that potentially can act as free radical scavengers, such as 4-tert-butylcatechol and TEMPO. 4-tert-butylicatechol is a radical scavenger generally used to control radical polymerisation. TEMPO contains a very stable radical and is also used as a radical scavenger (for example, Wright & English, 2003). For this investigation the reaction for compound 5.1 was chosen. The reaction was repeated using different concentrations of the scavengers. Note that different crops of starting material compound 5.0.2 were used for the experiments using 4-tert-butylicatechol and TEMPO.

<table>
<thead>
<tr>
<th>Amount of 4-tert-butylicatechol</th>
<th>Tetra-substituted product</th>
<th>Tri-substituted product</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 eq.</td>
<td>74.40%</td>
<td>25.60%</td>
</tr>
<tr>
<td>0.1 eq.</td>
<td>73.70%</td>
<td>26.30%</td>
</tr>
<tr>
<td>1 eq.</td>
<td>81.26%</td>
<td>18.74%</td>
</tr>
<tr>
<td>10 eq.</td>
<td>81.30%</td>
<td>18.70%</td>
</tr>
</tbody>
</table>

Table 5.2 Results of the reactions using 4-tert-butylicatechol as a radical scavenger. The relative amount of the different products in the crude reaction mixture was measured by HPLC. The combined amounts of the two major products, the tetra- and tri-substituted analogues, was normalised to 100%.
Reactions with 4-\textit{tert}-butylcatechol were performed using 0.1, 1 and 10 equivalents of the radical inhibitor (Table 5.2). A significant decrease (7%) in the amount of tri-substituted product respect to the tetra-substituted products was observed for the reactions using 1 or 10 equivalents of 4-\textit{tert}-butylcatechol. On the other hand, reactions using 0.1 equivalents did not show a significant decrease.

Reactions with TEMPO were performed using 1 and 10 equivalents of the radical inhibitor (Table 5.3).

<table>
<thead>
<tr>
<th>Amount of TEMPO</th>
<th>Tetra-substituted product</th>
<th>Tri-substituted product</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 eq.</td>
<td>88.07%</td>
<td>11.93%</td>
</tr>
<tr>
<td>1 eq.</td>
<td>90.37%</td>
<td>9.62%</td>
</tr>
<tr>
<td>10 eq.</td>
<td>90.51%</td>
<td>9.49%</td>
</tr>
</tbody>
</table>

Table 5.3 Results of the reactions using TEMPO as a radical scavenger. The relative amount of the different products in the crude reaction mixture was measured by HPLC. The combined amounts of the two major products, the tetra- and tri-substituted analogues, was normalised to 100%.

The reactions using TEMPO did show a small decrease in the production of tri-substituted product, however it had a much weaker effect than 4-\textit{tert}-butylcatechol.

It was observed, for both radical scavengers, that the amount of reagent used had little impact on the efficiency of the inhibition since similar results were obtained for the experiments using 1 or 10 equivalents.

The side reaction that produced the tri-substituted analogues occurred in all the reactions with 5.0.2, and these compounds, together with the targeted tetra-substituted, were consistently isolated from the reaction crudes using HPLC. The ratios of the formation of tri- and tetra-substituted analogues typically varied between 40 : 60 and 15 : 85 (tri/tetra).

The tetra-substituted analogues have an intense blue colour and the tri-substituted an intense orange colour. The different chromophoric characteristics of the compounds facilitated the HPLC analysis and purification of the mixtures. Tetra-substituted compounds eluted before for all the pairs of compounds except for 5.19 and 5.20, when the elution of the tri-substituted preceded the elution of the tetra-substituted analogue.
Tetra-substituted compounds were obtained in yields in the ranging from 6 to 22% and tri-substituted from 5 to 28%. The reactions for the analogues with the hydroxyl groups gave the poorest yields possibly due to the difficulties during the work-up.

All compounds were characterised by spectroscopic methods. The $^1$H NMR spectra for compounds 5.1 and 5.2 are depicted in figures 5.15 and 5.16 respectively. The integration of the aliphatic signals and the presence of one or three signals corresponding to the aromatic hydrogens were characteristic of tetra- and tri-substituted analogues.

**Figure 5.15** $^1$H NMR spectrum of compound 5.1 in CDCl$_3$. In the interest of clarity, only one hydrogen per CH$_2$ group is shown. $Ha$ was a singlet corresponding to the two equivalent aromatic hydrogens. The protons $Hb$ have a characteristic coupling with $Hc$ resulting in a triplet and quartet respectively. The chemical environment of protons $Hd$ and $He$, $He$ and $Hf$ and $Hg$ are similar and, as such, they appear in close proximity to each other as multiplets.

**Figure 5.16** $^1$H NMR spectrum of compound 5.2 in CDCl$_3$. In the interest of clarity, only one hydrogen per CH$_2$ group is shown. $Ha$, $Hb$ and $Hc$ each integrate one hydrogen. $Ha$ and $Hb$ are doublets resultant from the coupling with each other. The chemical environment of protons $Hh$, $Hi$ and $Hp$, $Hg$, $Hk$ and $Ho$ and $Hf$, $Hj$ and $Hn$ are similar and, as such, they appear in close proximity to each other as multiplets. $Hi$ and $Hm$, also similar, result in a multiplet signal.
5.5.3 Synthesis of the compounds 5.21 and 5.22

Reportedly, the substitution reaction at the anhydride groups of 5.0.2 can be conducted selectively without reaction at the bromines (Chaignon et al., 2007; Jones et al., 2007). In our hands, however, this reaction did not show the expected selectivity and complex mixtures were obtained. For this reason, compound 5.0.1 was used for the synthesis of compounds 5.21 and 5.22 where two different side chains were used (Figure 5.17). For this two-step synthesis compound 5.0.1 was treated with the first amine in acetic acid for 10 minutes at 120°C to obtain 5.0.21.1 in 81% yield. Treatment of 5.0.21.1 with the second amine, 1-(2-aminoethyl)piperidine or 3-amino-propanol, at 150°C for 10 minutes in the microwave afforded compounds 5.21 and 5.22 respectively, obtained after HPLC purification in 11% and 8% yields, respectively.

![Figure 5.17 Synthetic pathway for compound 5.22. Reaction conditions: a) Acetic acid, MW 120°C, 10 min, 81%; b) Amine as solvent, MW 150°C, 10 min, 8% (after HPLC purification).](image)

The work-up for compound 5.21 consisted of evaporation of the amine and HPLC purification of the crude mixture. For compound 5.22, the reaction mixture was diluted in water and partitioned with CHCl₃ and the crude was subsequently purified by HPLC. Compounds 5.21 and 5.22 were obtained by this method in 11% and 8% yields, respectively. The structure of the compounds was confirmed by spectroscopic methods. The ^1^H NMR spectrum for compound 5.22 is depicted in figure 5.18.
Chapter 5 – Design and synthesis of naphthalene diimide G-quadruplex targeting agents

Figure 5.18 \( ^1H \) NMR spectrum of compound 5.22 in MeOD. In the interest of clarity, only one hydrogen per CH\(_2\) group is shown.

5.5.4 Synthesis of the disubstituted analogues 5.23 and 5.24

The disubstituted compounds 5.23 and 5.24, previously reported (Sissi et al., 2007), were synthesised by treatment of commercially available naphthalene dianhydride with the correspondent amine used as solvent under microwave radiation at 120°C for 10 minutes (Figure 5.19).

Figure 5.19 Synthesis of compound 5.23. Reaction conditions; a) Amine as solvent, MW 120°C, 10 min, 58%.

The reaction mixtures were diluted with water and the products taken by filtration and washed. The products were crystalline solids and did not require further purification and were obtained in yields of 58% for 5.23 and 69% for 5.24. The characterisation of the compounds was carried out by spectroscopic methods and the spectra were in concordance with the reported values.
5.6 CONCLUSIONS

The synthesis of 22 novel and 2 previously reported naphthalene diimide ligands was accomplished. The tri- and tetra-substituted compounds represent a new family of G-quadruplex targeting agents. The structural differences of the components of this library in terms of number of side chains, length of the side chains and end groups, provides a comprehensive group of diverse compounds for the analysis of the structure-activity relationships (Chapters 6 and 7).

The synthetic method developed for the introduction of alkylamine substituents to the naphthalene diimide core (Thalacker et al., 2006) has been successfully adapted for the synthesis of compounds having a variety of tertiary amine and hydroxyl containing side chains.

Tri-substituted analogues were obtained as side products in the reaction towards the tetra-substituted naphthalene diimides. It was hypothesised that this side reaction could be of radical origin. Experiments using radical inhibitors were conducted and 4-tert-butylcatechol significantly reduced the formation of the tri-substituted analogue indicating that the generation of this subproduct can be of radical origin.

As a proof of concept, two of the ligands presenting different substituents at the imide and naphthalene positions were synthesised.
CHAPTER 6

BIOPHYSICAL EVALUATION OF
NAPHTHALENE DIIMIDE G-QUADRUPLEX
TARGETING AGENTS
6 BIOPHYSICAL EVALUATION OF NAPHTHALENE DIIMIDE G-QUADRUPLEX TARGETING AGENTS

6.1 ASSAYS FOR THE EVALUATION OF G-QUADRUPLEX LIGANDS

6.1.1 Background

For a general introduction into the biophysical methods used for evaluation of DNA binding ligands and the description of FRET and TRAP methods used within this chapter please refer to chapter 3.

6.1.2 Surface plasmon resonance (SPR)

The SPR studies described in this thesis were conducted by the laboratory of Professor W. David Wilson (Georgia State University, Atlanta, US).

In recent years, surface plasmon resonance (SPR) has become a widely spread method for measuring interactions between pairs of macromolecules and/or small molecules. In brief, the experiment involves the immobilisation of one of the molecules to the surface of a chip and exposure to a solution of the second molecule. The assay relies on measuring variations of the refractive index in the microenvironment that surrounds the surface of the chip, which are the result of the associative or dissociative interactions between the molecules under study.

The SPR technique can deliver qualitative and also quantitative information about the interaction of the molecules under study. Some of the parameters that can be obtained are stoichiometry, equilibrium constants $K_a$ and kinetic association and disassociation constants $k_a$ and $k_d$, between others. The values obtained by SPR techniques are
generally consistent with the values obtained by other traditional methods (Rich & Myszka, 2000).

SPR has been successfully applied to the study of duplex DNA/ligand interactions (Bailly et al., 2001) and more recently to G-quadruplex DNA/ligand interactions (Read et al., 2001; Harrison et al., 2003; Gonçalves et al., 2006; White et al., 2007).

The SPR method presents several advantages over other techniques traditionally used for quantitatively measuring interactions between small molecules and nucleic acids (Nguyen et al., 2007). Other methods often require the characterisation of some properties or the labelling of the small molecules before they can be evaluated. In SPR small molecules do not require any type of labelling and the technique can be applied to ligands that present a broad range of properties, such as large binding constants, weak spectroscopic signals or low interaction enthalpies, which often limit the applicability of other techniques. Furthermore, the sensitivity obtained by this method is excellent and the range of binding constants that are obtainable is broad, with binding constants from $10^4$ to $10^{11}$ M$^{-1}$ generally accurately obtained.

For a more technical description of the principle behind the assay, the experiment design or apparatus please refer to the manufacturer’s published literature (Biacore, Inc., NJ, USA).
6.2 MATERIALS AND METHODS

6.2.1 General

For the materials and methods for FRET and TRAP assays please refer to chapter 3.

6.2.2 Compound preparation

The solids (in free base form when applicable) were dissolved as described below and kept as stock solutions at -20°C. Compounds were dissolved in 4 mM HCl in water to obtain 1 mM solutions, except for compounds 5.19, 5.20 and 5.22 when 10 mM DMSO solutions were prepared instead. The stability of the compounds in solution was confirmed by periodical HPLC analysis.

6.2.3 Surface plasmon resonance (SPR)

The SPR studies were conducted as described previously (Read et al., 2001).

In brief, four-channel streptavidin-coated sensor chips (Biacore, Inc.) were preconditioned with five injections of 1 M NaCl in 50 mM NaOH followed by one continuous injection of Hepes buffer (0.01 M Hapes, 0.2 M KCl, 3 mM EDTA, 0.005% (v/v) surfactant P20, pH 7.4) until the signal stabilised to a reading of less than 1 RU/minute. The sequence mimic of the human telomeric G-quadruplex (5'-biotin-d[AG4(TTAG3)3]-3') was immobilised to the chip by manual injection of a 25 nM solution of the DNA in Hepes buffer at a flow of 2 µl/minute. The ligand binding experiments were conducted each time after regeneration of the surface. The kinetics experiments were conducted at flow rates of 50-100 µL/minute and the steady-state experiments were conducted at flow rates of 10-30 µL/minute. Association and dissociation curve fittings were performed using the supplier's software (BIA Evaluation Software, Biacore, Inc.) in the concentration range where the compounds only showed strong binding for the main binding site.
6.3 RESULTS OF THE BIOPHYSICAL EVALUATION OF THE
NAPHTHALENE DIIMIDE SERIES

6.3.1 Fluorescence resonance energy transfer assay (FRET)

The DNA binding abilities of compounds 5.1-5.24 and reference compound BRACO-19 (BR-19) with the sequences F21T, Ckit-1, Ckit-2 and Dup were assessed using the FRET assay. The values of $\Delta T_m$ at 0.5 $\mu$M were used for comparison (as opposed to 1 $\mu$M as with the acridone series; the reason is that at concentrations higher than 0.5 $\mu$M some of the ligands achieved saturation of the G-quadruplex binding sites) (Table 6.1).

<table>
<thead>
<tr>
<th>Comp. Code</th>
<th>FRET F21T $\Delta T_m$ [c=0.5 M] ($^\circ$C)</th>
<th>FRET Dup $\Delta T_m$ [c=0.5 M] ($^\circ$C)</th>
<th>FRET Ckit-1 $\Delta T_m$ [c=0.5 M] ($^\circ$C)</th>
<th>FRET Ckit-2 $\Delta T_m$ [c=0.5 M] ($^\circ$C)</th>
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<tr>
<td>5.1</td>
<td>33.2</td>
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</tr>
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<td>5.2</td>
<td>26.5</td>
<td>12.5</td>
<td>18.5</td>
<td>29</td>
</tr>
<tr>
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<td>28</td>
<td>7.5</td>
<td>18.1</td>
<td>29.7</td>
</tr>
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<td>5.4</td>
<td>17.2</td>
<td>4.2</td>
<td>13.1</td>
<td>18.7</td>
</tr>
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<td>35.2</td>
<td>4.2</td>
<td>24.7</td>
<td>36.2</td>
</tr>
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<td>17.8</td>
<td>28.5</td>
</tr>
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<td>30</td>
<td>4</td>
<td>20.6</td>
<td>31.2</td>
</tr>
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<td>5.8</td>
<td>21</td>
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<td>5.9</td>
<td>7.7</td>
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<td>7.2</td>
</tr>
<tr>
<td>BR-19</td>
<td>29</td>
<td>1.5</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Table 6.1 FRET data results for the naphthalene diimide ligands and reference compound BRACO-19. The values are the means of two experiments. The uncertainty in the $\Delta T_m$ values is $\pm 0.5 ^\circ$C.
Overall, the ligands showed extremely potent stabilisation ability for the G-quadruplex sequences (Figure 6.1). The maximum $\Delta T_m$ were 35.2°C for F21T, 24.7°C for Ckit-1 and 39.2°C for Ckit-2. However, some of the ligands also showed good binding with Dup and $\Delta T_m$ of up to 12.5°C were recorded.

There is a good correlation between the binding abilities of the naphthalene diimide ligands for the G-quadruplex sequences (Figure 6.2). This seems to suggest that the modifications affect the stabilisation ability of the ligands in a similar fashion for all three G-quadruplexes, which could mean that they share a similar binding mode.

The data for Dup does not correlate with the data for F21T if the whole series is considered but, interestingly, the combined sub-series of the di- and tri-substituted
analogues, which are the best binders for duplex, clearly correlate (Figure 6.3). This may suggest a different binding mode of tetra-substituted naphthalene diimide ligands with duplex DNA.

The differences obtained by side chain modification are discussed next based on the data for the F21T sequence. The conclusions are extrapolatable to the Ckit-2 sequence. Although there is also correlation between data for F21T and Ckit-1, the results for the latter will be treated separately as the exceptions are numerous.

The level of stabilisation obtained by some of the ligands of this series was very high and the effect achieved at 0.5 μM with compounds 5.1, 5.5 or 5.9 for example requires a concentration of BRACO-19 of 2.5 μM. At 1 μM, the melting of the G-quadruplex sequences was outside of instrument range for many of the strongest ligands.

Consistently for the whole series, the tetra-substituted ligands performed better than the tri- and these better than the disubstituted counterparts. There is a structural benefit in the use of four side chains regardless of the overall number of charges as this effect is observed too for compounds 5.13/5.14, 5.15/5.16 and 5.19/5.20, which are neutral or protonated to a lesser extent at the pH of the experiment, 7.4.

Compounds 5.1, 5.5 and 5.9 and 5.2, 5.6 and 5.10 have the highest ΔTm of all within the tetra- and tri-substituted series respectively. They all have side chain linkers of the same...
length (three carbons) and similar end groups (protonated at pH 7.4 tertiary amines). Their counterparts with shorter side chains, 5.3, 5.7 and 5.11 for the tetra-substituted and 5.4, 5.8 and 5.12 for the tri-substituted, all performed worse. Substitution of the tertiary amines for morpholino groups for compounds 5.13, 5.14, 5.15 and 5.16 reduces the $\Delta T_m$. However, the morpholino and also the hydroxyl analogues 5.19 and 5.20 displayed still good stabilisation ability (especially for F21T) and with the notable exception of telomestatin, no other neutral compounds show a similar degree of G-quadruplex affinity.

In the Ckit-1 experiments, the differences in stabilisation ability between tri- and tetra-substituted analogues were smaller and in some cases the tri-substituted performed better. A part from this difference, the major trends that are observed for the F21T and Ckit-2 are maintained for Ckit-1.

Compound 5.21 stands out of the rest of analogues because of its sequence selectivity towards F21T. The stabilisation obtained for the other G-quadruplexes and Dup is small. 5.21 is one of the two analogues having two types of side chains and the reasons for its selectivity could be related to this fact.

Most ligands showed some degree of interaction with Dup. Compounds 5.19 and 5.20, however, slightly destabilised this sequence. With the exception of the pair of compounds 5.3/5.4, the tri-substituted are stronger binders to the duplex DNA that the tetra-substituted analogues. The reason for the higher selectivity of the tetra-substituted analogues towards G-quadruplex DNA may be the increased steric hindrance caused by the four side chains in duplex DNA binding.

### 6.3.2 Competition FRET assay

Compounds 5.1-5.24 were evaluated in the competition FRET experiments.

As indicated by the traditional FRET experiments with Dup, the binding affinity of naphthalene diimide ligands for duplex DNA was high for several ligands, with $\Delta T_m$ values of up to 12.5°C. This was confirmed in the competition experiments, where most ligands were subject to competition by the duplex DNA (Figure 6.4).
Figure 6.4 FRET competition profiles for the naphthalene diimide ligands. X axes represent the different competition experiments: A: 1:0 ratio (G-quartets : pairs of bases); B: 1:1 ratio; C: 1:10 ratio; D: 1:100 ratio; E: 1:300 ratio. Y axes represent the percentage of stabilisation ability retained respect to the experiment with no competitor, which is column A in the X axes (retained stabilisation ability of 100%).
Tetra-substituted compounds showed better selectivity towards the F21T in the competition experiments than the other analogues (Figure 6.5).

![Graph showing FRET competition profiles for different subgroups of naphthalene diimides (NDIs) with varying numbers of side chains.](image)

**Figure 6.5** Comparison of average FRET competition profiles for the subgroups of naphthalene diimides (NDIs) having four, three or two side chains.

Tetra-substituted analogues retained 100% stabilisation ability at the 1:1 competition experiments, unlike the di- or tri-substituted analogues for which a reduction of the ability was observed (5-25%). Tetra-substituted analogues performed better at the 1:100 and 1:300 experiments with retention of the ability of 10-40% and 10-30% respectively. On the other hand most tri- and disubstituted ligands lost almost completely the stabilisation ability at these experiments.

In general, analogues having side chains with three methylene groups were more selective towards F21T in the competition experiments than the ones having two methylenes (Figure 6.6).

![Graph showing FRET competition profiles for NDIs with linker lengths of two or three carbons.](image)

**Figure 6.6** Comparison of FRET competition profiles for the subgroups of ligands having side chains with linker lengths of two or three carbons.
The selectivity of the ligands was also linked to the end group of the side chains (Figure 6.7)

Figure 6.7 Comparison of FRET competition profiles for the subgroups of ligands having side chains with different end groups. DMA: dimethylamine; DEA: diethylamine; Pyr: pyrrolidine; Mor: morpholine; OH: hydroxyl.

The ligands having side chains containing hydroxyl groups, compounds 5.19 and 5.20, were by far the most selective compounds, followed by the morpholino containing ligands, compounds 5.13-5.16. This is in agreement with the traditional FRET data with Dup. Amongst the ligands containing more basic tertiary amines, the ones containing diethylamino appendixes were significantly more selective than the rest. The effect of the hydroxyl or morpholino end groups has a negative effect in the net binding ability for F21T (Table 6.1). On the contrary, the ligands with diethylamino end groups are as strong binders as the other tertiary amine containing ligands.

6.3.3 Telomerase repeat amplification protocol assay (TRAP)

The TRAP evaluation of telomestatin and compound 5.11 were conducted by Dr. Mekala Gunaratnam and Ms. Mónica Beltrán in our laboratories.

A group of ligands representative of the diversity in the naphthalene library, compounds 5.1, 5.2, 5.5, 5.6, 5.9, 5.11 and 5.15 were evaluated as telomerase inhibitors using the modified TRAP assay. Three established G-quadruplex ligands, BRAC019, TMPyP4 and telomestatin were also evaluated with this method and, as previously reported, the
latter showed the lowest EC₅₀ value (Table 6.2). The naphthalene diimide ligands were tested at concentrations of up to 50 μM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TRAP EC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>25</td>
</tr>
<tr>
<td>5.2</td>
<td>17</td>
</tr>
<tr>
<td>5.5</td>
<td>21.3</td>
</tr>
<tr>
<td>5.6</td>
<td>27.9</td>
</tr>
<tr>
<td>5.9</td>
<td>21</td>
</tr>
<tr>
<td>5.11</td>
<td>12.3</td>
</tr>
<tr>
<td>5.15</td>
<td>&gt;50</td>
</tr>
<tr>
<td>BRAC019</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>TMPyP4</td>
<td>16.4 ± 11.9</td>
</tr>
<tr>
<td>Telomestatin</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

Table 6.2 TRAP EC₅₀ values for a selected group of naphthalene diimide ligands and reference ligands. TRAP evaluation was done by duplicate for the naphthalene diimide ligands (except for 5.11 when was done once) and at least three times for the reference compounds.

The results of the TRAP evaluation showed that the naphthalene diimide ligands are telomerase inhibitors in vitro at concentrations of the same order as BRACO-19 or TMPyP4.

No evident correlation could be observed between FRET and TRAP data but compound 5.15, the morphonline containing analogue, was the worst ligand in both FRET and TRAP in the group. Telomestatin was the best ligand in the new TRAP assay. The
recent discovery of the invalidity of the traditional TRAP assay (de Cian et al., 2007b), used for the evaluation of most of the reported ligands, limits the conclusions that can be obtained from these data until more ligands are evaluated with the newly modified TRAP assay.

### 6.3.4 Surface plasmon resonance (SPR)

*The SPR studies described in this thesis were conducted by the laboratory of Professor W. David Wilson (Georgia State University, Atlanta, US).*

Biosensor-SPR binding studies were conducted on a representative set of naphthalene diimide ligands, 5.1, 5.3, 5.5, 5.6, 5.9, 5.21 and 5.23, using an immobilised human telomeric G-quadruplex model.

The sensorgrams for all compounds reached a steady-state plateau between 100-200 seconds after initiation of compound injection. Analysis of the binding type consistently showed two different binding sites, a strong one and a second one around 50-fold weaker. Sensorgrams for the binding of compounds 5.5 and 5.23 are compared in figure 6.9.

![Sensorgrams for compounds 5.5 and 5.23](image)

*Figure 6.9 Sensorgrams for compounds 5.5 and 5.23.*
The association kinetics constants could not be determined because of surface absorption of the compounds in the initial period of injection. However, it is clear that the dissociation reaction, as seen in the sensorgrams for 5.5 and 5.23, was significantly slower for the tetra-substituted naphthalene diimides than for the disubstituted. The tri-substituted analogue 5.6 had a dissociation rate more similar to the tetra- than to the disubstituted naphthalene diimides.

The values for the main binding constants and dissociation constants for the naphthalene diimide ligands and a group of reference compounds (literature values) are tabulated in table 6.3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_a$ ($M^{-1}$)</th>
<th>$k_d$ ($s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>$2.4 \times 10^7$</td>
<td>0.023</td>
</tr>
<tr>
<td>5.3</td>
<td>$4.0 \times 10^6$</td>
<td>0.099</td>
</tr>
<tr>
<td>5.5</td>
<td>$3.1 \times 10^7$</td>
<td>0.019</td>
</tr>
<tr>
<td>5.6</td>
<td>$1.1 \times 10^7$</td>
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</tr>
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<tr>
<td>5.23</td>
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<td>BR-19</td>
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<tr>
<td>BSU6048</td>
<td>$3 \times 10^6$</td>
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</table>

Table 6.3 Surface plasmon resonance results for the naphthalene diimide series and a series of reference ligands with the human telomeric sequence. n.a.: not available.

Compound 5.5, the strongest binder in this series, showed a very strong binding site with $K_a=3.1 \times 10^7$ $M^{-1}$ and a weaker secondary site with a $K_a \sim 6 \times 10^5$ $M^{-1}$. According to this, compound 5.5 binds to the telomeric G-quadruplex with at least the same affinity than other G-quadruplex ligands such as BRACO-19, TmPyP4 and RHPS4 and with a clearly higher potency than most reported ligands, including disubstituted acridines such as BSU6048.

The SPR $K_a$ data correlates to some extent with the $\Delta T_m$ FRET data for F21T (Figure 6.10). This proves that the binding constants of ligands with G-quadruplex DNA influence the G-quadruplex stabilisation ability.
Chapter 6 – Biophysical evaluation of naphthalene diimide G-quadruplex targeting agents

Figure 6.10 Correlation between the $K_a$ obtained by SPR and the $\Delta T_m$ obtained by FRET, both assays using the human telomeric G-quadruplex, for the naphthalene diimide ligands and BRACO-19. The data correlates with at least a 5% level of significance.

The SPR data confirms that the tetra-substituted naphthalene diimides are amongst the strongest G-quadruplex binding compounds found to date.

The SPR data with other DNA sequences, including duplex DNA and G-quadruplex DNA from promoter regions, was not available at the time of writing and it will be reported elsewhere.

6.4 CONCLUSIONS

The naphthalene diimide ligands were evaluated in FRET, TRAP and SPR assays. FRET and SPR experiments showed some of the ligands to be amongst the stronger G-quadruplex binders reported to date. TRAP experiments showed the ligands to inhibit telomerase in vitro at concentrations of the same order as other G-quadruplex ligands.

The FRET assay showed very strong stabilisation of the telomeric and c-kit G-quadruplex structures at low concentrations (0.5 µM). Some of the compounds however, also showed some degree of interaction with duplex DNA, as confirmed by FRET competition experiments. Compounds with side chains having morpholino and hydroxyl groups showed remarkable potency when compared to other neutral or morpholino-containing ligands.
From the evaluation of the FRET data, it seems clear that tetra-substituted analogues containing side chains of three carbons in length and having tertiary amine end groups show the greater stabilisation ability for G-quadruplex DNA. The best compounds in terms of G-quadruplex/duplex DNA discriminatory ability are also tetra-substituted analogues with side chains with three carbons but having morpholino and hydroxyl end groups (and to a lesser extent diethylamino groups). Compounds containing combinations of these types of side chains are of interest for future investigations and may offer a compromise middle point between potency and selectivity.
CHAPTER 7

PRELIMINARY BIOLOGICAL EVALUATION OF NAPHTHALENE DHMIDE G-QUADRUPLEX TARGETING AGENTS
Chapter 7 - Preliminary biological evaluation of naphthalene diimide G-quadruplex targeting agents

7 PRELIMINARY BIOLOGICAL EVALUATION OF NAPHTHALENE DIIMIDE G-QUADRUPLEX TARGETING AGENTS

7.1 ASSAYS FOR THE BIOLOGICAL EVALUATION OF G-QUADRUPLEX LIGANDS

7.1.1 Background

For a general introduction into the biological methods used for the evaluation of anti-cancer drugs and the description of SRB, senescence detection and general cell culture methods used within this chapter please refer to chapter 4.

7.1.2 General

The compounds were evaluated against a small panel of cell lines: MCF7, human breast adenocarcinoma derived cell line; A549, human lung adenocarcinoma derived cell line; and WI38, derived from human lung fibroblasts. WI38 was used as a model for normal somatic tissue, necessary to assess the selectivity of the compounds between normal and cancerous cells.

7.1.3 Cell uptake

The impermeability of the outer membrane of the eukaryotic cells represents a major barrier for many small molecules that impede them to reach their intracellular targets. Cell uptake studies are sometimes necessary to confirm that a compound is reaching its target and to identify, when possible, the specific part of the cell where the agent accumulates. A common strategy to localise an agent in the intracellular domain is to modify it with fluorescent or radioactive tags that can be subsequently detected to reveal
its localisation. However, compounds that constitutively induce fluorescence can be detected in the intracellular domain without modifications (du Buy & Showacre, 1961).

The tri- and tetra-substituted naphthalene diimide compounds presented in this thesis showed fluorescent properties, in common with other naphthalene diimides (Thalacker et al., 2006). This allowed one to use their natural occurring fluorescence to detect their localisation within the cells using high resolution confocal microscopy.

Small molecules (MW < 5000 Da) can circulate unimpeded from the cytosol to the nucleus through the porous nuclear membrane (Lodish et al., 1998). If as hypothesised the naphthalene diimide ligands target DNA, once inside the cells they should be detected inside the nucleus, where DNA concentrates.

Cell uptake studies were conducted with MCF7 cells. These cells are relatively large, contain large nuclei and display a rounded morphology, which makes possible the visual identification of the different cell compartments.

7.1.4 Chromosome fusions

As explained in the introduction of this thesis (page 40), G-quadruplex targeting agents can displace key proteins from the telomeres resulting in the exposure of the free DNA ends. The uncapping of telomeres can result in the ends of telomeric DNA being recognised as DNA breaks by the cell and consequently processed as such by non-homologous end joining (NHEJ). The resulting cells would contain dicentric chromosomes and when they undergo mitosis chromosomes breakages occur, producing more free DNA ends. The successive rounds of mitosis would result in accumulation of chromosomal abnormalities and often end with the viability of the cell.

The ordered DNA arrangement in mitotic cells allows the karyotyping of cells for chromosomal analysis. The treatment of cells with colcemid arrests the mitotic cells in metaphase and allows the preparation of metaphase spreads that can be visualised by microscopy.
The exposure of cells to G-quadruplex ligands has shown in the past to increase the number of chromosomal fusions (Incles et al., 2004). The level of chromosomal fusions in cells under treatment with a representative group of naphthalene diimide ligands was evaluated.

7.1.5 Gene Expression

The gene expression assays described in this thesis were conducted by Dr. Mekala Gunaratnam and Mónica Beltrán in our laboratories.

The stabilisation of G-quadruplexes in promoter regions of genes can act as expression inhibitors, as discussed before (page 40). One of the genes containing G-quadruplex forming sequences with great interest for anti-cancer treatment, is the proto-oncogene receptor tyrosine kinase c-kit, which suffers gain-of-function mutations in many types of cancers.

Some 80% of gastrointestinal tumors (GIST) express the c-kit oncoprotein and direct inhibition with imatinib is therapeutically used in patients. The cell line GIST882, derived from a patient’s gastrointestinal stromal tumour, expresses constitutively activated c-kit. To evaluate if some of the ligands in the naphthalene diimide series were useful at inhibiting the expression of c-kit in this cell line, gene RNA expression analyses were conducted.

Furthermore, as explained in the introduction (page 40), some G-quadruplex targeting agents have been shown to decrease the level of intracellular telomerase activity, allegedly a consequence of the inhibition of the expression of c-myc, which regulates the expression of hTERT, or direct inhibition of the expression of the hTERT gene, which also contains a G-quadruplex forming sequence. The detection of diminished expression of c-myc and hTERT would support the G-quadruplex targeting mechanism of these ligands and these experiments were also conducted.
7.2 MATERIALS AND METHODS

7.2.1 General

General method descriptions for cell culture techniques can be found in chapter 4.

WI38 (American Type Culture Collection) cells were maintained as explained for the MCF7 cells in chapter 4 but using medium MEM Eagle (M2279, Sigma, UK) with added L-glutamine (2 mM), 1% essential amino acids and 10% foetal calf serum.

7.2.2 Cell uptake studies

Following counting of the cells as described before, 5x10^5 MCF7 cells were seeded in a well of a 8 chamber polystyrene vessel glass slide (BD Falcon, BD Biosciences, MA, USA) in 2 ml of indicator-free medium (GIBCO 31053, Invitrogen, UK) and incubated for 24 hours. The cells were then treated with the compound to test and incubated for 30 minutes. The medium was removed and the cells were washed with PBS (2 x 2 ml). The cells were then fixed by incubation at -20°C with methanol for 10 minutes and washed with PBS (2 x 2ml). The plastic cubette was dismounted and the slide covered with a coverslip using mounting medium (Vectashield, Vector Laboratories, CA, USA) and sealed using nail varnish. The slides were kept in the fridge in the dark during the short time prior to visualisation.

The same experiments were repeated without fixing the cells. Although the resulting slides were not optimal to take pictures they allowed the visual identification of the same ligand distribution in the cells as for the methanol fixed cells and permitted to discard the possibility of re-localisation of the ligands post-fixation.

Slide visualisations were performed in a LSM 510 META microscope (Zeiss, Germany) with a range of lenses (Zeiss, Germany). The localisation of the compounds within the cells was achieved by using both transmission and confocal fluorescent microscopy with 63X/1.4 NA or 40X/0.75 NA oil submersion lenses. The transmission light microscopy allowed the identification of the different compartments in the cells and the
fluorescent properties of the compounds (strong emission in the red region when excited with a laser of wavelength 543 nm) permitted the use of confocal microscopy to localise the compounds within the cells. Composite images using both transmitted light and fluorescence images were made using the software provided with the microscope system (Zeiss, Germany) to assign the source of the fluorescence.

7.2.3 Chromosome fusions

Following counting of the cells as described above, \(1 \times 10^5\) MCF7 cells were seeded in a 75 cm\(^2\) cell culture flask in 10 ml of medium and the compound to test. Several flasks for each experiment were needed to obtain enough cells for the slide preparation (around \(1 \times 10^6\) cells). The cells were re-treated on day 4 as described before. On day 7, the cells were treated with 100 µL of colcemid (GIBCO KARYO MAX®, Invitrogen, UK) and incubated for 1 hour. The medium was removed and the cells washed with PBS (2 ml), trypsinised and centrifuged at 1200 rpm for 15 minutes. The supernatant was removed except for the last few drops and the pellet was re-suspended by flicking the tube. Potassium chloride solution (0.075 M) was added until a total volume of 12 ml. The contents were mixed by inversion of the tube and then incubated for 20 minutes at room temperature followed by addition of 5 drops of freshly prepared fixative solution (3:1 methanol/acetic acid). The contents were mixed by inverting the tube 5 times and then centrifuged at 1200 rpm for 15 minutes. The supernatant was removed except for the last few drops and the pellet was re-suspended by flicking the tube. The dispersion was added into 9 ml of freshly prepared fixative solution using a Pasteur pipette and mixed by squeezing the pipette up and down 10 times. The mixture was centrifuged at 1200 rpm for 15 minutes. The supernatant was discarded and the pellet was re-suspended by vortexing whilst adding 5 ml of freshly prepared fixative solution. The mixture was centrifuged at 1200 rpm for 6 minutes. The supernatant was discarded and the pellet was re-suspended by vortexing whilst adding 3 ml of freshly prepared fixative solution. The mixture was centrifuged at 1200 rpm for 6 minutes. The supernatant was discarded except for the last few drops and the pellet was re-suspended by vortexing.

For the slide preparation 20 µL of the mixture were dropped onto a wet, pre-washed glass slide at arms length and the slide was breathed onto. The excess water from the
back of the slide was removed with a paper towel and the slide was placed on a hotplate at 80°C for 2 seconds. The slides were placed in an oven at 60°C overnight.

For the staining, the slides were submerged for 2 seconds in a tube containing saline solution (0.9% NaCl), then in a second tube containing saline solution and placed in a tube containing 5% Giemsa’s staining solution (Prod 350144M, BDH Gurr®, VWR, UK) in buffer pH 6.8 (Prod 331932D, BDH Gurr®, VWR, UK) for 3 minutes. The slides were rinsed by consecutive submersion in 2 tubes containing buffer pH 6.8. The excess water was removed using a paper towel and the slides were placed in an oven at 60°C overnight.

Slide visualisations were performed in a LSM 510 META microscope (Zeiss, Germany) with a range of lenses (Zeiss, Germany). Metaphase spreads were visualised using transmission light microscopy with a 63X/1.4 NA oil submersion lens. The level of chromosome fusions was expressed as a percentage of fused chromosomes per microscope field, averaged from at least 3 different fields representative of the total population.

### 7.2.4 Gene expression studies

The gene expression studies were conducted in an analogous manner as described by Gunaratnam (Gunaratnam et al., 2007).
7.3 RESULTS OF THE BIOLOGICAL EVALUATION OF THE NAPHTHALENE SERIES

7.3.1 Surforhodamine B assay (SRB)

The short-term cell growth inhibitory ability of the naphthalene diimide ligands was evaluated using the SRB assay. The compounds showed high potency, especially towards the cancer cell lines MCF7 and A549 when compared to the somatic cell line WI38 (Table 7.1).

<table>
<thead>
<tr>
<th>Compound Code</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCF7</td>
</tr>
<tr>
<td>5.1</td>
<td>105 ± 23</td>
</tr>
<tr>
<td>5.2</td>
<td>5.4 ± 1.3</td>
</tr>
<tr>
<td>5.3</td>
<td>9.2 ± 3</td>
</tr>
<tr>
<td>5.4</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>5.5</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>5.6</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>5.7</td>
<td>220 ± 40</td>
</tr>
<tr>
<td>5.8</td>
<td>164 ± 13</td>
</tr>
<tr>
<td>5.9</td>
<td>81 ± 15</td>
</tr>
<tr>
<td>5.10</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>5.11</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>5.12</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>5.13</td>
<td>437 *</td>
</tr>
<tr>
<td>5.14</td>
<td>800 *</td>
</tr>
<tr>
<td>5.15</td>
<td>6700 *</td>
</tr>
<tr>
<td>5.16</td>
<td>1610 *</td>
</tr>
<tr>
<td>5.17</td>
<td>296 ± 63</td>
</tr>
<tr>
<td>5.18</td>
<td>129 ± 41</td>
</tr>
<tr>
<td>5.19</td>
<td>8300 *</td>
</tr>
<tr>
<td>5.20</td>
<td>9200 *</td>
</tr>
<tr>
<td>5.21</td>
<td>27 ± 12</td>
</tr>
<tr>
<td>5.22</td>
<td>288 ± 111</td>
</tr>
<tr>
<td>5.23</td>
<td>135 *</td>
</tr>
<tr>
<td>5.24</td>
<td>119 *</td>
</tr>
<tr>
<td>BR-19</td>
<td>2500 *</td>
</tr>
</tbody>
</table>

Table 7.1 SRB IC<sub>50</sub> results for the evaluation of the naphthalene diimide ligands. The concentration of 10 μM was the higher tested. * Experiment performed by duplicate.
The compounds as a class showed potent cell growth arrest, especially in the cancer cell lines, although there is wide variation in behaviour with the most potent compounds such as 5.2, 5.3, 5.10-5.12 having IC$_{50}$ values of 5-10 nM. The MCF7 cell line was consistently the most sensitive.

![Figure 7.1](image1.png)

**Figure 7.1** Results for the SRB evaluation of compound 5.1 with cell line MCF7. On the Y axes, the percentage of cellular protein, which is measured by reading the absorbance of the associated SRB dye, normalised with the control experiment. On the X axes, the concentration of the different treatments.

The IC$_{50}$ values for the different cell lines showed a good correlation (Figures 7.2 and 7.3). The correlation of IC$_{50}$ values between different cell lines may suggest that the acute toxic effect of the compounds is a consequence of affecting the same molecular target in all three cell lines. This molecular target cannot be a distinctive viability mechanism of cancer cells, such as telomerase, but rather something that is constitutively present in cancerous but also somatic cells since this correlation is observed too for WI38 cells.

![Figure 7.2](image2.png)

**Figure 7.2** Correlation of IC$_{50}$ values of naphthalene diimide ligands with MCF7 and A549 cells.
Chapter 7 – Preliminary biological evaluation of naphthalene diimide G-quadr. targeting agents

Figure 7.3 Correlation of IC₅₀ values of naphthalene diimide ligands with MCF7 and WI38 cells.

The possible reasons for the selective acute toxicity towards cancer cells may be varied. One possibility is that the ligands are showing an additive effect of telomerase/telomere targeting and background toxicity, which would be observed in all cell lines but with more intensity on cancer cells. Moreover, it cannot be discarded that the selectivity is a consequence of the different rate of replication of the cancer and somatic cells or the different uptake by the different cells, which could influence the apparent potency.

Figure 7.4 Correlation analyses of FRET and IC₅₀ values for the naphthalene diimide ligands. The values correlate with at least a 5% level of significance.
The hypothesised mechanism of action of the naphthalene diimide ligands is G-quadruplex targeting. As shown by FRET experiments (page 131), the selectivity of most of these ligands between G-quadruplex and duplex DNA was not optimal. The possibility that some of the biological effects detected are a consequence of duplex DNA binding cannot be discarded. The potential correlation between FRET and $IC_{50}$ values was investigated (Figure 7.4). This analysis shows that the binding abilities of the naphthalene diimide ligands with both, G-quadruplex and duplex DNA, correlate to some extent with the toxicity found for the cell lines MCF7, A549 and WI38.

### 7.3.2 Senescence

The senescence experiments described in this chapter were conducted by Ms. Olga Greciano in our laboratories.

The onset of senescence was investigated in MCF7 cells treated for 1 week with sub-cytotoxic concentrations of a representative group of ligands, compounds 5.1, 5.6, 5.9 and 5.15. The results are tabulated in table 7.2.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Senescence (% of cells) after 1 week treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>5.1 (70 nM)</td>
<td>35.0 ± 3</td>
</tr>
<tr>
<td>5.6 (15 nM)</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>5.9 (75 nM)</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>5.15 (3.5 μM)</td>
<td>51 ± 3</td>
</tr>
</tbody>
</table>

Table 7.2 Senescence results for compounds 5.1, 5.6, 5.9 and 5.15 with cell line MCF7 after 1 week treatment.

All the compounds used in this study, including compound 5.15 which is significatively less active, showed a significant increase in the percentage of senescence cells after 1 week exposure to sub-cytotoxic concentrations. These results suggest that a large part of the cell growth inhibition caused by these compounds is related to the onset of senescence.
The senescence effects observed here are consistent with the observation that the compounds target telomeres and therefore their ability to selectively kill cancer cells seems to be at least in part a consequence of telomere targeting and telomerase uncapping effects.

Figure 7.5 Senescence staining for MCF7 cells treated with 15 nM of 5.6 for 1 week.

7.3.3 Cell uptake studies

A selection of compounds, representative of the structural diversity within the naphthalene diimide library, was evaluated in the cell uptake experiments. The selected molecules were compounds 5.1, 5.6, 5.9 and 5.15.

Compounds 5.1, 5.6 and 5.9 displayed similar results. All three compounds were shown to localise almost exclusively in the nucleus of MCF7 cells upon a 30 minutes exposure to a 0.5 μM ligand solution (Figure 7.6).

Figure 7.6 Pictures of the MCF7 cells uptake experiment with compound 5.1. a) Fluorescence image showing accumulation of compound 5.1 in the nucleus and particularly in the nucleoli of MCF7 cells upon exposure at 0.5 μM for 30 minutes; b) Composite image of the fluorescence image (a) and the transmitted light image (c), showing that the unambiguous localisation of the source of fluorescence is the nucleus of MCF7; c) Transmitted light image of the same microscopy field, where inner structures of the cells such as nucleus and nucleoli are recognisable.
Although some methodologies try to extract quantitative information from confocal imaging (Kask et al., 1999) this is generally not possible. For these experiments however, it is qualitatively clear that within the nucleus, the ligands showed preference for the nucleoli. The organelles were clearly visible using transmission light microscopy and composites images of the transmission and fluorescence pictures clearly indicated the origin of the fluorescence. This has been observed before for at least another two families of G-quadruplex targeting agents, RHPS4 and analogues (Cookson et al., 2005b) and CX-3543 (Whitten et al., 2007).

For the MCF7 cells exposed to compound 5.15, even at higher concentrations (50 μM), weaker and homogeneously distributed fluorescence was observed (Figure 7.7). The fluorescence signal from the cells was significantly more intense than the background signal, but within the cell, there seemed to be uniform distribution of the ligand. The fluorescent properties of the naphthalene diimides are conferred by the poly-aromatic core, hence it is expected that the different results for compounds 5.1, 5.6 and 5.9 with respect to compound 5.15, are exclusively due to the poor uptake of the latter.

![Figure 7.7](image)

**Figure 7.7** Pictures of the MCF7 cells uptake experiment with compound 5.15. a) Fluorescence image showing the low and diffuse emission detected after exposure of MCF7 cells to 50 μM of compound 5.15 for 30 minutes; b) Composite image of the fluorescence image (a) and the transmitted light image (c), showing the unspecific localisation of the ligand; c) Transmitted light image of the same microscopy field, where inner structures of the cells such as nucleus and nucleoli are recognisable.

Although compound 5.6, possessing only three side chains, is the most structurally different amongst the four evaluated ligands it was compound 5.15 the one that presented a remarkably different cell uptake. Compound 5.15 contains morpholine groups at the end of the side chains and this is the probable reason for its different behaviour. Compounds 5.1, 5.6 and 5.9 bare side chains with dimethylamine,
diethylamine and pyrrolidine respectively. The bases trimethylamine, triethylamine, \(N\)-methylpyrrolidine and \(N\)-methylmorpholine are structurally similar to the end groups of compounds 5.1, 5.6, 5.9 and 5.15 respectively. The p\(K_a\) of the conjugated acids of these bases (Williams, 2008) can serve as an estimation of the approximate p\(K_a\) of the correspondent conjugated acids of the naphthalene diimide ligands (Table 7.3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>p(K_a) of conjugated acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimethylamine</td>
<td>9.80</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>10.75</td>
</tr>
<tr>
<td>(N)-methylpyrrolidine</td>
<td>10.46</td>
</tr>
<tr>
<td>(N)-methylmorpholine</td>
<td>7.41</td>
</tr>
</tbody>
</table>

Table 7.3 p\(K_a\) values for tertiary amines structurally similar to the basic groups of compounds 5.1, 5.6, 5.9 and 5.15.

If, as expected, the p\(K_a\) values of these simpler amines are similar to those of the naphthalene diimide ligands, it should be expected that at least one of the basic groups of compounds 5.1, 5.6 and 5.9 will be almost completely protonated at pH 7.4, the pH of the medium. Compound 5.15, on the contrary, is expected to be partially protonated (± 50%) at pH 7.4.

### 7.3.3.1 Cell uptake mechanism

The cell membrane is permeable to small, uncharged molecules. Molecules of high molecular weight and molecules containing charges often require an active transport mechanism to be imported into the cytosol (Koepsell, 1998). The molecular weight (408-749) and the highly cationic character (up to four positive charges) of the naphthalene diimide ligands suggest that they probably require an active transport mechanism.

The images from the cell uptake studies showed the morpholino compound 5.15 to have poor cell permeability, as opposed to compounds 5.1, 5.6 and 5.9. The main difference in lipophilicity amongst these ligands is probably consequence of the different number of charges (differences in lipophilicity caused by differences in the length of the aliphatic carbon chains, for example, are smaller and can be overlooked for this discussion). These results suggest the existence of an active uptake mechanism for these
ligands that can discriminate between the highly protonated compounds, such as 5.1, 5.6 and 5.9 and the lesser protonated or neutral compounds, such as 5.15.

7.3.4 Chromosome fusions

G-quadruplex interacting agents can potentially cause telomere uncapping, which may result in end-to-end fusions of independent chromosomes. Chromosome spreads were prepared and visualised for MCF7 cells exposed to compounds 5.1, 5.6, 5.9 and 5.15 at the same concentrations that caused the onset of senescence.

![Figure 7.8 Chromosome spreads of MCF7 cells treated with compound 5.1.](image)

No significant increase in the number of chromosome fusions was observed in the treated cells when compared to the control untreated cells.

This result is not incompatible with the hypothesised targeting of the telomeres since the lack of chromosomal fusions could be consequence of a DNA damage response upon telomere uncapping that leads to the onset of senescence avoiding the generation of chromosomal abnormalities.

7.3.5 Gene expression studies

*The gene expression assays described in this thesis were conducted by Dr. Mekala Gunaratnam and Mónica Beltrán in our laboratories.*
Compound 5.11 was evaluated in gene expression studies with a gastrointestinal stromal tumor patient derived cell line, GIST882. The IC\textsubscript{50} of 5.11 with this cell line was 1.62 µM, as measured by the SRB assay.

The results showed 5.11 to inhibit 70% of the expression of c-kit mRNA after 24 hours exposure to a sub-cytotoxic concentration (1 µM), while it did not affect the expression of a control gene, GAPDH. This was accompanied by the inhibition of the expression of c-kit protein, which was completely inhibited with a treatment of 0.5 µM of 5.11 for 24 hours. The expression of actin, used as a control, was not affected.

Compound 5.11 was also found to inhibit the gene expression of both, c-myc and hTERT, following exposure at 0.5 µM for 24 hours. c-myc expression was reduced by approximately 30% whereas hTERT expression was diminished by approximately 20%. The same treatment did not affect the expression of k-ras, which also contains a G-quadruplex forming sequence.

Furthermore, 5.11 was found to completely abolish the intracellular telomerase activity in GIST882 cells exposed to 0.5 µM for one week, as measured by TRAP. This observation was concomitant with the abrogation of the expression of c-myc and/or hTERT.

These results are consistent with the gene expression inhibitory ability of G-quadruplex ligands in genes containing G-quadruplex forming sequences in their promoter regions.

Further experiments with other ligands and genes are undergoing and will be published elsewhere.

7.4 CONCLUSIONS

The biological evaluation showed the naphthalene diimide ligands to be a potent class of anti-proliferative agents, with most compounds being selectively toxic towards the cancerous cell lines, showing a typical preference of about 5-fold. The anti-proliferative effects observed seem to be related to the onset of senescence.
In the FRET and TRAP evaluation (pages 129 and 136 respectively) and cell toxicity studies (page 148), compound 5.15 displayed substantially different results to the rest of ligands (by extension, this discussion can be applicable to the other three morpholine containing ligands, compounds 5.13, 5.14 and 5.16). As proven by FRET and TRAP, the G-quadruplex binding abilities of the morpholino containing ligands are lower than for similar compounds containing different end groups. Their activity in cell culture is also weaker. The reason for the poor results in FRET and TRAP seems to be exclusively due to the weaker DNA binding abilities, as expected from previous results (chapter 3; for example, Harrison et al., 2004). This is probably a consequence of the lower basicity of the morpholino groups and consequently the neutral or weakly cationic character of the compounds at the neutral pH of the experiments. The low affinity of compound 5.15 for DNA is supported by the cell uptake pictures, which showed a lack of specificity in its localisation within the cells. The compound did not show preference for the nucleus, where the overwhelming majority of the cellular DNA is concentrated. In the toxicity studies however, the factors that affected the performance of compound 5.15 (and probably of compounds 5.13, 5.14 and 5.16) were probably a combination of poor cell uptake and low DNA affinity.

Compound 5.11 was shown to inhibit the expression of several genes that contain G-quadruplexes forming sequences in their promoter regions. This activity should also be considered as a possible mechanism of action of these ligands.
8 CONCLUSIONS

8.1 CONCLUSIONS FOR THE ACRIDONE SERIES

Some of the acridone ligands presented in this thesis showed very good activity in the FRET assays, both traditional and competition, where they displayed strong binding for G-quadruplex DNA, particularly the telomeric G-quadruplex, and very low affinity for duplex DNA. This activity is maximised for ligands containing protonated tertiary amines as end groups and side chains containing two carbon linkers, or three carbon linkers for the analogues without the carbonyl group. The hypothesised importance of the large planar moiety was confirmed, since the analogues not possessing this feature showed a decreased binding affinity.

None of the ligands showed inhibition of telomerase in the TRAP assay at concentrations of up to 100 μM. This result was somehow unexpected since other ligands with less tight binding to the telomeric G-quadruplex, such as BRACO-19, showed telomerase inhibition at lower concentrations and the correlation between FRET and TRAP data has been previously reported (Schultes et al., 2004). This suggests that the FRET data does not always successfully predict the telomerase inhibitory ability of G-quadruplex ligands as measured by the newly modified TRAP assay, although a larger number of ligands should be evaluated with it to support this conclusion.

The ligands as a group showed selective toxicity towards MCF7 and A549 cell lines and none of the disubstituted ligands showed toxicity for the somatic cell line IMR90. The IC₅₀ values did not clearly correlate with the DNA melting experiments values. This could be caused by differences in the uptake by cells or may imply that there is an alternative mechanism of action that contributes to the observed effect. In the long-term studies several compounds inhibited cell growth at sub-cytotoxic concentrations for at least three weeks and up to six weeks.
To try to obtain an insight into the mechanism of action, preliminary senescence and chromosomal spread visualisation studies were conducted. Some senescence was detected in both MCF7 and A549 treated cells, but the levels were low and possibly insufficient to explain the more pronounced effects on cell growth. The chromosome spreads did not show any sign of an abnormal level of chromosomal fusions.

The mechanism of action of the acridone ligands could not be unequivocally determined. Although the supposed mechanism of action is telomere related, the compounds also showed affinity for the \textit{c-kit} G-quadruplexes and their interaction with these, or other G-quadruplexes, and their effects on the gene expression of these targets may contribute to the biological effects.

Further evaluation of these compounds could include gene expression studies and further studies of mechanisms of cell growth arrest, such as more detailed senescence screening and apoptosis.

\textbf{8.2 CONCLUSIONS FOR THE NAPHTHALENE DIIMIDE SERIES}

The naphthalene diimide ligands, as a group, showed some of the tightest binding with G-quadruplex DNA reported to date, as the results for FRET and SPR analyses showed. The binding was of similar potency with the three G-quadruplex forming sequences analysed. The SAR analysis showed that ligands with four substituents were the stronger binders. The analogues containing side chains with three carbons in length and protonated tertiary amines were the best within the tetra- but also the tri-substituted sub-series.

Most ligands, to different extents, also showed some affinity towards duplex DNA as the traditional and competition FRET results showed. Although some analogues showed better selectivity, this enhancement was generally accompanied by a reduction in the net binding ability for the G-quadruplex sequences. The enhancement of the specificity of these ligands towards G-quadruplex DNA while maintaining their strong binding must be addressed in further development of this series. Furthermore, all G-quadruplex ligands must aim to selectively target a particular G-quadruplex or G-quadruplex type. The molecular optimisation of naphthalene diimide ligands can be based on the results
from crystallographic studies of complexes of ligands with G-quadruplexes. Several of these crystals have been recently obtained with several members of the naphthalene diimide library (Parkinson et al., 2008). The first of these structures is described in the next section.

The naphthalene diimide ligands were shown to inhibit telomerase in vitro in the TRAP assay with potencies similar to other well established G-quadruplex ligands.

Cell culture studies showed the naphthalene diimide ligands to possess, in general, very high cell growth inhibitory potency with some ligands in the low nanomolar range of activity. The ligands consistently showed selective toxicity towards the cancer cell lines when compared to the somatic cell line used as a control.

The mechanism of action of the ligands was investigated in cell culture using a number of experiments.

Senescence was detected in MCF7 cells exposed for one week to sub-cytotoxic concentrations of several naphthalene diimide compounds. The onset of senescence is a possible consequence of telomere damage and this result supports the notion that the naphthalene diimides are binding to the telomeres.

The cell uptake studies for four analogues showed that the ability of the ligands to cross the cellular membrane depends on the nature of the end groups. The morpholino analogue, less basic than the rest of the tested compounds, showed poor uptake in MCF7 cells. This could be due to the different cationic character of the molecules. Furthermore, the morpholino analogue was found to homogeneously distribute within the cell, whereas the other three analogues showed clear preference for the nucleus and particularly nucleoli. The localisation in the nucleus is consistent with the DNA targeting abilities of the ligands and the fact that the morpholino analogue did not show this preference is consistent with the weaker DNA binding ability showed by this compound in the FRET assays.

The reason for the specificity for the localisation in the nucleoli is not clear although there is growing evidence of the biological relevance of G-quadruplex structures in the
nucleolus. The G-quadruplex ligand RHPS4 and analogues were similarly detected to localise in nucleoli (Cookson et al., 2005b). Another G-quadruplex targeting agent, CX-3543, currently in clinical trials, was also found to accumulate in the nucleoli (Whitten et al., 2007). Ribosomal RNA (rRNA) genes of ribosomal DNA (rDNA), which accumulate in the nucleolus, were found to contain a high number of putative G-quadruplex forming sequences (Whitten et al., 2007). CX-3543 was found to inhibit the synthesis of rRNA, to disrupt the complexes between rDNA and nucleolin (a nucleolar protein) and cause cell growth inhibition in cancer cells (Whitten et al., 2007).

Furthermore, a recent study in yeast detected a dramatic downregulation of the nucleolar-function genes upon exposure to a G-quadruplex targeting porphyrin (Hershman et al., 2008). This was hypothesised to occur as a response to the direct inhibition of rRNA, which is rich in putative G-quadruplex forming sequences.

The gene expression studies conducted showed that naphthalene diimide ligands can inhibit the expression of c-kit, c-myc and hTERT. This result is consistent with gene expression inhibition by G-quadruplex stabilisation at the promoter regions.

As has been discussed here, the naphthalene diimide ligands have several possible mechanisms of action. Both telomere targeting and gene expression inhibition have been confirmed experimentally. The accumulation of the ligands in the nucleoli is intriguing and part of their activity could be related to rDNA or rRNA targeting. Furthermore, the affinity of these ligands for duplex DNA and its consequences cannot be overlooked since this interaction may also have a role in the overall activity of the ligands.

8.2.1 Structural studies

The crystallographic studies discussed in this section were conducted by Dr. Gary Parkinson in our laboratories.

NMR and crystallographic studies have been extensively used to obtain three dimensional representations of nucleic acid structures and their complexes with ligands. Several structures of G-quadruplex DNA bound to ligands have been obtained, including the complex of the Oxytricha nova telomeric G-quadruplex and a
disubstituted acridine compound (Haider et al., 2002), the complex of daunomycin with an intermolecular G-quadruplex (Clark et al., 2003) and importantly for the telomerase drug discovery program a complex between the human telomeric G-quadruplex and TMPyP4 (Parkinson et al., 2007) and more recently with BRACO-19 (Campbell et al., 2008).

A series of naphthalene diimides described in this thesis have been employed to obtain crystals of complexes with a number of DNA sequences. At the time of writing, several of these structures were being solved. The first of these structures to be obtained is briefly presented. The crystallographic and computing methods utilised to obtain this and the forthcoming structures will be shortly published (Parkinson et al., 2008).

![Figure 8.1 View of the asymmetric unit of the crystal obtained for the complex of the 23-mer with 5.22. For clarity the ligands have been represented using thick cylinders, the guanine residues involved in the formation of G-quartets in black and the rest of DNA in grey. The planes of the G-quartets, aromatic core of the ligands and bases of loops involved in the stacking of ligands are all perpendicular to the paper.](image)

The structure is a complex of a 23-mer from the human telomeric sequence and similar to the 22-mer from the human telomeric G-quadruplex crystal structure (Parkinson et
with the ligand \textit{5.22}. The asymmetric unit of the crystal contains two G-quadruplexes, two molecules of ligand and 4 molecules of ligand shared with contiguous unit cells (Figure 8.1). The stoichiometry is $2 : 6$ (DNA/ligand).

The core structure is very similar to the all parallel structure described for the native 22-mer (Parkinson et al., 2002), which supports the notion that the all parallel structure is relevant for drug design.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.2}
\caption{Two views of the two ligands stacked between the two G-quadruplexes in the complex of the 23-mer with \textit{5.22}. For clarity the ligands have been coloured in pink and cyan and the DNA in grey.}
\end{figure}
The ligand binds in three different ways to the G-quadruplex. There are two ligands in between the G-quadruplexes, stacked to each other by π-π interactions. The side chains in these two ligands seem to be positioned so they increase the interaction with each other, potentially forming some hydrogen bonds (Figure 8.2).

The ligands that bind to both ends of the G-quadruplex do so by stacking interactions (Figure 8.3). The side chains of the four ligands stacked to the G-quartets face the grooves but the end groups are twisted upwards and do not establish any clear interaction apart from with water molecules. This seems to suggest that a longer side chain could improve the interactions of the ligand with the grooves (Gary Parkinson, personal communication).

The third mode of binding is to the loops where the bases form a duplex type Watson-Crick base with bases from the loops of the contiguous cell. The ligand stacks to this pair via π-π interactions. On the other end, a non-canonical pair of bases (both strands run in parallel) also stacks with the ligand. The loops that are not bound to ligand keep the same geometry as in the previous structure for the native 22-mer whereas the ones that have ligand bound adopt a different one to accommodate the ligand (Figure 8.4).

**Figure 8.3** View of the ligand stacked to the 3' end of one of the G-quadruplexes in the complex of the 23-mer with the 5.22. For clarity the ligand has been represented thick cylinders, the guanine residues of the 3' G-quartet in black and the rest of DNA in grey.
Chapter 8 – Conclusions

The mode of binding that 5.22 displays with the loops resembles the intercalation binding mode to duplex DNA.

Figure 8.4 View of the ligand stacked to the pair of bases formed by the loops for the complex of the 23-mer with 5.22. Only the bases from one of the unit cells are represented, in the left. For clarity the ligands have been represented using thick cylinders, the guanine residues involved in the formation of G-quartets in black and the rest of DNA in grey.

8.3 FINAL REMARKS

The work described in this thesis was based on the hypotheses that G-quadruplex stabilising agents can be used to selectively treat cancer taking advantage of two potential cancer-selective mechanisms of action: 1- G-quadruplex formation at telomeres of telomerase positive cancer cells and consequent telomere disruption and cell growth inhibition; and 2- G-quadruplex formation at the promoter region of the c-kit oncogene and consequent inhibition of its expression.

The results presented herein support the first hypothesis. Cancer-selective toxicity was found for most ligands and for the case of the naphthalene diimides this finding was accompanied by the detection of high levels of senescence, which support the telomere targeting mechanism. The second hypothesis was also corroborated in c-kit positive cells for which an inhibition of c-kit mRNA and protein expression was concomitant with the growth inhibitory effect.
CHAPTER 9

EXPERIMENTAL PROCEDURES
9 EXPERIMENTAL PROCEDURES

9.1 GENERAL METHODS

Reagents, solvents and chemicals were purchased from Alfa Aesar, Avocado Organics, GOSS, Lancaster Synthesis, Novabiochem or Sigma-Aldrich and were used as supplied without further purification. All the organic solvents used for the reactions were anhydrous. Microwave irradiation was performed with an Initiator microwave (Biotage, Sweden). Reactions were monitored when possible using LC/MS (as described below) or TLC. TLC analysis was carried out on silica gel (Merck 60F-254) with visualisation at 254 and 366 nm. Work-up of an organic solution in the usual manner refers to stepwise drying with magnesium sulphate, filtration and then evaporation of the filtrate in vacuo. Flash chromatography was carried out with BDH silica gel (BDH 153325P).

HPLC analyses were performed using a Gilson apparatus combining a 322 PUMP and an Agilent 1100 SERIES detector. The column was C18 5μ (100 x 4.6 mm) (41622271 (W), YMC, Japan). The flow was 1 ml/min. Four different methods were used:

Method A. Description: aqueous solvent: 0.1% formic acid in water; organic solvent: 0.1% formic acid in acetonitrile; gradient: 100% aqueous for 5 min after injection, to 75% aqueous over 17.5 min and to 40% aqueous over 3 min.

Method B. Description: aqueous solvent: 0.1% formic acid in water; organic solvent: 0.1% formic acid in methanol; gradient: 100% aqueous for 5 min after injection, to 75% aqueous over 17.5 min and to 40% aqueous over 3 min.

Method C. Description: aqueous solvent: 0.1% trifluoroacetic acid in water; organic solvent: 0.1% trifluoroacetic acid in methanol; gradient: 75% aqueous for 5 min after injection and to 25% aqueous over 40 min.
Method D. Description: aqueous solvent: 0.1% trifluoroacetic acid in water; organic solvent: 0.1% trifluoroacetic acid in acetonitrile; gradient: 75% aqueous for 5 min after injection and to 25% aqueous over 40 min.

LC/MS were performed using a Waters system combining a 2695 separation module, a Micromass ZQ spectrometer and a 2996 photodiode array detector. Method description: mobile phase: 50:50 (0.1% formic acid in water):(0.1% formic acid in acetonitrile); run time: 3 min isocratic; mode: electrospray positive (ES+); MS running conditions: 3 min run time; cone: 25; offset: 1; skimmer: 1.5; RF lens: 0.1; source heater: 150°C; gas: 400 l/h.

HPLC purifications were performed using a Gilson apparatus combining a 322 PUMP and a UV/VIS-155 detector. The preparative column was C18 5μ (100 x 20 mm) (201022272 (W), YMC, Japan). The flow was 10 ml/min. Two different methods were used:

Method E. It was employed, when necessary, for purifying compounds 2.1-2.19. Description: aqueous solvent: 0.1% trifluoroacetic acid in water; organic solvent: 0.1% trifluoroacetic acid in methanol; gradient: 75% aqueous for 5 min after injection and to 25% aqueous over 40 min. Compound isolation was achieved by fraction basification with ammonia followed by filtration of the precipitate.

Method F. It was employed for purifying compounds 5.1-5.22. Description: aqueous solvent: 0.1% formic acid in water; organic solvent: 0.1% formic acid in acetonitrile; gradient: 10% aqueous for 5 min after injection, to 60% aqueous over 25 min and to 40% aqueous over 10 min. Compound isolation was achieved by two different methods: method 1, consisting of fraction basification with ammonia followed by chloroform extraction and work-up in the usual manner (for compounds 5.1-5.18, 5.21 and 5.22); method 2, consisting of fraction basification with ammonia followed by concentration in vacuo until constant weight (for compounds 5.19 and 5.20).

Melting points (mp) were recorded on a Stuart Scientific SMP1 melting point apparatus and are uncorrected. “mp d250” (e.g.) refers to decomposition observed at 250°C. IR
spectra were recorded using a Perkin Elmer SPECTRUM 1000 FT-IR spectrometer. NMR spectra were recorded at 400 MHz ($^1$H NMR) and 100 MHz ($^{13}$C NMR) on a Bruker spectrometer in CDCl$_3$, MeOD or DMSO-d$_6$ using the residual solvent peaks as internal standards, or TMS when stated as TMS=0. Coupling constant $J$ values are given in hertz (Hz) designated as s (singlet), br s (broad singlet), d (doublet), t (triplet), dd (doublet of doublets), ddd (doublet of doublet of doublets), td (triple of doublets), tt (triplet of triplets), 4q (quartet), 5q (quintuplet) and m (multiplet). Signal assignments were done using 2D NMR (COSY) for the $^1$H NMR spectra and $^{13}$C DEPT for the $^{13}$C NMR spectra. High resolution mass spectra (HRMS) and elemental analysis (CHN) services were provided by The School of Pharmacy. HRMS were conducted upon a Micromass Q-TTOF Ultima Global tandem mass spectrometer run under electrospray ionisation (ESI) or matrix assisted laser desorption/ionisation (MALDI) modes. CHN were conducted upon a Carlo Erba CHN1108 elemental analyser.
9.2 EXPERIMENTAL PROCEDURES FOR THE ACRIDONE SERIES

2-Bromobenzene-1,3-dioic acid (2.0.1.1): 2-bromo-1,3-dimethylbenzene (50 g, 0.275 mol) was added to water (500 ml). The mixture was heated up to 80°C and KMnO₄ (125 g, 0.825 mol) was added. The mixture was heated at reflux for 24 h. The solution was then separated by filtration of the black precipitate, which was washed several times with water. The aqueous fractions were evaporated, suspended in 5% ammonia solution (20 ml) and acidified with concentrated HCl. The white solid was taken by filtration and washed with 0.1 M aqueous HCl. Yield 2.0.1.1 (20.22 g, 30%): mp 217-218°C; IR $\nu$ (cm$^{-1}$): 2786, 2623, 2502, 1673, 1579, 1393, 1239; $^1$H NMR (DMSO-d$_6$) $\delta$: 7.52 (t, 1H, $J$=7.6 Hz), 7.70 (d, 2H, $J$=7.6 Hz), 13.36 (br s, 2H); $^{13}$C NMR (DMSO-d$_6$) $\delta$: 116.16 (C), 127.79 (CH), 130.69 (2xCH), 136.70 (2xC), 167.74 (2xC=O); MS (ES) calcd. C$_6$H$_5$BrO$_4$ [M+H]$^+$ 246.02. Found: 246.88.

2-(2-Carboxyphenylamino)benzene-1,3-dioic acid (2.0.1.2): Anthranilic acid (4 g, 29.8 mmol), 2.0.1.1 (7.30 g, 29.8 mmol), anhydrous potassium carbonate (12.35 g, 89.4 mmol) and copper powder (380 mg, 5.96 mmol) were added to anhydrous DMF (180 ml) under nitrogen atmosphere. The mixture was stirred at 120°C overnight. The reaction was left to reach RT and 3% ammonia in water (150 ml) and 2 spoonfuls of activated carbon were added, prior to filtration through celite. The solution was evaporated and dissolved in 5% aqueous ammonia (15 ml). The solution was acidified with concentrated HCl and extracted with EtOAc (3 x 10 ml). The organics were treated in the usual manner to obtain a dense oil. Ether (20 ml) was added and the pale yellow precipitate was taken by filtration and washed with ether. Yield 2.0.1.2 (6.71 g, 75%): mp 255-256°C; $^1$H NMR (DMSO-d$_6$) $\delta$: 6.78 (d, 1H, $J$=8.4 Hz), 6.79 (t, 1H, $J$=7.5 Hz), 7.18 (t, 1H, $J$=7.7 Hz), 7.27 (m, 1H), 7.83 (dd, 1H, $J$=7.9, 1.5 Hz), 7.92 (d, 2H, $J$=7.7 Hz), 10.68 (s, 1H), 12.98 (br s, 3H); $^{13}$C NMR (DMSO-d$_6$) $\delta$: 113.51 (CH), 115.21 (C), 118.57 (CH), 121.68 (CH), 125.15 (2xC), 131.41 (CH), 133.32 (CH), 134.28 (2xC),
Chapter 9 – Experimental procedures

140.46 (C), 146.62 (C), 167.70 (2xC=O), 168.66 (C=O); MS (ES) calcd. C_{15}H_{11}NO_5 [M+H]^+ 302.05. Found: 301.96.

9,10-Dihydro-9-oxoacridine-4,5-dicarboxylic acid (2.0.1): Compound 2.0.1.2 (3.93 g, 13.05 mmol) was dissolved in concentrated sulphuric acid (48 ml) and the mixture was heated at 50°C for 2 h. The warm mixture was added onto ice (50 ml) dropwise. The precipitate was taken by filtration and washed with hot acetic acid (5 ml), hot water (5 ml) and hot acetone (5 ml) to give a yellow solid. Yield 2.0.1 (2.65 g, 72%): IR \nu (cm^{-1}) 3183, 1713, 1681, 1613, 1591, 1523, 1417, 1149; ^1H NMR (DMSO-d_6) \delta: 7.42 (t, 2H, \ J=7.7 \text{ Hz}), 8.50 (dd, 2H, \ J=7.5, 1.7 \text{ Hz}), 8.56 (dd, 2H, \ J=7.9, 1.7 \text{ Hz}), 13.83 (br s, 2H), 13.99 (s, 1H); ^13C NMR (DMSO-d_6) \delta: 116.24 (2xC), 120.99 (2xCH), 121.60 (2xC), 132.01 (2xCH), 137.29 (2xCH), 140.51 (2xC), 167.89 (2xC=O), 176.20 (C=O); MS (ES) calcd. C_{15}H_{11}NO_5 [M-H]^+ 282.24. Found: 281.88.

2-(Phenylamino)benzene-1,3-dioic acid (2.0.2.1): 2-bromobenzoic acid (3 g, 14.9 mmol), anthranilic acid (2.25 g, 16.4 mmol), potassium carbonate anhydrous (6 g, 44 mmol) and copper powder (0.2 g, 2.9 mmol) were added to anhydrous DMF (20 ml) under nitrogen atmosphere. The mixture was heated at reflux overnight. Cold water (200 ml) and 2 spoonfuls of activated carbon were added and the solution filtered through celite. The filtrate was acidified with concentrated HCl and the white precipitate was taken by filtration and washed with hot ethanol (10 ml). Yield 2.0.2.1 (3.5 g, 91%): mp 252-253°C; ^1H NMR (DMSO-d_6) \delta: 6.95 (ddd, 2H, \ J=7.7, 6.3, 2.0 \text{ Hz}), 7.41-7.47 (m, 4H), 7.90 (dd, 2H, \ J=7.9, 1.4 \text{ Hz}), 10.80 (br s, 1H), 13.00 (br s, 2H); ^13C NMR (DMSO-d_6) \delta: 117.02 (C), 117.44 (2xCH), 117.50 (2xC), 119.84 (2xCH), 129.81 (C), 131.65 (2xCH), 133.20 (CH), 143.47 (CH), 168.23 (2xC=O); MS (ES) calcd. C_{14}H_{11}NO_4 [M-H]^+ 256.25. Found: 256.61.
9,10-Dihydro-9-oxoacridine-4-carboxylic acid (2.0.2): Compound 2.0.2.1 (0.5 g, 1.94 mmol) was dissolved in concentrated sulphuric acid (10 ml) and the mixture was heated at 80°C for 2 h. The warm mixture was added onto ice (50 ml) dropwise. The precipitate was taken by filtration and washed with hot water (5 ml), aqueous ethanol (5 ml) and ether (5 ml) to give a yellow solid. Yield 2.0.2 (0.16 g, 34%): mp 321-323°C; IR $\nu$ (cm$^{-1}$): 3223, 2871, 1689, 1564, 1519, 1214, 1138; $^1$H NMR (DMSO-d$_6$) $\delta$: 7.36 (m, 2H), 7.78 (m, 2H), 8.24 (d, 1H, $J=7.9$ Hz), 8.44 (d, 1H, $J=7.05$ Hz), 8.53 (d, 1H, $J=7.7$ Hz), 11.95 (s, 1H); $^{13}$C NMR (DMSO-d$_6$) $\delta$: 114.83 (C), 118.43 (CH), 120.05 (CH), 120.47 (C), 121.49 (C), 122.14 (CH), 125.74 (CH), 132.25 (CH), 133.91 (CH), 136.71 (CH), 139.76 (C), 141.05 (C), 168.97 (C=O), 176.35 (C=O); MS (ES) calcd. C$_{14}$H$_9$NO$_3$ [M-H]$^-$ 238.23. Found: 238.55.

General procedure A: Compounds 2.0.3, 2.0.27, 2.0.30, 2.0.33 and 2.0.39 were prepared according to general procedure A. A suspension of the appropriate nitroaniline in the appropriate chloroacyl chloride was heated at 50°C for 16 h. Diethyl ether (10 ml) was added to the resulting solution to give a precipitate that was filtered and washed with ether.

3-Chloro-N-(4-nitrophenyl)propanamide (2.0.3): A suspension of 4-nitroaniline (2 g, 14.5 mmol) in 3-chloropropionyl chloride (10 ml) was treated according to general procedure A. The target compound was obtained as a pale green solid. Yield 2.0.3 (2.63 g, 80%): mp 169-171°C; IR $\nu$ (cm$^{-1}$) 3347, 1703, 1593, 1499, 1317, 1295, 1110, 862; $^1$H NMR (DMSO-d$_6$) $\delta$: 2.91 (t, 2H, $J=6.2$ Hz), 3.90 (t, 2H, $J=6.2$ Hz), 7.85 (d, 2H, $J=9.3$ Hz), 8.23 (d, 2H, $J=9.2$ Hz), 10.69 (s, 1H); $^{13}$C NMR (DMSO-d$_6$) $\delta$: 39.30 (CH$_2$), 40.35 (CH$_3$), 118.75 (2xCH$_2$), 125.04 (2xCH), 142.16 (C), 144.94 (C), 168.98 (C=O); MS (ES) calcd. C$_9$H$_9$ClN$_2$O$_3$ [M+H]$^+$ 229.03. Found: 228.93.

2-Chloro-N-(4-nitrophenyl)acetamide (2.0.27): A suspension of 4-nitroaniline (1 g, 7.25 mmol) in
chloroacetyl chloride (5 ml) was treated according to general procedure A. The target compound was obtained as a pale yellow solid. Yield **2.0.27** (1.32 g, 85%): mp 181-183°C; IR λ (cm⁻¹) 3101, 1737, 1683, 1564, 1494, 1333; ¹H NMR (CDCl₃) δ: 4.24 (s, 2H), 7.78 (d, 2H, J=9.1 Hz), 8.26 (d, 2H, J=9.1 Hz), 8.50 (br s, 1H); ¹³C NMR (CDCl₃) δ: 42.81 (CH₂), 119.48 (2xCH), 125.12 (2xCH), 139.60 (C), 147.30 (C), 164.14 (C=O); MS (ES) calcd. C₈H₇ClN₂O₃ [M-H]⁻ 213.01. Found: 212.89.

**4-Chloro-N-(4-nitrophenyl)butanamide (2.0.30):**

A suspension of 4-nitroaniline (1 g, 7.25 mmol) in 4-chlorobutiryl chloride (5 ml) was treated according to general procedure A. The target compound was obtained as a pale green solid. Yield **2.0.30** (790 mg, 45%): mp 105-107°C; IR λ (cm⁻¹) 3358, 1700, 1593, 1541, 1494, 1478, 1317; ¹H NMR (CDCl₃) δ: 2.22 (5q, 2H, J=6.3 Hz), 2.64 (t, 2H, J=7.0 Hz), 3.68 (t, 2H, J=6.1 Hz), 7.56 (br s, 1H), 7.71 (d, 2H, J=9.2 Hz), 8.22 (d, 2H, J=9.2 Hz); ¹³C NMR (CDCl₃) δ: 27.48 (CH₂), 34.18 (CH₂), 44.22 (CH₂), 118.99 (2xCH), 125.14 (2xCH), 143.43 (C), 143.56 (C), 170.32 (C=O); MS (ES) calcd. C₁₀H₁₁ClN₂O₃ [M+H]⁺ 243.05. Found: 243.07.

**5-Chloro-N-(4-nitrophenyl)pentanamide (2.0.33):** A suspension of 4-nitroaniline (1 g, 7.25 mmol) in 5-chloropentaryl chloride (3 ml) was treated according to general procedure A. The target compound was obtained as a pale green solid. Yield **2.0.33** (1.23 g, 66%): mp 121-123°C; IR λ (cm⁻¹) 3284, 1678, 1547, 1507, 1337, 854; ¹H NMR (CDCl₃) δ: 1.91 (m, 4H), 2.48 (t, 2H, J=6.9 Hz), 3.60 (t, 2H, J=6.0 Hz), 7.42 (br s, 1H), 7.70 (d, 2H, J=9.1 Hz), 8.22 (d, 2H, J=9.1 Hz); ¹³C NMR (CDCl₃) δ: 22.49 (CH₂), 31.74 (CH₂), 36.76 (CH₂), 44.44 (CH₂), 118.98 (2xCH), 119.01 (C), 125.13 (2xCH), 143.49 (C), 170.77 (C=O); MS (ES) calcd. C₁₁H₁₃ClN₂O₃ [M+H]⁺ 257.69. Found: 257.28.

**3-Chloro-N-(3-nitrophenyl)propanamide (2.0.39):** A suspension of 3-nitroaniline (5 g, 36.2 mmol) in 3-chloropropionyl chloride (20 ml) was treated according to
general procedure A. The target compound was obtained as a pale brown solid. Yield 2.0.39 (6.75 g, 79%): mp 96-98°C; IR λ (cm⁻¹) 3281, 1736, 1670, 1597, 1519, 1348; ¹H NMR (CDCl₃) δ: 2.88 (t, 2H, J=6.3 Hz), 3.91 (t, 2H, J=6.3 Hz), 7.51 (t, 1H, J=8.2 Hz), 7.53 (br s, 1H), 7.94 (dd, 1H, J=8.0, 1.4 Hz), 7.99 (ddd, 1H, J=8.2, 2.1, 0.7 Hz), 8.40 (t, 1H, J=2.1 Hz); ¹³C NMR (CDCl₃) δ: 39.51 (CH₂), 40.50 (CH₂), 114.74 (CH), 119.31 (CH), 125.60 (CH), 129.95 (CH), 138.44 (C), 148.54 (C), 168.24 (C=O); MS (ES) calcd. C₉H₅ClN₂O₃ [M+H]⁺ 229.03. Found: 228.98.

N-(3-Chloropropyl)-4-nitrobenzenamine (2.0.18):
A suspension of 2.0.3 (4 g, 17.5 mmol) in a 2M solution of borane-dimethyl sulfide complex in THF (17.5 ml, 35 mmol) under nitrogen atmosphere was stirred for 2 h at RT, and 1 h at 60°C. Water (50 ml) was added to the yellow mixture and stirred for 2 h at RT. The organics were treated in the usual manner to obtain a green oil that crystallised overnight. Yield 2.0.18 (3.4 g, 91%): mp 65-67°C; IR λ (cm⁻¹) 3333, 1599, 1464, 1266, 1108; ¹H NMR (CDCl₃) δ: 2.12 (5q, 2H, J=6.2 Hz), 3.46 (t, 2H, J=6.7 Hz), 3.66 (t, 2H, J=6.1 Hz), 4.57 (br s, 1H), 6.56 (d, 2H, J=9.2 Hz), 8.10 (d, 2H, J=9.2 Hz); ¹³C NMR (CDCl₃) δ: 31.39 (CH₂), 40.46 (CH₂), 42.12 (CH₂), 111.09 (2xCH), 126.46 (2xCH), 138.26 (C), 152.94 (C); MS (ES) calcd. C₉H₁₁ClN₂O₂ [M+H]⁺ 215.05. Found: 214.94.

General procedure B: Compounds 2.0.4, 2.0.6, 2.0.8, 2.0.10, 2.0.12, 2.0.14, 2.0.19, 2.0.21, 2.0.23, 2.0.25, 2.0.28, 2.0.31, 2.0.34 and 2.0.40 were prepared according to general procedure B. A solution of the appropriate chlorinated compound in neat amine (unless otherwise stated) was stirred at RT for 24 h. The solvent was evaporated in vacuo and the crude was suspended in 0.5M aqueous HCl and washed twice with EtOAc. The aqueous phase was basified to pH 9 using 3% aqueous ammonia and extracted with EtOAc. The organics were treated in the usual manner to give the target compound. Purification was performed by silica gel chromatographic columns using hexane/EtOAc mixtures when required.

N-(4-Nitrophenyl)-3-(pyrrolidin-1-yl)propanamide (2.0.4): A solution of 2.0.3 (14.31 g, 63 mmol) in neat pyrrolidine (100 ml) was treated
Chapter 9 – Experimental procedures

according to general procedure B. The target compound was obtained as a yellow solid. Yield 2.0.4 (9.38 g, 57%): mp 174-176°C; IR \( \lambda \) (cm\(^{-1}\)) 2806, 1679, 1547, 1504, 1320; \(^1\)H NMR (DMSO-d\(_6\)) \( \delta \): 1.67 (m, 4H), 2.46 (m, 4H), 2.54 (t, 2H, \( J=6.8 \) Hz), 2.72 (t, 2H, \( J=6.8 \) Hz), 7.82 (d, 2H, \( J=9.2 \) Hz), 8.21 (d, 2H, \( J=9.2 \) Hz), 10.68 (s, 1H); \(^1^3\)C NMR (DMSO-d\(_6\)) \( \delta \): 23.73 (2xCH\(_2\)), 34.51 (CH\(_2\)), 51.07 (CH\(_2\)), 53.11 (2xCH\(_2\)), 118.94 (2xCH), 125.10 (2xCH), 142.93 (C), 144.80 (C), 171.30 (C=O); MS (ES) calcd. C\(_{13}\)H\(_{17}\)N\(_3\)O\(_3\) [M+H]+ 264.13. Found: 264.05.

3-(Morpholine-4-yl)-N-(4-nitrophenyl)propanamide (2.0.6): A solution of 2.0.3 (1 g, 4.37 mmol) in neat morpholine (10 ml) was treated according to general procedure B. The target compound was obtained as a yellow solid. Yield 2.0.6 (1.05 g, 86%): mp 96-98°C; IR \( \lambda \) (cm\(^{-1}\)) 1692, 1594, 1552, 1459, 1325, 1108; \(^1\)H NMR (CDCl\(_3\), TMS=0) \( \delta \): 2.60 (t, 2H, \( J=5.9 \) Hz), 2.65 (m, 4H), 2.79 (t, 2H, \( J=5.9 \) Hz), 3.83 (m, 4H), 7.68 (d, 2H, \( J=9.2 \) Hz), 8.11 (d, 2H, \( J=9.2 \) Hz), 11.29 (s, 1H); \(^1^3\)C NMR (CDCl\(_3\)) \( \delta \): 32.04 (CH\(_2\)), 52.35 (2xCH\(_2\)), 53.37 (CH\(_2\)), 66.54 (2xCH\(_2\)), 118.38 (2xCH), 124.52 (2xCH), 142.39 (C), 144.22 (C), 170.58 (C=O); MS (ES) calcd. C\(_{13}\)H\(_{17}\)N\(_3\)O\(_4\) [M+H]+ 280.12. Found: 280.07.

3-(Dimethylamino)-N-(4-nitrophenyl)propanamide (2.0.8): A solution of 2.0.3 (1 g, 4.37 mmol) in 2M dimethylamine in THF (10 ml, 4.5 eq.) was treated according to general procedure B. The target compound was obtained as a yellow solid. Yield 2.0.8 (1.01 g, 98%): mp 205-207°C; IR \( \lambda \) (cm\(^{-1}\)) 3273, 2781, 1683, 1556, 1495, 1328, 1263, 1108, 850; \(^1\)H NMR (CDCl\(_3\)) \( \delta \): 2.42 (s, 6H), 2.55 (t, 2H, \( J=5.9 \) Hz), 2.70 (t, 2H, \( J=5.9 \) Hz), 7.67 (d, 2H, \( J=9.2 \) Hz), 8.13 (d, 2H, \( J=9.2 \) Hz), 11.65 (s, 1H); \(^1^3\)C NMR (CDCl\(_3\)) \( \delta \): 33.15 (CH\(_2\)), 44.05 (2xCH\(_3\)), 54.45 (CH\(_2\)), 118.78 (2xCH), 124.58 (2xCH), 142.49 (C), 144.50 (C), 171.09 (C=O); MS (ES) calcd. C\(_{11}\)H\(_{15}\)N\(_3\)O\(_3\) [M+H]+ 238.11. Found: 237.97.

3-(4-Methylpiperazin-1-yl)-N-(4-nitrophenyl)propanamide (2.0.10): A solution of 2.0.3 (1.5 g, 6.57 mmol) in neat \( N-\)
methylpiperazine (5 ml) was treated according to general procedure B. The target compound was obtained as a pale brown solid. Yield 2.0.10 (741 mg, 39%): mp 220-221°C; IR $\lambda$ (cm$^{-1}$) 1693, 1494, 1556, 1489, 1457; $^1$H NMR (DMSO-d$_6$) $\delta$: 2.67 (m, 11H), 3.01 (m, 2H), 3.34 (m, 2H), 7.89 (d, 2H, $J$=9.2 Hz), 8.22 (d, 2H, $J$=9.2 Hz), 10.87 (s, 1H); $^{13}$C NMR (DMSO-d$_6$) $\delta$: 43.61 (2xCH$_2$), 45.79 (CH$_3$), 51.98 (2xCH$_2$), 53.31 (CH$_2$), 55.16 (CH$_2$), 118.38 (2xCH), 124.52 (2xCH), 142.89 (C), 144.22 (C), 170.58 (C=O); MS (ES) calcd. C$_{14}$H$_{20}$N$_4$O$_3$ [M+H]$^+$ 293.15. Found: 293.14.

![N-(4-Nitrophenyl)-3-(piperidin-1-yl)propanamide (2.0.12): A solution of 2.0.3 (525 mg, 2.30 mmol) in neat piperidine (5 ml) was treated according to general procedure B. The target compound was obtained as a pale yellow solid. Yield 2.0.12 (577 mg, 90%): mp 169-170°C; IR $\lambda$ (cm$^{-1}$) 1738, 1674, 1545, 1503, 1320; $^1$H NMR (CDCl$_3$) $\delta$: 1.58 (m, 2H), 1.72 (m, 4H), 2.55 (t, 2H, $J$=5.8 Hz), 2.56 (m, 4H), 2.69 (t, 2H, $J$=5.8 Hz), 7.69 (d, 2H, $J$=9.2 Hz), 8.19 (d, 2H, $J$=9.2 Hz), 12.09 (s, 1H); $^{13}$C NMR (CDCl$_3$) $\delta$: 24.09 (CH$_3$), 26.32 (2xCH$_2$), 32.45 (CH$_2$), 53.53 (2xCH$_2$), 53.99 (CH$_2$), 118.79 (2xCH), 125.14 (2xCH), 142.90 (C), 144.86 (C), 171.38 (C=O); MS (ES) calcd. C$_{14}$H$_{19}$N$_3$O$_3$ [M+H]$^+$ 278.14. Found: 278.06.](image)

3-($N$-Cyclohexylamino)-$N$-(4-nitrophenyl)propanamide (2.0.14): A solution of 2.0.3 (1 g, 4.4 mmol) in cyclohexylamine (2 ml) was treated according to general procedure B. The target compound was obtained as a pale brown solid. Yield 2.0.14 (1.28 g, 50%): mp 69-70°C; IR $\lambda$ (cm$^{-1}$) 2919, 1848, 1738, 1673, 1506, 1325, 854; $^1$H NMR (CDCl$_3$) $\delta$: 1.10-1.38 (m, 6H), 1.69 (m, 1H), 1.80 (m, 2H), 2.01 (m, 2H), 2.51 (t, 2H, $J$=5.7 Hz), 2.55 (tt, 1H, $J$=10.5, 3.8 Hz), 3.03 (t, 2H, $J$=5.7 Hz), 7.68 (d, 2H, $J$=9.2 Hz), 8.19 (d, 2H, $J$=9.2 Hz), 11.90 (s, 1H); $^{13}$C NMR (CDCl$_3$) $\delta$: 24.92 (2xCH$_2$), 25.91 (CH$_3$), 33.76 (2xCH$_2$), 36.39 (CH$_2$), 41.98 (CH$_2$), 56.28 (CH), 118.83 (2xCH), 125.12 (2xCH), 140.00 (C), 144.73 (C), 171.76 (C=O); MS (ES) calcd. C$_{15}$H$_{21}$N$_3$O$_3$ [M+H]$^+$ 292.35. Found: 291.90.
4-Nitro-N-(3-(pyrrolidin-1-yl)propyl) benzenamine (2.0.19): A solution of 2.0.18 (469 mg, 2.19 mmol) in neat pyrrolidine (10 ml) was treated according to general procedure B. The target compound was obtained as a dark brown solid. Yield 2.0.19 (545 mg, 100%): mp 59-60°C; IR $\lambda$ (cm$^{-1}$) 1597, 1505, 1471, 1284, 1108; $^1$H NMR (CDCl$_3$) $\delta$: 1.81 (m, 4H), 1.84 (5q, 2H, $J$=6.2 Hz), 2.54 (m, 4H), 2.65 (t, 2H, $J$=6.1 Hz), 3.30 (m, 2H), 6.44 (d, 2H, $J$=9.2 Hz), 6.49 (br s, 1H), 8.07 (d, 2H, $J$=9.2 Hz); $^{13}$C NMR (CDCl$_3$) $\delta$: 23.55 (2xCH$_2$), 26.63 (CH$_2$), 43.62 (CH$_2$), 54.09 (2xCH$_2$), 55.12 (CH$_2$), 110.68 (2xCH), 110.72 (C), 126.51 (2xCH), 153.92 (C); MS (ES) calcd. C$_{13}$H$_{19}$N$_3$O$_2$ [M+H]$^+$ 250.15. Found: 250.17.

N-(3-(Morpholine-4-yl)propyl)-4-nitrobenzenamine (2.0.21): A solution of 2.0.18 (1 g, 4.67 mmol) in neat morpholine (6.5 ml) was treated according to general procedure B. The target compound was obtained as an orange solid. Yield 2.0.21 (0.93 g, 75%): mp 63-64°C; IR $\lambda$ (cm$^{-1}$) 3301, 1600, 1588, 1498, 1447, 1280, 1104; $^1$H NMR (CDCl$_3$) $\delta$: 1.85 (5q, 2H, $J$=6.1 Hz), 2.51 (m, 4H), 2.54 (t, 2H, $J$=6.1 Hz), 3.31 (m, 2H, $J$=5.6 Hz), 3.76 (m, 4H, $J$=4.6 Hz), 6.24 (br s, 1H), 6.49 (d, 2H, $J$=9.2 Hz), 8.08 (d, 2H, $J$=9.2 Hz); $^{13}$C NMR (CDCl$_3$) $\delta$: 24.28 (CH$_2$), 43.30 (CH$_2$), 53.70 (2xCH$_2$), 57.73 (CH$_2$), 67.08 (2xCH$_2$), 110.63 (C), 110.69 (2xCH), 126.51 (2xCH), 153.67 (C); MS (ES) calcd. C$_{13}$H$_{19}$N$_3$O$_3$ [M+H]$^+$ 266.14. Found: 266.08.

N-(3-(Dimethylamino)propyl)-4-nitrobenzenamine (2.0.23): A solution of 2.0.18 (1 g, 4.67 mmol) in 2M dimethylamine in THF (10.7 ml, 5 eq.) was treated according to general procedure B. The target compound was obtained as a yellow solid. Yield 2.0.23 (874 mg, 84%): mp 63-65°C; IR $\lambda$ (cm$^{-1}$) 3242, 1595, 1456, 1299; $^1$H NMR (CDCl$_3$) $\delta$: 2.19 (5q, 2H, $J$=6.0 Hz), 2.84 (s, 6H), 3.24 (t, 2H, $J$=6.7 Hz), 3.47 (m, 2H), 6.65 (d, 2H, $J$=9.2 Hz), 6.91 (br s, 1H), 8.06 (d, 2H, $J$=9.2 Hz); $^{13}$C NMR (CDCl$_3$) $\delta$: 43.74 (CH$_2$), 52.98 (2xCH$_3$), 63.43 (CH$_2$),
77.20 (CH$_2$), 110.89 (2xCH), 126.44 (2xCH), 137.50 (C), 153.58 (C); MS (ES) calcd. C$_{11}$H$_{17}$N$_3$O$_2$ [M+H]$^+$ 224.13. Found: 224.12.

N-(3-(4-Methylpiperazin-1-yl)propyl)-4-nitrobenzenamine (2.0.25): A solution of 2.0.18 (200 mg, 0.93 mmol) in neat N-methyl piperazine (5 ml) was treated according to general procedure B.

The target compound was obtained as a white solid. Yield 2.0.25 (250 mg, 97%): mp 68-69°C; $^1$H NMR (CDCl$_3$) $\delta$: 1.81 (5q, 2H, J=6.0 Hz), 2.31 (s, 3H), 2.52 (br s, 8H), 2.53 (t, 2H, J=6.0 Hz), 3.27 (m, 2H, J=5.5 Hz), 6.45 (d, 2H, J=9.2 Hz), 6.60 (br s, 1H), 8.05 (d, 2H, J=9.2 Hz); $^{13}$C NMR (CDCl$_3$) $\delta$: 24.28 (CH$_2$), 43.61 (CH$_2$), 46.02 (CH$_3$), 53.14 (2xCH$_2$), 55.38 (2xCH$_2$), 57.41 (CH$_2$), 110.58 (2xCH), 126.47 (2xCH), 137.21 (C), 153.84 (C); MS (ES) calcd. C$_{14}$H$_{22}$N$_4$O$_2$ [M+H]$^+$ 279.36. Found: 279.24.

N-(4-Nitrophenyl)-2-(pyrrolidin-1-yl) acetamide (2.0.28): A solution of 2.0.27 (500 mg, 2.33 mmol) in neat pyrrolidine (5 ml) was treated according to general procedure B. The target compound was obtained as a dark green solid. Yield 2.0.28 (218 mg, 37%): mp 51-52°C; IR $\nu$ (cm$^{-1}$) 1697, 1606, 1503, 1489, 1337, 1109; $^1$H NMR (CDCl$_3$) $\delta$: 1.81 (m, 4H), 2.66 (m, 4H), 3.27 (s, 2H), 7.73 (d, 2H, J=9.2 Hz), 8.15 (d, 2H, J=9.2 Hz), 9.53 (br s, 1H); $^{13}$C NMR (CDCl$_3$) $\delta$: 23.93 (2xCH$_2$), 54.50 (2xCH$_2$), 59.59 (CH$_2$), 118.75 (2xCH), 124.86 (2xCH), 143.17 (C), 143.45 (C), 169.76 (C=O); MS (ES) calcd. C$_{12}$H$_{18}$N$_3$O$_3$ [M+H]$^+$ 250.27. Found: 250.01.

N-(4-Nitrophenyl)-4-(pyrrolidin-1-yl) butanamide (2.0.31): A solution of 2.0.30 (435 mg, 1.79 mmol) in neat pyrrolidine (5 ml) was treated according to general procedure B. The target compound was obtained as a yellow solid. Yield 2.0.31 (268 mg, 54%): mp 155-156°C; IR $\nu$ (cm$^{-1}$) 3291, 1623, 1598, 1473, 1300, 1105, 833; $^1$H NMR (DMSO-d$_6$) $\delta$: 1.76 (5q, 2H, J=6.4 Hz), 1.82 (m, 4H), 3.06 (m, 4H), 3.27 (t, 2H, J=6.8 Hz), 3.36 (t, 2H, J=6.8 Hz), 6.64 (d, 2H, J=9.4 Hz), 7.43 (t, 1H, J=5.4 Hz), 7.43 (d, 2H, J=9.4 Hz), 7.43 (t, 1H, J=5.4 Hz).
7.98 (d, 2H, J=9.3 Hz); $^{13}$C NMR (DMSO-d$_6$) δ: 23.69 (2xCH$_2$), 25.53 (CH$_2$), 44.42 (2xCH$_2$), 45.20 (CH$_2$), 45.75 (CH$_2$), 126.19 (2xCH), 126.32 (C), 135.32 (2xCH), 154.54 (C), 169.77 (C=O); MS (ES) calcd. C$_{14}$H$_{19}$N$_3$O$_3$ [M+H]$^+$ 278.14. Found: 278.10.

A-(4-Nitrophenyl)-5-(pyrrolidin-1-yl)pentanamide (2.0.34): A solution of 2.0.33 (400 mg, 1.55 mmol) in neat pyrrolidine (5 ml) was treated according to general procedure B. The target compound was obtained as a yellow solid. Yield 2.0.34 (154 mg, 34%): mp 64-65°C; IR λ (cm$^{-1}$) 2942, 1737, 1695, 1594, 1553, 1496, 1327; $^1$H NMR (CDCl$_3$) δ: 1.71 (m, 4H), 1.80 (5q, 2H, J=7.0 Hz), 1.91 (5q, 2H, J=7.0 Hz), 2.41 (m, 4H), 3.88 (m, 4H), 7.75 (d, 2H, J=9.2 Hz), 8.10 (d, 2H, J=9.2 Hz); $^{13}$C NMR (CDCl$_3$) δ: 23.26 (2xCH$_2$), 23.26 (CH$_2$), 28.06 (CH$_2$), 37.09 (CH$_2$), 54.03 (2xCH$_2$), 55.67 (CH$_2$), 119.10 (2xCH), 124.77 (2xCH), 142.95 (C), 144.35 (C), 172.38 (C=O); MS (ES) calcd. C$_{15}$H$_{21}$N$_3$O$_3$ [M+H]$^+$ 292.15. Found: 292.07.

A-(3-Nitrophenyl)-3-(pyrrolidin-1-yl) propanamide (2.0.40): A solution of 2.0.39 (2 g, 8.8 mmol) in neat pyrrolidine (10 ml) was treated according to general procedure B. The target compound was obtained as a dark brown oil. Yield 2.0.40 (300 mg, 13%): IR λ (cm$^{-1}$) 1738, 1650, 1524, 1348, 1216; $^1$H NMR (CDCl$_3$) δ: 1.92 (m, 4H), 2.55 (t, 2H, J=5.8 Hz), 2.70 (m, 4H), 2.86 (t, 2H, J=5.8 Hz), 7.43 (t, 1H, J=8.2 Hz), 7.84 (ddd, 1H, J=8.1, 2.0, 0.9 Hz), 7.87 (ddd, 1H, J=8.2, 2.2, 0.9 Hz), 8.29 (t, 1H, J=2.1 Hz), 11.78 (br s, 1H); $^{13}$C NMR (CDCl$_3$) δ: 23.72 (2xCH$_2$), 34.44 (CH$_2$), 51.13 (CH$_2$), 53.07 (2xCH$_2$), 114.20 (CH), 117.98 (CH), 125.25 (CH), 129.58 (CH), 139.98 (C), 148.56 (C), 171.28 (C=O); MS (ES) calcd. C$_{13}$H$_{17}$N$_3$O$_3$ [M+H]$^+$ 264.30. Found: 264.34.

3-(1H-Imidazol-1-yl)-N-(4-nitrophenyl)propanamide (2.0.16): A solution of 2.0.3 (630 mg, 2.76 mmol) and imidazole (1.5 g, 22 mmol, 8 eq.) in DMF (5 ml) was treated in the microwave at 150°C for 5 min. Water (10 ml) was added and the precipitate was taken by filtration and washed with water (10 ml). The target compound was obtained as a yellow solid.
Yield 2.0.16 (592 g, 83%): mp 207-209°C; IR λ (cm\(^{-1}\)) 1691, 1592, 1491, 1322; \(^1\)H NMR (DMSO-\(d_6\)) δ: 2.89 (t, 2H, J=6.7 Hz), 4.29 (t, 2H, J=6.7 Hz), 6.86 (m, 1H), 7.16 (m, 1H), 7.61 (m, 1H), 7.80 (d, 2H, J=9.3 Hz), 8.22 (d, 2H, J=9.3 Hz), 10.61 (s, 1H); \(^{13}\)C NMR (DMSO-\(d_6\)) δ: 37.86 (CH\(_2\)), 41.70 (CH\(_2\)), 118.68 (2xCH), 119.18 (CH), 124.96 (2xCH), 128.32 (CH), 137.24 (CH), 142.12 (C), 144.89 (C), 169.57 (C=O); MS (ES) calcd. C\(_{12}\)H\(_{12}\)N\(_4\)O\(_3\) [M+H]\(^+\) 261.25. Found: 260.90.

3-(4-Nitrophenylcarbamoyl)propanoic acid (2.0.36): To a hot solution of 4-nitroaniline (1.38 g, 10 mmol) in toluene (50 ml), succinic anhydride (1.35 g, 13.5 mmol) was added. The mixture was heated at reflux for 1 h and the precipitate was filtered while hot and washed with hot toluene (50 ml). The target compound was obtained as a yellow solid. Yield 2.0.36 (1.4 g, 59%): mp 201-203°C; IR λ (cm\(^{-1}\)) 3338, 1695, 1595, 1489, 1328, 1145; \(^1\)H NMR (DMSO-\(d_6\)) δ: 2.55 (t, 2H, J=6.6 Hz), 2.64 (t, 2H, J=6.6 Hz), 7.83 (d, 2H, J=9.2 Hz), 8.21 (d, 2H, J=9.2 Hz), 10.61 (s, 1H), 12.20 (s, 1H); \(^{13}\)C NMR (DMSO-\(d_6\)) δ: 28.44 (CH\(_2\)), 31.18 (CH\(_2\)), 118.43 (2xCH), 124.88 (2xCH), 141.87 (C), 145.29 (C), 171.10 (C=O), 173.55 (C=O); MS (ES) calcd. C\(_{10}\)H\(_{10}\)N\(_2\)O\(_5\) [M-H]\(^-\) 237.20. Found: 237.31.

\(N\)-(4-Nitrophenyl)-4-oxo-4-(pyrrolidin-1-yl)butanamide (2.0.37): A solution of 2.0.36 (1 g, 4.2 mmol), HOBt (675 mg, 5.04 mmol, 1.2 eq.), EDCI (962 mg, 5.04 mmol, 1.2 eq.) and pyrrolidine (596 mg, 8.4 mmol) in DMF (10 ml) was stirred at RT overnight. MeOH (20 ml) was added and the precipitate was taken by filtration. The organics were evaporated in vacuo and suspended in MeOH (10 ml), sonicated and the remaining solid taken by filtration. The solids were combined and passed through a plug of silica using DCM:MeOH 15:1 as eluent. The solvents were removed in vacuo to obtain a yellow solid. Yield 2.0.37 (1.05 g, 86%): mp 200-202°C; IR λ (cm\(^{-1}\)) 3271, 1737, 1693, 1624, 1497; \(^1\)H NMR (CDCl\(_3\)) δ: 1.91 (5q, 2H, J=6.8 Hz), 2.01 (5q, 2H, J=6.7 Hz), 2.71-2.79 (m, 4H), 3.45 (t, 2H, J=6.8 Hz), 3.51 (t, 2H, J=6.9 Hz), 7.69 (d, 2H, J=9.2 Hz), 8.14 (d, 2H, J=9.2 Hz), 10.07 (s, 1H); \(^{13}\)C NMR (CDCl\(_3\)) δ: 24.33 (CH\(_2\)), 26.01 (CH\(_2\)), 30.48 (CH\(_2\)), 33.07 (CH\(_2\)), 46.22 (CH\(_2\)), 46.78 (CH\(_2\)), 118.87
(2xCH), 124.89 (2xCH), 143.04 (C), 144.48 (C), 170.84 (C=O), 171.63 (C=O); MS (ES) calcd. C_{14}H_{17}N_{3}O_{4} [M-H]^+ 290.13. Found: 290.15.

**1-Methyl-4-nitro-N-(3-(pyrrolidin-1-yl)propyl)-1H-pyrrole-2-carboxamide (2.0.43):** A solution of 1-methyl-4-nitro-1H-pyrrole-2-carboxylic acid (150 mg, 0.88 mmol), 3-(pyrrolidin-1-yl)propan-1-amine (135 mg, 1.05 mmol, 1.2 eq.), HOBT (142 mg, 1.06 mmol, 1.2 eq.) and EDCI (202 mg, 1.06 mmol, 1.2 eq.) in acetonitrile (10 ml) was stirred overnight at RT. The mixture was evaporated *in vacuo*, suspended in chloroform (10 ml), washed with 2% aqueous ammonia (2 x 5 ml) and extracted with saturated NH_{4}Cl (2 x 10 ml). The aqueous phase was neutralised with ammonia and extracted with chloroform (3 x 5 ml). The organics were treated in the usual manner to obtain a yellow solid. Yield 2.0.43 (180 mg, 73%): mp 116-117°C; IR $\tilde{\nu}$ (cm$^{-1}$) 3124, 2969, 2792, 1737, 1646, 1518, 1299; $^1$H NMR (CDCl$_3$) $\delta$: 1.73 (5q, 2H, $J=5.6$ Hz), 1.86 (m, 4H), 2.56 (m, 4H), 2.68 (t, 2H, $J=5.7$ Hz), 3.46 (td, 2H, $J=5.7, 4.8$ Hz), 3.98 (s, 3H), 6.91 (d, 1H, $J=1.9$ Hz), 7.49 (d, 1H, $J=1.9$ Hz), 9.04 (s, 1H); $^{13}$C NMR (CDCl$_3$) $\delta$: 23.36 (2xCH$_2$), 25.55 (CH$_2$), 37.71 (CH$_2$), 40.68 (CH$_2$), 54.13 (2xCH$_2$), 56.28 (CH$_3$), 106.33 (CH), 126.08 (CH), 126.98 (C), 134.80 (C), 160.14 (C=O); MS (ES) calcd. C$_{13}$H$_{20}$N$_{4}$O$_{3}$ [M+H]$^+$ 281.33. Found: 281.24.

**General procedure C.** Compounds 2.0.5, 2.0.7, 2.0.9, 2.0.11, 2.0.13, 2.0.15, 2.0.17, 2.0.20, 2.0.22, 2.0.24, 2.0.26, 2.0.29, 2.0.32, 2.0.35, 2.0.38, 2.0.41 and 2.0.44 were prepared according to general procedure C. A suspension of the appropriate nitro compound, ammonium formate (4 eq) and Pd/C (5% in mass related to the nitro compound) in absolute ethanol (2 ml) was treated in the microwave for 3 min at 120°C. The clear mixture was filtered through a plug of celite. The organics were concentrated and the crude suspended in 3% aqueous ammonia. The mixture was extracted with chloroform and the organics treated in the usual manner to obtain the target compound. Decomposition was observed to occur in some cases, so these compounds were used immediately after synthesis without further purification.
$N$-(4-Aminophenyl)-3-(pyrrolidin-1-yl)propanamide (2.0.5): Compound 2.0.4 (100 mg, 0.38 mmol) was treated according to general procedure C. The target compound was obtained as a clear oil that changed to pink overnight. Yield 2.0.5 (69 mg, 78%): IR $\nu$ (cm$^{-1}$) 2956, 2804, 1646, 1610, 1514, 1239; $^1$H NMR (CDCl$_3$) $\delta$: 1.86 (m, 4H), 2.50 (t, 2H, $J$=5.9 Hz), 2.65 (m, 4H), 2.82 (t, 2H, $J$=5.9 Hz), 3.59 (br s, 2H), 6.64 (d, 2H, $J$=8.6 Hz), 7.27 (d, 2H, $J$=8.5 Hz), 10.82 (s, 1H); $^{13}$C NMR (CDCl$_3$) $\delta$: 23.65 (2xCH$_2$), 34.53 (CH$_2$), 51.45 (CH$_2$), 53.12 (2xCH$_2$), 115.54 (2xCH), 121.32 (2xCH), 130.45 (C), 142.53 (C), 170.44 (C=O); MS (ES) calcd. C$_{13}$H$_{19}$N$_3$O $[M+H]^+$ 234.15. Found: 234.09.

3-(Morpholine-4-yl)-$N$-(4-aminophenyl)propanamide (2.0.7): Compound 2.0.6 (125 mg, 0.45 mmol) was treated according to general procedure C. The target compound was obtained as a yellow oil. Yield 2.0.7 (62 mg, 55%): IR $\nu$ (cm$^{-1}$) 3329, 3241, 2856, 1634, 1519, 1107; $^1$H NMR (CDCl$_3$) $\delta$: 1.71 (t, 2H, $J$=5.9 Hz), 1.79 (m, 4H), 1.92 (t, 2H, $J$=5.9 Hz), 3.00 (m, 4H), 5.85 (d, 2H, $J$=8.7 Hz), 6.51 (d, 2H, $J$=8.7 Hz), 9.53 (br s, 1H); $^{13}$C NMR (CDCl$_3$) $\delta$: 32.13 (CH$_2$), 52.71 (2xCH$_2$), 54.15 (CH$_2$), 66.90 (2xCH$_2$), 115.30 (2xCH), 121.08 (2xCH), 129.91 (C), 142.79 (C), 169.70 (C=O); MS (ES) calcd. C$_{13}$H$_{19}$N$_3$O $[M+H]^+$ 250.2. Found: 250.2.

3-(Dimethylamino)-$N$-(4-aminophenyl)propanamide (2.0.9): Compound 2.0.8 (106 mg, 0.45 mmol) was treated according to general procedure C. The target compound was obtained as a red oil. Yield 2.0.9 (58 mg, 62%): IR $\nu$ (cm$^{-1}$) 3300, 2947, 2782, 1644, 1518, 1266; $^1$H NMR (CDCl$_3$) $\delta$: 2.24 (s, 6H), 2.36 (t, 2H, $J$=5.6 Hz), 2.52 (t, 2H, $J$=6.0 Hz), 3.50 (br s, 2H), 6.53 (d, 2H, $J$=8.7 Hz), 7.19 (d, 2H, $J$=8.7 Hz), 10.42 (br s, 1H); $^{13}$C NMR (CDCl$_3$) $\delta$: 33.21 (CH$_2$), 44.27 (2xCH$_3$), 54.98 (CH$_2$), 115.10 (2xCH), 121.37 (2xCH), 129.80 (C), 142.70 (C), 170.11 (C=O); MS (ES) calcd. C$_{11}$H$_{17}$N$_3$O $[M+H]^+$ 208.14. Found: 208.09.
3-(4-Methylpiperazin-1-yl)-N-(4-aminophenyl)propanamide (2.0.11): Compound 2.0.10 (515 mg, 1.76 mmol) was treated according to general procedure C. The target compound was obtained as a red oil. Yield 2.0.11 (178 mg, 38%): IR λ (cm\(^{-1}\)) 2804, 1739, 1508, 1361, 1221; \(^1\)H NMR (CDCl\(_3\)) δ: 2.33 (s, 3H), 2.48 (t, 2H, \(J=5.9\) Hz), 2.69 (t, 2H, \(J=5.9\) Hz), 2.46-2.68 (m, 8H), 3.63 (br s, 2H), 6.63 (d, 2H, \(J=8.7\) Hz), 7.31 (d, 2H, \(J=8.7\) Hz), 10.56 (s, 1H); \(^1^3\)C NMR (CDCl\(_3\)) δ: 32.32 (CH\(_2\)), 45.87 (CH\(_3\)), 52.16 (2xCH\(_2\)), 53.60 (CH\(_3\)), 55.17 (2xCH\(_2\)), 115.28 (2xCH), 121.00 (2xCH), 130.12 (C), 142.62 (C), 169.87 (C=O); MS (ES) calcd. C\(_{14}\)H\(_{22}\)N\(_4\)O \([M+H]^+\) 263.2. Found: 263.1.

N-(4-Aminophenyl)-3-(piperidin-1-yl)propanamide (2.0.13): Compound 2.0.12 (58 mg, 0.21 mmol) was treated according to general procedure C. The target compound was obtained as a red oil. Yield 2.0.13 (62 mg, 89%): IR λ (cm\(^{-1}\)) 2929, 1739, 1646, 1518; \(^1\)H NMR (CDCl\(_3\)) δ: 1.52 (m, 2H), 1.67 (5q, 4H, \(J=5.6\) Hz), 2.47 (t, 2H, \(J=5.9\) Hz), 2.51 (br s, 4H), 2.63 (t, 2H, \(J=5.8\) Hz), 3.41 (br s, 2H), 6.64 (d, 2H, \(J=8.7\) Hz), 7.32 (d, 2H, \(J=8.7\) Hz), 11.00 (s, 1H); \(^1^3\)C NMR (CDCl\(_3\)) δ: 24.03 (CH\(_2\)), 26.01 (2xCH\(_2\)), 32.28 (CH\(_2\)), 53.38 (2xCH\(_2\)), 54.21 (CH\(_2\)), 115.20 (2xCH), 120.81 (2xCH), 130.22 (C), 142.50 (C), 170.11 (C=O); MS (ES) calcd. C\(_{14}\)H\(_{21}\)N\(_3\)O \([M+H]^+\) 248.2. Found: 248.2.

N-(4-Aminophenyl)-3-(N-cyclohexylamino)propanamide (2.0.15): Compound 2.0.14 (1 g, 3.45 mmol) was treated according to general procedure C. The target compound was obtained as a red oil. Yield 2.0.15 (897 mg, 100%): IR λ (cm\(^{-1}\)) 2919, 2850, 1644, 1516, 1261; \(^1\)H NMR (CDCl\(_3\)) δ: 1.03-1.31 (m, 6H), 1.61 (m, 1H), 1.72 (m, 2H), 1.92 (m, 2H), 2.40 (t, 2H, \(J=5.7\) Hz), 2.45 (tt, 1H, \(J=10.3, 3.7\) Hz), 2.92 (t, 2H, \(J=5.8\) Hz), 3.57 (br s, 2H), 6.60 (d, 2H, \(J=8.7\) Hz), 7.28 (d, 2H, \(J=8.7\) Hz), 10.56 (s, 1H); \(^1^3\)C NMR (CDCl\(_3\)) δ: 24.79 (2xCH\(_2\)), 25.89 (CH\(_2\)), 33.49 (2xCH\(_2\)), 36.31 (CH\(_2\)), 42.32 (CH\(_2\)), 56.15 (CH), 115.26 (2xCH), 121.09 (2xCH), 130.13 (C), 142.51 (C), 170.62 (C=O); MS (ES) calcd. C\(_{18}\)H\(_{23}\)N\(_3\)O \([M+H]^+\) 262.2. Found: 262.2.
**N-(4-Aminophenyl)-3-(1H-imidazol-1-yl)-propanamide (2.0.17):** Compound **2.0.16** (900 mg, 3.47 mmol) was treated according to general procedure C. The target compound was obtained as a red oil. Yield **2.0.17** (594 mg, 71%): IR \( \lambda \) (cm\(^{-1}\)) 1648, 1510, 1250; \(^1\)H NMR (CDCl\(_3\)) \( \delta \): 2.71 (t, 2H, \( J=6.4 \) Hz), 3.85 (br s, 2H), 4.30 (t, 2H, \( J=6.4 \) Hz), 6.59 (d, 2H, \( J=8.7 \) Hz), 6.94 (m, 1H), 6.97 (m, 1H), 7.23 (d, 2H, \( J=8.7 \) Hz), 7.37 (m, 1H), 8.71 (br s, 1H); \(^13\)C NMR (CDCl\(_3\)) \( \delta \): 38.60 (CH\(_2\)), 43.12 (CH\(_2\)), 115.28 (2xCH), 119.51 (CH), 122.08 (2xCH), 128.72 (CH), 129.12 (C), 136.91 (CH), 143.46 (C), 167.58 (C=O); MS (ES) calcd. C\(_{12}\)H\(_{14}\)N\(_4\)O [M+H]\(^+\) 231.1. Found: 231.1.

**4-Amino-N-(3-(pyrrolidin-1-yl)propyl)benzenamine (2.0.20):** Compound **2.0.19** (585 mg, 2.3 mmol) was treated according to general procedure C. The target compound was obtained as a yellow oil. Yield **2.0.20** (267 mg, 53%): IR \( \lambda \) (cm\(^{-1}\)) 2944, 2797, 1624, 1516; \(^1\)H NMR (CDCl\(_3\)) \( \delta \): 1.71 (m, 4H), 1.72 (5q, 2H, \( J=6.6 \) Hz), 2.43 (m, 4H), 2.48 (td, 2H, \( J=7.0 \), 1.8 Hz), 3.04 (td, 2H, \( J=6.6 \), 2.0 Hz), 3.39 (br s, 3H), 6.41 (m, 2H), 6.50 (m, 2H); \(^13\)C NMR (CDCl\(_3\)) \( \delta \): 23.31 (2xCH\(_2\)), 28.38 (CH\(_2\)), 44.14 (CH\(_2\)), 54.06 (2xCH\(_2\)), 54.66 (CH\(_2\)), 114.26 (2xCH), 116.70 (2xCH), 137.37 (C), 141.72 (C); MS (ES) calcd. C\(_{13}\)H\(_{21}\)N\(_3\) [M+H]\(^+\) 220.17. Found: 220.13.

**N-(3-(Morpholine-4-yl)propyl)-4-aminobenzenamine (2.0.22):** Compound **2.0.21** (468 mg, 1.76 mmol) was treated according to general procedure C. The target compound was obtained as a red oil. Yield **2.0.22** (160 mg, 38% yield): IR \( \lambda \) (cm\(^{-1}\)) 3337, 1853, 1661, 1516, 1261, 1115; \(^1\)H NMR (CDCl\(_3\)) \( \delta \): 1.74 (t, 2H, \( J=6.7 \) Hz), 2.42 (m, 4H), 2.43 (t, 2H, \( J=6.8 \) Hz), 3.09 (t, 2H, \( J=6.6 \) Hz), 3.39 (br s, 3H), 6.47 (d, 2H, \( J=8.7 \) Hz), 6.57 (d, 2H, \( J=8.7 \) Hz); \(^13\)C NMR (CDCl\(_3\)) \( \delta \): 25.57 (CH\(_2\)), 43.98 (CH\(_2\)), 53.51 (2xCH\(_2\)), 57.27 (CH\(_2\)), 66.74 (2xCH\(_2\)), 114.12 (2xCH), 116.55 (2xCH), 137.45 (C), 141.45 (C); MS (ES) calcd. C\(_{13}\)H\(_{21}\)N\(_3\)O [M+H]\(^+\) 236.2. Found: 236.1.
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\[
\text{\textbf{\textit{N-(3-(Dimethylamino)propyl)-4-aminobenzenamine (2.0.24):}}}
\]

Compound 2.0.23 (393 mg, 1.76 mmol) was treated according to general procedure C. The target compound was obtained as a yellow oil. Yield 2.0.24 (125 mg, 37%): IR \(\nu\) (cm\(^{-1}\)) 3372, 2947, 1602, 1515, 1305; \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 1.75 (5\(^\circ\), 2H, \(J=6.9\) Hz), 2.23 (s, 6H), 2.38 (t, 2H, \(J=7.0\) Hz), 3.09 (t, 2H, \(J=6.7\) Hz), 3.42 (br s, 3H), 6.50 (d, 2H, \(J=8.6\) Hz), 6.59 (d, 2H, \(J=8.7\) Hz); \(^1^3\)C NMR (CDCl\(_3\)) \(\delta\): 42.78 (CH\(_2\)), 51.65 (2xCH\(_3\)), 60.23 (CH\(_2\)), 74.81 (CH\(_2\)), 114.83 (2xCH), 117.01 (2xCH), 133.29 (C), 144.13 (C); MS (ES) calcd. C\(_{11}\)H\(_{19}\)N\(_3\) \([\text{M+H}]^+\) 194.2. Found: 194.2.

\[
\text{\textbf{\textit{N-(3-(4-Methylpiperazin-1-yl)propyl)benzene-1,4-diamine (2.0.26):}}}
\]

Compound 2.0.25 (250 mg, 0.90 mmol) was treated according to general procedure C. The target compound was obtained as a red oil. Yield 2.0.26 (200 mg, 89%): IR \(\nu\) (cm\(^{-1}\)) 2804, 1514, 1273, 1152; \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 1.74 (5\(^\circ\), 2H, \(J=6.6\) Hz), 2.27 (s, 3H), 2.44 (t, 2H, \(J=6.8\) Hz), 2.30-2.55 (m, 8H), 3.08 (t, 2H, \(J=6.5\) Hz), 3.25 (br s, 3H), 6.47 (d, 2H, \(J=8.7\) Hz), 6.57 (d, 2H, \(J=8.7\) Hz); \(^1^3\)C NMR (CDCl\(_3\)) \(\delta\): 25.83 (CH\(_2\)), 44.25 (CH\(_2\)), 45.81 (CH\(_3\)), 53.00 (2xCH\(_2\)), 55.02 (2xCH\(_2\)), 56.97 (CH\(_2\)), 114.12 (2xCH), 116.61 (2xCH), 137.32 (C), 141.62 (C); MS (ES) calcd. C\(_{14}\)H\(_{24}\)N\(_4\) \([\text{M+H}]^+\) 249.2. Found: 249.1.

\[
\text{\textbf{\textit{N-(4-Aminophenyl)-2-(pyrrolidin-1-yl)acetamide (2.0.29):}}}
\]

Compound 2.0.28 (218 mg, 0.87 mmol) was treated according to general procedure C. The target compound was obtained as a red oil. Yield 2.0.29 (110 mg, 57%): \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 1.80 (m, 4H), 2.64 (m, 4H), 3.21 (s, 2H), 3.56 (br s, 2H), 6.62 (d, 2H, \(J=8.7\) Hz), 7.31 (d, 2H, \(J=8.7\) Hz), 8.86 (br s, 1H); \(^1^3\)C NMR (CDCl\(_3\)) \(\delta\): 24.07 (2xCH\(_2\)), 54.59 (2xCH\(_2\)), 59.70 (CH\(_2\)), 115.42 (2xCH), 121.35 (2xCH), 129.35 (C), 143.04 (C), 168.69 (C=O); MS (ES) calcd. C\(_{12}\)H\(_{17}\)N\(_3\)O \([\text{M+H}]^+\) 220.1. Found: 220.1.
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N-(4-Aminophenyl)-4-(pyrrolidin-1-yl)butanamide (2.0.32): Compound 2.0.31 (210 mg, 0.76 mmol) was treated according to general procedure C. The target compound was obtained as a red oil. Yield 2.0.32 (83 mg, 44%): IR \(\tilde{\nu}(\text{cm}^{-1})\) 2946, 2794, 1644, 1516; \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 1.73 (m, 4H), 1.81 (5q, 2H, \(J=6.8\) Hz), 2.48 (m, 6H), 3.60 (br s, 2H), 6.54 (d, 2H, \(J=8.7\) Hz), 7.20 (d, 2H, \(J=8.7\) Hz), 9.17 (br s, 1H); \(^13\)C NMR (CDCl\(_3\)) \(\delta\): 23.19 (2xCH\(_2\)), 24.07 (CH), 35.85 (CH\(_2\)), 53.62 (2xCH\(_2\)), 55.47 (CH\(_2\)), 114.95 (2xCH), 121.44 (2xCH), 129.60 (C), 142.76 (C), 171.09 (C=O); MS (ES) calcd. C\(_{14}\)H\(_{21}\)N\(_3\)O [M+H]+ 248.2. Found: 248.2.

N-(4-Aminophenyl)-5-(pyrrolidin-1-yl)pentanamide (2.0.35): Compound 2.0.34 (250 mg, 0.86 mmol) was treated according to general procedure C. The target compound was obtained as a red oil. Yield 2.0.35 (200 mg, 89%): IR \(\tilde{\nu}(\text{cm}^{-1})\) 2935, 2793, 1645, 1515, 1257; \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 1.55 (5q, 2H, \(J=7.5\) Hz), 1.71 (5q, 2H, \(J=7.4\) Hz), 1.73 (m, 4H), 2.29 (t, 2H, \(J=7.4\) Hz), 2.43 (t, 2H, \(J=7.5\) Hz), 2.45 (m, 4H), 3.59 (br s, 2H), 6.57 (d, 2H, \(J=8.6\) Hz), 7.21 (d, 2H, \(J=8.6\) Hz), 7.83 (br s, 1H); \(^13\)C NMR (CDCl\(_3\)) \(\delta\): 23.24 (2xCH\(_2\)), 23.66 (CH\(_2\)), 28.26 (CH\(_2\)), 36.86 (CH\(_2\)), 54.02 (2xCH\(_2\)), 55.83 (CH\(_2\)), 115.12 (2xCH), 122.18 (2xCH), 129.28 (C), 143.11 (C), 171.30 (C=O); MS (ES) calcd. C\(_{15}\)H\(_{23}\)N\(_3\)O [M+H]+ 262.2. Found: 262.2.

N-(4-Aminophenyl)-4-oxo-4-(pyrrolidin-1-yl)butanamide (2.0.38): Compound 2.0.37 (107 mg, 0.37 mmol) was treated according to general procedure C. The target compound was obtained as a white solid. Yield 2.0.38 (77 mg, 80%): IR \(\tilde{\nu}(\text{cm}^{-1})\) 3248, 2960, 1739, 1606, 1365, 1220; \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 1.84 (5q, 2H, \(J=6.8\) Hz), 1.94 (5q, 2H, \(J=6.6\) Hz), 2.67 (m, 4H), 3.42 (t, 2H, \(J=6.7\) Hz), 3.45 (t, 2H, \(J=6.8\) Hz), 3.52 (br s, 2H), 6.60 (d, 2H, \(J=8.7\) Hz), 7.29 (d, 2H, \(J=7.3\) Hz), 8.65 (br s, 1H); \(^13\)C NMR (CDCl\(_3\)) \(\delta\): 24.26 (CH\(_2\)), 25.90 (CH\(_2\)), 30.33 (CH\(_2\)), 32.19 (CH\(_2\)), 45.81 (CH\(_2\)), 46.49 (CH\(_2\)), 115.20 (2xCH), 121.46...
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(2xCH), 129.98 (C), 142.70 (C), 170.52 (C=O), 170.64 (C=O); MS (ES) calcd. C<sub>14</sub>H<sub>19</sub>N<sub>3</sub>O<sub>2</sub> [M+H]<sup>+</sup> 262.1. Found: 262.1.

**N-(3-Aminophenyl)-3-(pyrrolidin-1-yl)propanamide (2.0.41):** Compound 2.0.40 (157 mg, 0.60 mmol) was treated according to general procedure C. The target compound was obtained as a yellow oil. Yield 2.0.41 (88 mg, 63%): IR λ (cm<sup>-1</sup>) 2952, 2804, 1607, 1550, 1451; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 1.81 (m, 4H), 2.45 (t, 2H, J=6.0 Hz), 2.58 (m, 4H), 2.76 (t, 2H, J=6.0 Hz), 3.70 (br s, 2H), 6.33 (dd, 1H, J=7.2, 2.0 Hz), 6.59 (dd, 1H, J=8.0 Hz), 6.98 (t, 1H, J=8.0 Hz), 7.10 (m, 1H), 10.92 (br s, 1H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ: 23.42 (2xCH<sub>2</sub>), 34.60 (CH<sub>3</sub>), 51.07 (CH<sub>2</sub>), 52.83 (2xCH<sub>3</sub>), 106.19 (CH), 109.29 (CH), 110.19 (CH), 129.24 (CH), 139.51 (C), 147.12 (C), 170.64 (C=O); MS (ES) calcd. C<sub>13</sub>H<sub>19</sub>N<sub>3</sub>O [M+H]<sup>+</sup> 234.1. Found: 234.2.

**4-Amino-1-methyl-N-(3-(pyrrolidin-1-yl)propyl)-1H-pyrrole-2-carboxamide (2.0.44):** Compound 2.0.43 (87 mg, 0.31 mmol) was treated according to general procedure C. The target compound was obtained as a brown oil. Yield 2.0.44 (78 mg, 100%): IR λ (cm<sup>-1</sup>) 3320, 2939, 1634, 1530, 1448, 1284; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 1.73 (5q, 2H, J=6.1 Hz), 1.82 (m, 4H), 2.55 (m, 4H), 2.62 (t, 2H, J=6.1 Hz), 2.82 (br s, 2H), 3.43 (td, 2H, J=6.0, 5.2 Hz), 3.82 (s, 3H), 6.00 (d, 1H, J=2.1 Hz), 6.24 (d, 1H, J=2.1 Hz), 7.68 (br s, 1H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ: 23.47 (2xCH<sub>2</sub>), 26.87 (CH<sub>2</sub>), 36.05 (CH<sub>3</sub>), 39.48 (CH<sub>2</sub>), 54.06 (2xCH<sub>2</sub>), 55.65 (CH<sub>2</sub>), 102.52 (CH), 115.12 (CH), 124.07 (C), 129.58 (C), 161.71 (C=O); MS (ES) calcd. C<sub>13</sub>H<sub>22</sub>N<sub>4</sub>O [M+H]<sup>+</sup> 251.2. Found: 251.1.

**General procedure D:** Compounds 2.1-2.19 were prepared according to general procedure D. A solution of 2.0.1 (or 2.0.2 for compound 2.19), PyBOP (3 eq.) and the appropriate amine (2.5-4 eq.) in a mixture of anhydrous DMF (3 ml) and acetonitrile (1 ml) under nitrogen atmosphere was stirred at RT for 24 h. EtOAc (30 ml) was added and the precipitate taken by filtration, washed with EtOAc (3 x 5 ml) and ether (3 x 5 ml). If the addition of EtOAc did not produce a precipitate, the solution was concentrated in vacuo, dissolved in the minimum amount of DMF and EtOAc added.
HPLC purification (as described in the general methods) was performed for compounds that showed a purity lower than 95%.

\[ \text{N}^2,\text{N}^4\text{-Bis(4-(3-(pyrrolidin-1-yl)propanamido)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.1):} \]

Compounds 2.0.1 (20 mg, 0.071 mmol) and 2.0.5 (69 mg, 1.82 mmol,) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.1 (42.8 mg, 84.5%): mp d250°C; IR \( \lambda (\text{cm}^{-1}) \) 3283, 1644, 1610, 1510, 1433, 1406, 1311, 1238, 1022, 832; \(^1\)H NMR (DMSO-\(d_6\)) \( \delta \): 1.89 (m, 8H), 2.79 (t, 4H, \( J=6.9 \) Hz), 3.10 (m, 8H), 3.33 (m, 4H), 7.46 (t, 2H, \( J=7.7 \) Hz), 7.61 (d, 4H, \( J=9.1 \) Hz), 7.67 (d, 4H, \( J=9.1 \) Hz), 8.34 (dd, 2H, \( J=7.5, 1.4 \) Hz), 8.50 (dd, 2H, \( J=8.1, 1.3 \) Hz), 10.29 (s, 2H), 10.62 (s, 2H), 13.26 (s, 1H); \(^{13}\)C NMR (DMSO-\(d_6\)) \( \delta \): 23.02 (4xCH\(_2\)), 35.52 (2xCH\(_2\)), 51.28 (2xCH\(_2\)), 53.28 (4xCH\(_2\)), 119.19 (4xCH), 120.58 (2xC), 120.63 (2xC), 121.06 (2xCH), 121.29 (4xCH), 129.78 (2xCH), 133.66 (2xCH), 134.05 (2xCH), 134.57 (2xC), 139.25 (2xC), 165.56 (2xC=O), 169.60 (2xC=O), 176.19 (C=O); HRMS (ESI+) calcd. C\(_{41}\)H\(_{33}\)N\(_7\)O\(_5\) [M+H]\(^+\) 714.3398. Found: 714.3395.

\[ \text{N}^4,\text{N}^6\text{-Bis(4-(3-(morpholine-4-yl)propanamido)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.2):} \]

Compounds 2.0.1 (45 mg, 0.16 mmol) and 2.0.7 (99.6 mg, 0.40 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.2 (78 mg, 65%): mp d260°C; IR \( \lambda (\text{cm}^{-1}) \) 3298, 1644, 1610, 1555, 1513, 1407, 1310, 1128, 981, 840; \(^1\)H NMR (DMSO-\(d_6\)) \( \delta \): 2.42 (m, 8H), 2.49 (t, 4H, \( J=7.2 \) Hz), 2.64 (t, 4H, \( J=7.0 \) Hz), 3.59 (m, 8H), 7.45 (t, 2H, \( J=7.7 \) Hz), 7.59 (d, 4H, \( J=9.0 \) Hz), 7.65 (d, 4H, \( J=9.0 \) Hz), 8.33 (dd, 2H, \( J=7.5, 1.2 \) Hz), 8.50 (dd, 2H, \( J=8.0, 1.0 \) Hz), 10.05 (s, 2H), 10.59 (s, 2H), 13.27 (s, 1H); \(^{13}\)C NMR (DMSO-\(d_6\)) \( \delta \): 33.75 (2xCH\(_2\)), 52.96
(4xCH₂), 54.10 (2xCH₂), 66.11 (4xCH₂), 119.18 (4xCH), 119.71 (2xC), 120.55 (2xC), 121.03 (2xC), 121.26 (4xCH), 129.70 (2xC), 133.62 (2xC), 134.04 (2xC), 135.44 (2xC), 138.52 (2xC), 165.51 (2xC=O), 169.78 (2xC=O), 176.38 (C=O); HRMS (ESI+) calcd. C₄₁H₄₅N₇O₇ [M+H]^+ 746.3297. Found: 746.3269.

\[ \text{N}^4,\text{N}^5\text{-Bis}(4\text{-}(3\text{-}(\text{dimethylamino})\text{propanamido})\text{phenyl})\text{-}9,10\text{-dihydro}-9\text{-}\text{oxoacridine}-4,5\text{-dicarboxamide} \quad (2.3) \]

Compounds 2.0.1 (45 mg, 0.16 mmol) and 2.0.9 (83 mg, 0.40 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.3 (75 mg, 70%): mp d305°C; IR \( \lambda \) (cm\(^{-1}\)) 3299, 1659, 1643, 1611, 1517, 1408, 1313, 840; \(^1\)H NMR (DMSO-\(d_6\)) \( \delta \): 2.70 (s, 12H), 2.76 (m, 4H), 3.23 (m, 4H), 7.47 (t, 2H, \( J=7.7 \) Hz), 7.60 (d, 4H, \( J=8.9 \) Hz), 7.68 (d, 4H, \( J=9.0 \) Hz), 8.33 (dd, 2H, \( J=7.5, 1.3 \) Hz), 8.51 (dd, 2H, \( J=8.0, 1.1 \) Hz), 10.21 (s, 2H), 10.62 (s, 2H), 13.25 (s, 1H); \(^13\)C NMR (DMSO-\(d_6\)) \( \delta \): 34.42 (2xCH₂), 44.73 (4xCH₂), 54.90 (2xCH₂), 119.17 (4xCH), 120.61 (2xC), 120.99 (2xC), 121.24 (4xCH), 121.25 (2xC), 129.75 (2xC), 133.60 (2xC), 134.05 (2xC), 135.47 (2xC), 139.27 (2xC), 165.51 (2xC=O), 169.75 (2xC=O), 176.18 (C=O); HRMS (ESI+) calcd. C₃₇H₃₉N₇O₅ [M+H]^+ 662.3085. Found: 662.3095.

\[ \text{N}^4,\text{N}^5\text{-Bis}(4\text{-}(3\text{-}(4\text{-methylpiperazin-1-yl})\text{propanamido})\text{phenyl})\text{-}9,10\text{-dihydro}-9\text{-}\text{oxoacridine}-4,5\text{-dicarboxamide} \quad (2.4) \]

Compounds 2.0.1 (48 mg, 0.17 mmol) and 2.0.11 (178 mg, 0.68 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.4 (131 mg, 100%): mp d210°C; IR \( \lambda \) (cm\(^{-1}\)) 1646, 1607, 1508, 1309, 826, 751; \(^1\)H NMR (DMSO-\(d_6\)) \( \delta \): 2.52 (t, 4H, \( J=6.9 \) Hz), 2.58 (s, 6H), 2.66 (br s, 8H), 2.76 (t, 4H, \( J=6.9 \) Hz), 2.93 (br s, 8H), 7.46 (t, 2H, \( J=7.7 \) Hz), 7.60 (d, 4H, \( J=9.0 \) Hz), 7.66 (d, 4H, \( J=9.0 \) Hz), 8.33 (dd, 2H, \( J=7.5, 1.1 \) Hz), 8.51 (dd, 2H, \( J=8.0, 1.0 \) Hz), 10.02 (s, 2H), 10.59 (s, 2H), 13.27 (s, 1H); \(^13\)C NMR (DMSO-\(d_6\)) \( \delta \): 33.59 (2xCH₂), 43.17 (2xCH₂), 50.16 (4xCH₂), 52.87 (2xCH₂), 52.97 (4xCH₂), 119.24 (4xCH), 120.66 (2xC), 120.90 (2xC), 121.27 (4xCH), 121.32 (2xC),
129.87 (2xCH), 133.73 (2xC), 134.16 (2xCH), 135.37 (2xC), 139.30 (2xC), 165.58 (2xC=O), 169.37 (2xC=O), 176.19 (C=O); HRMS (ESI+) calcd. C_{43}H_{49}N_{9}O_{5} [M+H]^+ 772.3929. Found: 772.3905.

$N^4,N^5$-Bis(4-(3-(piperidin-1-yl)propanamido)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.5): Compounds 2.0.1 (22 mg, 0.08 mmol) and 2.0.13 (77 mg, 0.32 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.5 (29.8 mg, 50%): mp 220-221°C; IR λ (cm⁻¹) 1738, 1506, 1365, 1216, 833; $^1$H NMR (DMSO-d₆) δ: 1.48 (br s, 4H), 1.63 (br s, 8H), 2.66 (br s, 4H), 2.81 (br s, 8H), 3.01 (br s, 4H), 7.46 (t, 2H, J=7.7 Hz), 7.59 (d, 4H, J=8.8 Hz), 7.67 (d, 4H, J=8.8 Hz), 8.33 (d, 2H, J=7.0 Hz), 8.51 (d, 2H, J=7.8 Hz), 10.19 (s, 2H), 10.60 (s, 2H), 13.26 (s, 1H); $^{13}$C NMR (DMSO-d₆) δ: 22.46 (2xCH₂), 23.91 (4xCH₂), 32.08 (2xCH₂), 52.89 (4xCH₂), 53.02 (2xCH₂), 119.26 (4xCH), 120.66 (2xCH), 120.90 (2xC), 121.31 (4xCH), 123.85 (2xC), 129.86 (2xCH), 133.83 (2xC), 134.15 (2xCH), 135.21 (2xC), 139.29 (2xC), 165.58 (2xC=O), 168.70 (2xC=O), 176.18 (C=O); HRMS (ESI+) calcd. C_{43}H_{47}N_{9}O_{5} [M+H]^+ 742.3712. Found: 742.3701.

$N^4,N^5$-Bis(4-(3-(N-cyclohexylamino)propanamido)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.6): Compounds 2.0.1 (55 mg, 0.19 mmol) and 2.0.15 (200 mg, 0.77 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.6 (15 mg, 10%): mp d230°C; $^1$H NMR (DMSO-d₆) δ: 1.02-1.28 (m, 12H), 1.56 (m, 2H), 1.68 (m, 4H), 1.85 (m, 4H), 2.46 (t, 4H, J=6.5 Hz), 2.88 (t, 4H, J=6.3 Hz), 7.45 (t, 2H, J=7.4 Hz), 7.58 (d, 4H, J=7.8 Hz), 7.65 (d, 4H, J=8.7 Hz), 8.33 (d, 2H, J=6.5 Hz), 8.50 (d, 2H, J=7.5 Hz), 10.24 (s, 2H), 10.57 (s, 2H), 13.27 (s, 1H); $^{13}$C NMR (DMSO-d₆) δ: 24.07 (4xCH₂), 25.19 (2xCH₂), 30.21 (4xCH₂), 34.25 (2xCH₂), 48.45 (2xCH₂), 55.74 (2xCH), 119.24 (4xCH), 120.01 (2xC), 120.63 (2xCH), 121.22 (4xCH), 121.31 (2xC), 124.45 (2xC), 129.63 (2xCH), 133.83 (2xC), 133.88 (2xCH), 135.20 (2xC),
165.55 (2xC=O), 168.81 (2xC=O), 176.21 (C=O); HRMS (ESI+) calcd. C_{45}H_{51}N_7O_5 [M+H]^+ 770.4025. Found: 770.4020.

\[ N^4,N^5\text{-Bis(4-(3\text{-}(1H-imidazol-1-yl)propanamido)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.7):} \]

Compounds 2.0.1 (18 mg, 0.06 mmol) and 2.0.17 (44 mg, 0.19 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.7 (20 mg, 47%): mp d280°C; \^H NMR (DMSO-d_6) δ: 2.78 (t, 4H, \text{J}=6.7 Hz), 4.28 (t, 4H, \text{J}=6.1 Hz), 6.88 (s, 2H), 7.03 (t, 2H, \text{J}=7.5 Hz), 7.15 (s, 2H), 7.29 (d, 4H, \text{J}=8.8 Hz), 7.69 (d, 4H, \text{J}=8.8 Hz), 8.08 (dd, 2H, \text{J}=7.0, 1.5 Hz), 8.38 (dd, 2H, \text{J}=8.0, 1.7 Hz), 10.07 (s, 2H), 10.61 (s, 2H), 13.27 (s, 1H); \^C NMR (DMSO-d_6) δ: 37.63 (2xCH_2), 42.08 (2xCH_2), 119.34 (4xCH), 120.60 (2xCH), 121.12 (2xCH), 121.28 (4xCH), 124.48 (2xCH), 126.71 (2xCH), 128.26 (2xCH), 129.76 (2xCH), 132.17 (2xCH), 133.88 (2xCH), 134.03 (2xCH), 135.09 (2xCH), 139.29 (2xCH), 165.56 (2xC=O), 168.19 (2xC=O), 176.35 (C=O); HRMS (ESI+) calcd. C_{39}H_{33}N_9O_7 [M+H]^+ 708.2678. Found: 708.2686.

\[ N^4,N^5\text{-Bis(4-(4-oxo-4-(pyrrolidin-1-yl)butanamido)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.8):} \]

Compounds 2.0.1 (21 mg, 0.074 mmol) and 2.0.38 (77 mg, 0.295 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.8 (15 mg, 26%): mp d195°C; \^H NMR (DMSO-d_6) δ: 1.77 (m, 4H), 1.89 (m, 4H), 2.57 (m, 8H), 3.30 (m, 4H), 3.45 (m, 4H), 7.45 (t, 2H, \text{J}=7.5 Hz), 7.59 (d, 4H, \text{J}=8.6 Hz), 7.64 (d, 4H, \text{J}=8.7 Hz), 8.33 (d, 2H, \text{J}=7.2 Hz), 8.50 (d, 2H, \text{J}=8.0 Hz), 9.96 (br s, 2H), 10.56 (br s, 2H), 13.27 (br s, 1H); \^C NMR (DMSO-d_6) δ: 23.85 (2xCH_2), 25.49 (2xCH_2), 28.86 (2xCH_2), 30.98 (2xCH_2), 45.17 (2xCH_2), 45.67 (CH_2), 118.98 (4xCH), 120.61 (2xCH), 121.05 (2xCH), 121.19 (4xCH), 121.27 (2xCH), 129.72 (2xCH), 133.43 (2xCH), 134.02 (2xCH), 135.65 (2xCH), 139.25 (2xCH), 165.46 (2xC=O), 169.35 (2xC=O), 170.28 (2xC=O), 176.19 (C=O); HRMS (ESI+) calcd. C_{43}H_{43}N_7O_7 [M+H]^+ 770.3297. Found: 770.3312.
$N^6,N^7$-Bis(4-(3-(pyrrolidin-1-yl)propylamino)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.9): Compounds 2.0.1 (86 mg, 0.305 mmol) and 2.0.20 (267 mg, 1.22 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.9 (46 mg, 22%): mp d160°C; IR $\lambda$ (cm$^{-1}$) 1609, 1514, 1431, 1313, 835; $^1$H NMR (DMSO-d$_6$) $\delta$: 1.70 (m, 8H), 1.74 (5q, 4H, $J=7.0$ Hz), 2.51-2.58 (m, 12H), 3.04 (t, 4H, $J=6.6$ Hz), 6.56 (d, 4H, $J=8.7$ Hz), 7.42 (d, 4H, $J=8.7$ Hz), 7.45 (t, 2H, $J=9.2$ Hz), 8.28 (d, 2H, $J=7.3$ Hz), 8.47 (dd, 2H, $J=8.0$, 1.2 Hz), 10.30 (s, 2H), 13.35 (s, 1H); $^{13}$C NMR (DMSO-d$_6$) $\delta$: 23.00 (4xCH$_2$), 27.60 (2xCH$_2$), 41.29 (2xCH$_2$), 53.36 (2xCH$_2$), 53.48 (4xCH$_2$), 111.60 (4xCH), 120.60 (2xCH), 121.27 (2xC), 122.58 (4xCH), 124.04 (2xC), 127.21 (2xC), 129.39 (2xCH), 133.76 (2xCH), 138.46 (2xC), 146.04 (2xC), 164.98 (2xC=O), 176.27 (C=O); HRMS (ESI+) calcd. C$_{41}$H$_{47}$N$_7$O$_3$ [M+H$^+$] 686.3813. Found: 686.3796.

$N^6,N^7$-Bis(4-(3-(morpholine-4-yl)propylamino)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.10): Compounds 2.0.1 (48 mg, 0.17 mmol) and 2.0.22 (160 mg, 0.68 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.10 (35.4 mg, 29%): mp d270°C; IR $\lambda$ (cm$^{-1}$) 1738, 1610, 1515, 1365, 1216, 831; $^1$H NMR (DMSO-d$_6$) $\delta$: 1.90 (5q, 4H, $J=7.0$ Hz), 2.99-3.13 (m, 16H), 3.78 (m, 8H), 6.61 (d, 4H, $J=8.7$ Hz), 7.41-7.45 (m, 6H), 8.28 (d, 2H, $J=6.8$ Hz), 8.48 (d, 2H, $J=7.3$ Hz), 10.29 (s, 2H), 13.32 (s, 1H); $^{13}$C NMR (DMSO-d$_6$) $\delta$: 25.86 (2xCH$_2$), 40.46 (2xCH$_2$), 51.64 (4xCH$_2$), 54.73 (2xCH$_2$), 63.91 (4xCH$_2$), 111.93 (4xCH), 120.68 (2xC), 121.36 (2xCH), 122.80 (4xCH), 124.96 (2xC), 127.64 (2xC), 131.16 (2xC), 133.93 (2xC), 139.35 (2xC), 145.65 (2xC), 165.17 (2xC=O), 176.32 (C=O); HRMS (ESI+) calcd. C$_{41}$H$_{47}$N$_7$O$_5$ [M+H$^+$] 718.3712. Found: 718.3708.
\text{N}^4,\text{N}^5\text{-Bis(4-(3-(dimethylamino)propylamino)phenyl)}-9,10\text{-dihydro-9-oxoacridine-4,5-dicarboxamide (2.11):}\\
\text{Compounds 2.0.1 (48 mg, 0.17 mmol) and 2.0.24 (125 mg, 0.68 mmol) were treated according to general procedure D to obtain the target compound as a brown powder. Yield 2.11 (61.5 mg, 59%): mp 278-279°C; IR }\nu\text{ (cm}^{-1}\text{): 1738, 1506, 1365, 1216, 833; }^1\text{H NMR (DMSO-d}_6\text{)} \delta: 1.87 (5q, 4H, }J=7.5\text{ Hz), 2.72 (s, 12H), 3.05 (t, 4H, }J=7.9\text{ Hz), 3.10 (t, 4H, }J=6.5\text{ Hz), 6.61 (d, 4H, }J=8.7\text{ Hz), 7.41-7.45 (m, 6H), 8.29 (d, 2H, }J=6.8\text{ Hz), 8.48 (d, 2H, }J=7.5\text{ Hz), 10.30 (s, 2H), 13.32 (s, 1H); }^{13}\text{C NMR (DMSO-d}_6\text{)} \delta: 23.96 (2xCH}_2\text{), }40.21 (2xCH}_2\text{), }42.58 (4xCH}_3\text{), }55.24 (2xCH}_2\text{), }111.83 (4xCH), 120.60 (2xCH), 121.27 (2xCH), 122.66 (4xCH), 124.00 (2xCH), 127.59 (2xCH), 129.51 (2xCH), 133.85 (2xCH), 139.25 (2xCH), 145.55 (2xCH), 165.06 (2xCH=O), 176.22 (C=O); HRMS (ESI+) calcd. C}_{37}\text{H}_{42}\text{N}_7\text{O}_3\text{[M+H]+ }634.3500. \text{ Found: 634.3474.}

\text{N}^4,\text{N}^5\text{-Bis(4-(3-(4-methylpiperazin-1-yl)propylamino)phenyl)}-9,10\text{-dihydro-9-oxoacridine-4,5-dicarboxamide (2.12):}\\
\text{Compounds 2.0.1 (9.5 mg, 0.033 mmol) and 2.0.26 (25 mg, 0.10 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.12 (5.4 mg, 22%): mp 186-187°C; IR }\nu\text{ (cm}^{-1}\text{): 1738, 1515, 1365, 1216, 1020, 838; }^1\text{H NMR (DMSO-d}_6\text{)} \delta: 1.84 (m, 4H), 2.77 (s, 6H), 2.91 (m, 4H), 3.11 (t, 4H, }J=6.7\text{ Hz), 3.25 (m, 16H), 6.66 (d, 4H, }J=8.8\text{ Hz), 7.44 (t, 2H, }J=7.7\text{ Hz), 7.47 (d, 4H, }J=8.9\text{ Hz), 8.29 (d, 2H, }J=7.1\text{ Hz), 8.48 (dd, 2H, }J=8.0, 1.2\text{ Hz), 10.32 (s, 2H), 13.31 (s, 1H); }^{13}\text{C NMR (DMSO-d}_6\text{)} \delta: 25.79 (2xCH}_2\text{), }41.42 (2xCH}_2\text{), }45.43 (2xCH}_2\text{), }52.46 (4xCH}_2\text{), }54.52 (4xCH}_2\text{), }55.62 (2xCH}_2\text{), }111.57 (4xCH), 120.52 (2xCH), 121.22 (2xCH), 121.48 (2xCH), 122.57 (4xCH), 127.16 (2xCH), 129.32 (2xCH), 133.66 (2xCH), 139.21 (2xCH), 146.06 (2xCH), 164.92 (2xCH=O), 176.23 (C=O); HRMS (ESI+) calcd. C}_{43}\text{H}_{53}\text{N}_9\text{O}_3\text{[M+H]+ }744.4344. \text{ Found: 744.4332.}
\( \text{N}^\text{\alpha}, \text{N}^\text{\beta}-\text{Bis(4-(2-(pyrrolidin-1-yl)acetamido)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.13):} \)

Compounds 2.0.1 (35 mg, 0.125 mmol) and 2.0.29 (110 mg, 0.5 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.13 (48 mg, 56%): mp 211-212°C; IR \( \lambda \) (cm\(^{-1}\)) 1738, 1645, 1609, 1509, 830; \(^1\)H NMR (DMSO-d\(_6\)) \( \delta \): 1.90 (br s, 8H), 3.10 (br s, 8H), 3.88 (s, 4H), 7.47 (t, 2H, \( J=7.7 \) Hz), 7.62 (d, 4H, \( J=8.9 \) Hz), 7.70 (d, 4H, \( J=8.8 \) Hz), 8.34 (d, 2H, \( J=6.6 \) Hz), 8.51 (d, 2H, \( J=7.8 \) Hz), 10.24 (s, 2H), 10.65 (s, 2H), 13.25 (s, 1H); \(^{13}\)C NMR (DMSO-d\(_6\)) \( \delta \): 23.37 (4xCH\(_2\)), 53.64 (4xCH\(_2\)), 59.28 (2xCH\(_2\)), 119.66 (4xCH), 120.60 (2xCH), 121.01 (2xC), 121.16 (4xCH), 121.30 (2xC), 128.28 (2xC), 129.78 (2xC), 133.92 (2xC), 134.05 (2xCH), 134.86 (2xC), 165.55 (2xC=O), 169.33 (2xC=O), 176.20 (C=O); HRMS (ESI+) calcd. \( \text{C}_{39}\text{H}_{39}\text{N}_{7}\text{O}_{5} \) [M+H]\(^{\text{+}}\) 686.3085. Found: 686.3059.

\( \text{N}^\text{\alpha}, \text{N}^\text{\beta}-\text{Bis(4-(4-(pyrrolidin-1-yl)butanamido)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.14):} \)

Compounds 2.0.1 (29 mg, 0.102 mmol) and 2.0.32 (63 mg, 0.255 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.14 (68.4 mg, 90%): mp 255-257°C; IR \( \lambda \) (cm\(^{-1}\)) 1646, 1607, 1508, 1307, 830; \(^1\)H NMR (DMSO-d\(_6\)) \( \delta \): 1.69 (m, 8H), 1.77 (5q, 4H, \( J=7.2 \) Hz), 2.36 (t, 4H, \( J=7.4 \) Hz), 2.47 (m, 12H), 7.45 (t, 2H, \( J=7.7 \) Hz), 7.59 (d, 4H, \( J=8.8 \) Hz), 7.64 (d, 4H, \( J=9.0 \) Hz), 8.32 (d, 2H, \( J=7.2 \) Hz), 8.50 (d, 2H, \( J=7.9 \) Hz), 9.89 (s, 2H), 10.56 (s, 2H), 13.27 (s, 1H); \(^{13}\)C NMR (DMSO-d\(_6\)) \( \delta \): 22.88 (4xCH\(_2\)), 23.19 (2xCH\(_2\)), 38.84 (2xCH\(_2\)), 53.11 (4xCH\(_2\)), 54.45 (2xCH\(_2\)), 119.20 (4xCH), 120.56 (2xCH), 121.16 (2xC), 121.24 (4xCH), 121.31 (2xC), 126.39 (2xC), 129.76 (2xCH), 133.59 (2xC), 134.02 (2xCH), 135.53 (2xC), 165.55 (2xC=O), 170.48 (2xC=O), 176.20 (C=O); HRMS (ESI+) calcd. \( \text{C}_{43}\text{H}_{47}\text{N}_{7}\text{O}_{5} \) [M+H]\(^{\text{+}}\) 742.3712. Found: 742.3690.
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\[ \text{N}^4,\text{N}^5\text{-Bis(4-(5-(pyrrolidin-1-yl)pentanamido)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.15):} \]

Compounds 2.0.1 (22 mg, 0.078 mmol) and 2.0.35 (81 mg, 0.31 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.15 (50.4 mg, 84%): mp d220°C; IR λ (cm\(^{-1}\)) 1646, 1606, 1508, 1307, 829, 751; \(^1\)H NMR (DMSO-d\(_6\)) δ: 1.49 (5q, 4H, \(J=7.4\) Hz), 1.62 (5q, 4H, \(J=7.6\) Hz), 1.68 (m, 8H), 2.32 (t, 4H, \(J=7.3\) Hz), 2.46 (m, 12H), 7.45 (t, 2H, \(J=7.7\) Hz), 7.60 (d, 4H, \(J=8.9\) Hz), 7.64 (d, 4H, \(J=9.0\) Hz), 8.33 (d, 2H, \(J=7.1\) Hz), 8.50 (d, 2H, \(J=7.9\) Hz), 9.90 (s, 2H), 10.60 (s, 2H), 13.27 (s, 1H); \(^13\)C NMR (DMSO-d\(_6\)) δ: 22.82 (2xCH\(_2\)), 22.85 (4xCH\(_2\)), 27.04 (2xCH\(_2\)), 35.94 (2xCH\(_2\)), 53.21 (4xCH\(_2\)), 54.77 (2xCH\(_2\)), 119.23 (4xCH), 120.54 (2xCH), 121.15 (2xC), 121.22 (4xCH), 121.32 (2xC), 126.26 (2xC), 129.74 (2xCH), 133.59 (2xC), 134.01 (2xCH), 135.50 (2xC), 165.55 (2xC=O), 170.76 (2xC=O), 176.18 (C=O); HRMS (ESI+) calcd. C\(_{45}\)H\(_{51}\)N\(_7\)O\(_5\) [M+H]\(^+\) 770.4025. Found: 770.4021.

\[ \text{N}^4,\text{N}^5\text{-Bis(3-(3-(pyrrolidin-1-yl)propanamido)phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (2.16):} \]

Compounds 2.0.1 (43 mg, 0.152 mmol) and 2.0.41 (88 mg, 0.38 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.16 (79.3 mg, 72%): mp 202-204°C; IR λ (cm\(^{-1}\)) 1738, 1365, 1228, 1216, 835; \(^1\)H NMR (DMSO-d\(_6\)) δ: 1.68 (m, 8H), 2.49 (m, 12H), 2.73 (m, 4H), 7.28 (t, 2H, \(J=8.0\) Hz), 7.42 (dd, 4H, \(J=8.0, 1.6\) Hz), 7.46 (t, 2H, \(J=7.7\) Hz), 7.95 (s, 2H), 8.34 (d, 2H, \(J=7.3\) Hz), 8.51 (d, 2H, \(J=7.9\) Hz), 10.19 (s, 2H), 10.67 (s, 2H), 13.17 (s, 1H); \(^13\)C NMR (DMSO-d\(_6\)) δ: 23.06 (4xCH\(_2\)), 35.89 (2xCH\(_2\)), 51.45 (2xCH\(_2\)), 53.35 (4xCH\(_2\)), 111.68 (2xCH), 114.93 (2xCH), 115.83 (2xCH), 120.71 (2xC), 120.91 (2xCH), 121.31 (2xC), 128.77 (2xCH), 129.98 (2xCH), 134.55 (2xC), 138.75 (2xC), 139.32 (2xC), 139.45 (2xC), 165.90 (2xC=O), 170.06 (2xC=O), 176.20 (C=O); HRMS (ESI+) calcd. C\(_{41}\)H\(_{43}\)N\(_7\)O\(_5\) [M+H]\(^+\) 714.3398. Found: 714.3423.
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\(N,N'-\text{Bis}(5-(3-(\text{pyrrolidin-1-yl})\text{propylcarbamoyl})-1\text{-methyl-1H-pyrrol-3-yl})-9,10\text{-dihydro-9-oxoacridine-4,5-dicarboxamide (2.17)}:\) Compounds 2.0.1 (20 mg, 0.07 mmol) and 2.0.44 (70 mg, 0.28 mmol) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.17 (19 mg, 36%): mp 165-167°C; \(^1\)H NMR (DMSO-d\(_6\)) \(\delta:\) 1.69 (5\text{q}, 4\text{H}, \text{J}=7.1 \text{Hz}), 1.71 (\text{m}, 8\text{H}), 2.50 (\text{m}, 4\text{H}), 2.54 (\text{m}, 8\text{H}), 3.22 (\text{m}, 4\text{H}, \text{J}=6.4 \text{Hz}), 3.86 (\text{s}, 6\text{H}), 6.88 (\text{d}, 2\text{H}, \text{J}=1.5 \text{Hz}), 7.36 (\text{d}, 2\text{H}, \text{J}=1.4 \text{Hz}), 7.42 (\text{t}, 2\text{H}, \text{J}=7.7 \text{Hz}), 8.17 (\text{t}, 2\text{H}, \text{J}=5.1 \text{Hz}), 8.25 (\text{d}, 2\text{H}, \text{J}=6.1 \text{Hz}), 8.47 (\text{dd}, 2\text{H}, \text{J}=0.8, 1.1 \text{Hz}), 10.65 (\text{s}, 2\text{H}), 13.46 (\text{s}, 1\text{H}); \(^13\)C NMR (DMSO-d\(_6\)) \(\delta:\) 22.88 (4\text{xCH}_2), 27.75 (2\text{xCH}_2), 35.95 (2\text{xCH}_3), 36.83 (2\text{xCH}_2), 53.10 (2\text{xCH}_2), 53.27 (4\text{xCH}_2), 104.21 (2\text{xCH}), 114.52 (2\text{C}), 118.66 (2\text{CH}), 121.15 (2\text{C}), 121.31 (2\text{C}), 121.35 (2\text{C}), 122.35 (2\text{CH}), 123.22 (2\text{C}), 129.41 (2\text{CH}), 133.49 (2\text{CH}), 161.05 (2\text{C}=\text{O}), 164.00 (2\text{xC}=\text{O}), 176.39 (\text{C}=\text{O}); HRMS (ESI\(^+\)) calcd. C\(_{41}\)H\(_{49}\)N\(_9\)O\(_5\) [M+H\(^+\)] 748.3929. Found: 748.3939.

\(N,N'-\text{Bis}(3-(\text{dimethylamino})\text{propyl})-9,10\text{-dihydro-9-oxoacridine-4,5-dicarboxamide (2.18):}\) Compound 2.0.1 (43 mg, 0.152 mmol) and \(N,N\text{-dimethylpropane-1,3-diamine (61 mg, 0.59 mmol)}\) were treated according to general procedure D to obtain the target compound as a yellow powder. Yield 2.18 (45 mg, 65%): mp 262-264°C; IR \(\lambda\) (cm\(^{-1}\)) 3307, 1737, 1518, 1372; \(^1\)H NMR (DMSO-d\(_6\)) \(\delta:\) 1.78 (5\text{q}, 4\text{H}, \text{J}=6.8 \text{Hz}), 2.25 (\text{s}, 12\text{H}), 2.43 (\text{t}, 4\text{H}, \text{J}=6.7 \text{Hz}), 3.38 (\text{t}, 4\text{H}, \text{J}=5.8 \text{Hz}), 7.36 (\text{t}, 2\text{H}, \text{J}=7.7 \text{Hz}), 8.16 (\text{d}, 2\text{H}, \text{J}=7.1 \text{Hz}), 8.42 (\text{d}, 2\text{H}, \text{J}=7.6 \text{Hz}), 8.95 (\text{br s}, 2\text{H}), 13.62 (\text{s}, 1\text{H}); \(^13\)C NMR (DMSO-d\(_6\)) \(\delta:\) 23.30 (2\text{xCH}_2), 53.67 (4\text{xCH}_3), 58.89 (2\text{xCH}_2), 60.76 (2\text{xCH}_2), 113.45 (2\text{C}), 115.97 (2\text{C}), 116.80 (2\text{CH}), 119.65 (2\text{CH}), 121.17 (2\text{CH}), 134.79 (2\text{C}), 168.67 (2\text{xC}=\text{O}), 171.36 (\text{C}=\text{O}); HRMS (ESI\(^+\)) calcd. C\(_{23}\)H\(_{33}\)N\(_3\)O\(_3\) [M+H\(^+\)] 452.2656. Found: 452.2645.
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N-(4-(3-(Pyrrolidin-1-yl)propanamido)phenyl)-9,10-dihydro-9-oxoacridine-4-carboxamide (2.19): Compound 2.0.2 (20 mg, 0.084 mmol), 2.0.5 (39.1 mg, 0.17 mmol) and PyBOP (52.45 mg, 0.101 mmol) were dissolved in DMF (3 ml) and acetonitrile (1 ml) and stirred overnight at RT. A saturated solution of Na₂CO₃ (10 ml) was added and the product extracted with EtOAc (3 x 10 ml). The organics were partitioned with 1M HCl (2 x 10ml) and the aqueous phase neutralised with saturated Na₂CO₃ and extracted with EtOAc (3 x 10ml). The organics were treated in the usual manner and the crude purified by silica chromatography using DCM:MeOH:TEA 9.25:0.25:0.5 to give 2.19 as a yellow solid. Yield 2.19 (17 mg, 44%): mp d265°C; ¹H NMR (MeOD) δ: 1.95 (m, 4H), 2.72 (t, 2H, J=6.9 Hz), 3.00 (m, 4H), 3.18 (t, 2H, J=6.9 Hz), 7.30 (t, 1H, J=7.5, 0.9 Hz), 7.35 (t, 1H, J=7.7 Hz), 7.54 (d, 2H, J=9.0 Hz), 7.58 (d, 1H, J=7.3 Hz), 7.66 (d, 2H, J=8.9 Hz), 7.73 (m, 1H), 8.29 (dd, 1H, J=3.4, 1.0 Hz), 8.31 (dd, 1H, J=4.1, 1.3Hz), 8.54 (dd, 1H, J=8.1, 1.4 Hz); ¹³C NMR (MeOD) δ: 24.32 (2xCH₂), 36.38 (CH₂), 52.94 (CH₂), 54.98 (2xCH₂), 116.02 (C), 116.70 (C), 117.58 (C), 119.34 (CH), 121.46 (2xCH), 122.00 (C), 123.08 (2xCH), 123.54 (CH), 126.24 (C), 127.30 (CH), 131.63 (CH), 131.96 (CH), 134.68 (CH), 135.53 (C), 135.59 (CH), 141.86 (C), 164.64 (C=O), 164.90 (C=O), 173.40 (C=O); MS (ES) calcd. C₂₇H₂₆N₄O₃ [M+H]⁺ 455.52. Found: 455.21.
9.3 HPLC ANALYSIS DATA FOR THE ACRIDONE FINAL COMPOUNDS

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Table 9.1 HPLC analysis data for the acridone final compounds 2.1-2.19.
9.4 EXPERIMENTAL PROCEDURES FOR THE NAPHTHALENE DIIMIDE SERIES

1,3,4,5,6,8,9,10-Octachloro-4,5,9,10-tetrahydropyrene (5.0.1.1): Pyrene (25 g, 124 mmol) and I₂ (0.75 g, 2.95 mmol) were dissolved in 1,2,4-trichlorobenzene (250 ml) in a 500 ml three-necked flask equipped with a mechanical stirrer. Chlorine was bubbled through the solution via a wide mouth glass cannula at the minimum flow possible. After 45 min at RT the temperature was raised to 50°C and after another 45 min to 110°C. The mixture was stirred for 4 h then the heating and chlorine flow were stopped. The mixture was left to cool to RT and then in an ice bath. The solid in the mixture was filtered, washed with toluene (2 x 50 ml) and dried in vacuo. A first crop (6.93 g) of the product was obtained as a pale green powder. The filtrate was left to stand at RT for 48 h and a second crop was filtered and washed with toluene (2 x 50 ml) and dried (5.28 g). Overall yield 5.0.1.1 (12.21 g, 25.3 mmol, 20.4%): mp 295-298°C; ¹H NMR (CDCl₃) δ: 5.81 (s, 4H), 7.66 (s, 2H); ¹³C NMR (CDCl₃) δ: 52.91 (4xCH), 128.06 (2xC), 128.65 (4xC), 131.80 (2xCH), 136.56 (4xC); CHN: calcd. C 39.88%, H 1.26%; found C 39.69%, H 1.08%; HRMS (ESI⁺) calcd. C₁₆H₁₆Cl₈ [M+H]⁺ 478.8056. Found: 478.8672.

1,3,4,6,8,9-Hexachloropyrene (5.0.1.2.a) and 1,3,4,6,8,10-Hexachloropyrene (5.0.1.2.b) regio-isomers: To a suspension of 5.0.1.1 (10.65 g, 22.1 mmol) in ethanol (85 ml) in a three-necked flask equipped with a mechanical stirrer, KOH (7.69 g, 137 mmol) was added slowly. The mixture was then heated at reflux for 5 h. The mixture was then left to cool down and it was filtered while still warm (50°C). The solid obtained was washed with boiling water (2 x 20 ml) and ethanol (20 ml). The pale yellow solid was dried under air flow. Combined yield 5.0.1.2.a and 5.0.1.2.b (8.67 g, 21.2 mmol, 96%) as an isomeric mixture: mp> 350°C; CHN: calcd. C 47.00%, H 0.99%; found C 46.78%, H 0.79%; HRMS (MALDI) calcd. C₁₆H₄Cl₆ [M] 407.8415. Found: 407.7534.
2,5,7,10-Tetrachloropyrene-3,8-quinone (5.0.1.3.a):
Fuming HNO$_3$ (12.7 ml) was added to a two-necked flask equipped with a thermometer in a -5°C bath. The mixture of isomers 5.0.1.2.a and 5.0.1.2.b (4.33 g, 10.6 mmol) was added portionwise over a 30 min period with good stirring and maintaining the temperature under 5°C. After completion of the addition the mixture was stirred for another 15 min at 0°C and then filtered to obtain a dark orange solid that was washed with acetic acid (5 x 10 ml) and water (2 x 10ml). The product was purified by sublimation (1-2 mbar, 250°C) to obtain an orange solid. Yield 5.0.1.3.a (0.74 g, 2.0 mmol, 18.9%): mp 315-320°C; $^1$H NMR (CDCl$_3$) $\delta$: 7.06 (s, 2H), 8.46 (s, 2H); $^{13}$C NMR (CDCl$_3$) $\delta$: 125.29 (2xC), 127.45 (2xC), 131.07 (2xC), 131.27 (2xCH), 133.80 (2xCH), 139.35 (2xC), 144.36 (2xC), 176.96 (2xC=O); CHN: calcd. C 51.94%, H 1.09%; found C 51.49%, H 0.39%.

2,6-Dichloro-1,4,5,8-naphthalenetetracarboxylic acid dianhydride (5.0.1): In a two-necked flask equipped with a condenser and a thermometer, compound 5.0.1.3.a (500 mg, 1.35 mmol) was dissolved in conc. sulfuric acid (7 ml). The flask was heated at 100°C and fuming nitric acid (0.775 ml) was added dropwise maintaining the temperature at 120°C. The mixture was then cooled down to 70°C and poured onto ice (50 ml). The yellow solid formed was filtered and washed with cold acetic acid (5 ml). The product was purified by crystallisation from acetic acid. Yield 5.0.1 (157 mg, 0.47 mmol, 34.5%): mp >350°C; $^1$H NMR (DMSO-d$_6$) $\delta$: 8.69 (s, 2H); $^{13}$C NMR (DMSO-d$_6$) $\delta$: 121.47 (2xC), 124.79 (2xC), 128.86 (2xC), 134.58 (2xCH), 138.27 (2xC), 159.90 (2xC=O), 165.46 (2xC=O); CHN: calcd. C 49.89%, H 0.60%; found C 49.46%, H 0.38%.
2,6-Dibromo-1,4,5,8-naphthalenetetracarboxylic acid dianhydride (5.0.2): Naphthalene dianhydride (1 g, 3.72 mmol) was dissolved in fuming sulphuric acid (20% SO₃, 38 ml). A solution of dibromoisocyanuric acid (1.07 g, 3.72 mmol) in fuming sulphuric acid (18.5 ml) was added dropwise into it, with stirring, over a 4 h period. The mixture was stirred for a further 1 h and then poured onto ice (500 ml). The yellow precipitate was filtered, washed with 0.5 M HCl in water (2 x 10 ml) and dried in vacuo. Yield 5.0.2 (1.30 g, 3.05 mmol, 82%): mp > 350°C; CHN: calcd. C 39.48%, H 0.47%; found C 39.50%, H 0.47%.

General procedure E. Compounds 5.1-5.12, 5.17 and 5.18 were prepared according to general procedure E. Compound 5.0.2 was suspended in the appropriate amine in a microwave reaction vessel. The tube was flushed with nitrogen, sealed and treated at 150°C for 10 min in the microwave. The amine was then evaporated in vacuo. The crude mixture was purified by HPLC (as described in general methods) to obtain the target compound and its analogue with three side chains.
$N,N'$-Bis(3-(dimethylamino)propylamino)-2,6-bis(3-(dimethylamino)propylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.1) and $N,N'$-Bis(3-(dimethylamino)propylamino)-2-(3-(dimethylamino)propylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.2): Compound 5.0.2 (100 mg, 0.234 mmol) and $N,N$-dimethyl-1,3-propanediamine (0.5 ml) were treated according to general procedure E. 5.1 and 5.2 were obtained as a blue and an orange solid respectively. Yield 5.1 (23.1 mg, 0.036 mmol, 15.5%), 5.2 (12.6 mg, 0.024 mmol, 10.0%). 5.1: $^1$H NMR (CDCl$_3$) $\delta$: 1.90 (5q, 4H, $J=7.4$ Hz), 1.94 (5q, 4H, $J=7.0$ Hz), 2.26 (s, 12H), 2.27 (s, 12H), 2.44 (m, 8H), 3.57 (m, 4H), 4.22 (m, 4H), 8.16 (s, 2H), 9.41 (t, 2H, $J=5.1$ Hz); $^{13}$C NMR (CDCl$_3$) $\delta$: 26.10 (2xCH$_2$), 27.51 (2xCH$_2$), 38.71 (2xCH$_2$), 41.25 (2xCH$_2$), 45.41 (4xCH$_3$), 45.52 (4xCH$_3$), 56.99 (2xCH$_2$), 57.32 (2xCH$_2$), 101.93 (2xC), 118.37 (2xCH), 121.17 (2xC), 125.79 (2xC), 149.19 (2xC), 163.05 (2xC=O), 166.12 (2xC=O); HRMS (ES+) calcd. C$_{34}$H$_{52}$N$_8$O$_4$ [M+H]$^+$ 637.4190. Found: 637.4199. 5.2: $^1$H NMR (CDCl$_3$) $\delta$: 1.90 (5q, 2H, $J=7.2$ Hz), 1.91 (5q, 2H, $J=7.5$ Hz), 1.96 (5q, 2H, $J=6.9$ Hz), 2.24 (s, 6H), 2.26 (s, 6H), 2.27 (s, 6H), 2.41-2.47 (m, 6H), 3.67 (m, 2H), 4.23 (m, 4H), 8.27 (s, 1H), 8.32 (d, 1H, $J=7.8$ Hz), 8.63 (d, 1H, $J=7.8$ Hz), 10.21 (t, 1H, $J=5.5$ Hz); $^{13}$C NMR (CDCl$_3$) $\delta$: 26.00 (CH$_2$), 26.08 (CH$_2$), 27.48 (CH$_2$), 38.68 (CH$_2$), 39.25 (CH$_2$), 41.32 (CH$_2$), 45.36 (2xCH$_3$), 45.41 (2xCH$_3$), 45.48 (2xCH$_3$), 56.65 (CH$_2$), 57.22 (CH$_2$), 57.31 (CH$_2$), 99.88 (C), 119.42 (C), 119.97 (CH), 123.56 (C), 124.36 (CH), 126.18 (C), 127.93 (C), 129.57 (C), 131.22 (CH), 152.44 (C), 162.99 (C=O), 163.05 (C=O), 163.39 (C=O), 166.12 (C=O); HRMS (ES+) calcd. C$_{29}$H$_{40}$N$_6$O$_4$ [M+H]$^+$ 537.3189. Found: 537.3217.
$\text{N,N'}$-Bis(2-(dimethylamino)ethylamino)-2,6-bis(2-(dimethylamino)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.3) and $\text{N,N'}$-Bis(3-(dimethylamino)ethylamino)-2-(3-(dimethylamino)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.4): Compound 5.0.2 (100 mg, 0.234 mmol) and $\text{N,N'}$-dimethyl-1,2-ethanediameine (0.6 ml) were treated according to general procedure E. 5.3 and 5.4 were obtained as a blue and an orange solid respectively. Yield 5.3 (12.4 mg, 0.021 mmol, 9.1%), 5.4 (11.8 mg, 0.024 mmol, 10.2%). 5.3: $^1$H NMR (CDCl$_3$) δ: 2.36 (s, 12H), 2.37 (s, 12H), 2.63 (t, 4H, $J=7.1$ Hz), 2.71 (t, 4H, $J=6.3$ Hz), 3.56 (m, 4H), 4.31 (t, 4H, $J=1.1$ Hz), 8.06 (s, 2H), 9.40 (t, 2H, $J=4.8$ Hz); $^{13}$C NMR (CDCl$_3$) δ: 38.14 (2xCH$_2$), 41.05 (2xCH$_2$), 45.59 (4xCH$_3$), 45.79 (4xCH$_3$), 56.98 (2xCH$_2$), 58.20 (2xCH$_2$), 101.93 (2xCH), 118.29 (2xCH), 121.12 (2xCH), 125.62 (2xCH), 148.91 (2xCH), 163.08 (2xCH=O), 165.84 (2xCH=O); HRMS (ES+) calcd. C$_{30}$H$_{44}$N$_8$O$_4$ [M+H]$^+$ 581.3564. Found: 581.3558. 5.4: $^1$H NMR (CDCl$_3$) δ: 2.35 (s, 6H), 2.37 (s, 12H), 2.64-2.68 (m, 4H), 2.73 (t, 2H, $J=6.3$ Hz), 3.65 (m, 2H), 4.31 (t, 2H, $J=6.8$ Hz), 4.35 (t, 2H, $J=7.1$ Hz), 8.20 (s, 1H), 8.32 (d, 1H, $J=7.8$ Hz), 8.63 (d, 1H, $J=7.8$ Hz), 10.20 (t, 1H, $J=4.6$ Hz); $^{13}$C NMR (CDCl$_3$) δ: 38.02 (CH$_2$), 38.56 (CH$_2$), 41.24 (CH$_2$), 45.54 (2xCH$_3$), 45.74 (2xCH$_3$), 45.75 (2xCH$_3$), 56.91 (CH$_2$), 56.96 (CH$_2$), 58.04 (CH$_2$), 100.13 (C), 120.08 (C), 120.08 (CH), 123.64 (C), 124.50 (CH), 126.14 (C), 127.87 (C), 129.61 (C), 131.29 (CH), 152.18 (C), 163.12 (C=O), 163.13 (C=O), 163.44 (C=O), 165.95 (C=O); HRMS (ES+) calcd. C$_{26}$H$_{34}$N$_6$O$_4$ [M+H]$^+$ 495.2720. Found: 495.2705.
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**N,N′-Bis(3-(diethylamino)propylamino)-2,6-bis(3-(diethylamino)propylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.5) and N,N′-Bis(3-(diethylamino)propylamino)-2-(3-(diethylamino)propylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.6):** Compound 5.0.2 (100 mg, 0.234 mmol) and N,N-diethyl-1,3-ethanediamine (0.6 ml) were treated according to general procedure E. 5.5 and 5.6 were obtained as a blue and an orange solid respectively. Yield 5.5 (29.3 mg, 0.039 mmol, 16.7%), 5.6 (20.0 mg, 0.032 mmol, 13.8%).

5.5: \( ^1H \) NMR (CDCl\(_3\)) δ: 1.03 (t, 12H, \( J=1.4 \) Hz), 1.04 (t, 12H, \( J=1.5 \) Hz), 1.85-1.97 (m, 8H), 2.53-2.63 (m, 24H), 3.56 (m, 4H), 4.20 (t, 4H, \( J=1.6 \) Hz), 8.16 (s, 2H), 9.42 (t, 2H, \( J=5.3 \) Hz); \(^{13}C\) NMR (CDCl\(_3\)) δ: 11.69 (4xCH\(_3\)), 25.28 (2xCH\(_2\)), 27.14 (2xCH\(_2\)), 38.98 (2xCH\(_2\)), 41.45 (2xCH\(_2\)), 46.72 (4xCH\(_2\)), 46.89 (4xCH\(_2\)), 50.26 (2xCH\(_2\)), 50.44 (2xCH\(_2\)), 101.93 (2xC), 118.38 (2xCH), 125.35 (2xC), 148.26 (2xC), 149.20 (2xC), 163.10 (2xC=O), 166.11 (2xC=O); HRMS (ES+) calcd. C\(_{42}\)H\(_{68}\)N\(_8\)O\(_4\) [M+H]\(^+\) 749.5442. Found: 749.5436.

5.6: \( ^1H \) NMR (CDCl\(_3\)) δ: 1.00-1.06 (m, 18H), 1.86-1.98 (m, 6H), 2.53-2.64 (m, 18H), 3.65 (m, 2H), 4.20 (m, 4H), 8.23 (s, 1H), 8.31 (d, 1H, \( J=7.8 \) Hz), 8.63 (d, 1H, \( J=7.8 \) Hz), 10.19 (t, 1H, \( J=5.2 \) Hz); \(^{13}C\) NMR (CDCl\(_3\)) δ: 11.57 (2xCH\(_3\)), 11.62 (2xCH\(_3\)), 25.18 (CH\(_2\)), 25.27 (CH\(_2\)), 27.24 (CH\(_2\)), 38.89 (CH\(_2\)), 39.48 (CH\(_2\)), 41.54 (CH\(_2\)), 46.64 (2xCH\(_2\)), 46.68 (2xCH\(_2\)), 46.84 (2xCH\(_2\)), 50.03 (CH\(_2\)), 50.38 (CH\(_2\)), 50.39 (CH\(_2\)), 99.82 (C), 119.40 (C), 119.93 (CH), 123.55 (C), 124.33 (CH), 126.17 (C), 127.91 (C), 129.55 (C), 131.20 (CH), 152.37 (C), 162.98 (C=O), 163.03 (C=O), 163.38 (C=O), 166.06 (C=O); HRMS (ES+) calcd. C\(_{35}\)H\(_{52}\)N\(_8\)O\(_4\) [M+H]\(^+\) 621.4128. Found: 621.4108.
$N,N'$-Bis(3-(diethylamino)ethylamino)-2,6-bis(3-(diethylamino)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.7) and $N,N'$-Bis(3-(diethylamino)ethylamino)-2-(3-(diethylamino)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.8): Compound 5.0.2 (100 mg, 0.234 mmol) and $N,N$-diethyl-1,2-ethanediameine (0.6 ml) were treated according to general procedure E. 5.7 and 5.8 were obtained as a blue and an orange solid respectively. Yield 5.7 (22.2 mg, 0.032 mmol, 13.7%), 5.8 (26.6 mg, 0.046 mmol, 19.6%).

5.7: $^1$H NMR (CDCl$_3$) δ: 1.09 (t, 24H, $J$=1.4 Hz), 2.61-2.69 (m, 16H), 2.75 (m, 4H), 2.83 (t, 4H, $J$=6.3 Hz), 3.51 (m, 4H), 4.26 (m, 4H), 8.05 (s, 2H), 9.47 (t, 2H, $J$=4.8 Hz); $^{13}$C NMR (CDCl$_3$) δ: 11.99 (4xCH$_3$), 12.31 (4xCH$_3$), 37.79 (2xCH$_2$), 41.36 (2xCH$_2$), 47.13 (4xCH$_2$), 47.71 (4xCH$_2$), 49.72 (2xCH$_2$), 51.66 (2xCH$_2$), 101.85 (2xC), 118.33 (2xCH), 121.04 (2xC), 125.56 (2xC), 148.87 (2xC), 163.03 (2xC=O), 165.65 (2xC=O); HRMS (ES+) calcd. C$_{38}$H$_{60}$N$_8$O$_4$ [M+H]$^+$ 693.4816. Found: 693.4813.

5.8: $^1$H NMR (CDCl$_3$) δ: 1.04-1.11 (m, 18H), 2.61-2.69 (m, 12H), 2.76 (m, 4H), 2.84 (t, 2H, $J$=6.2 Hz), 3.60 (m, 2H), 4.23-4.31 (m, 4H), 8.16 (s, 1H), 8.27 (d, 1H, $J$=7.8 Hz), 8.58 (d, 1H, $J$=7.8 Hz), 10.26 (t, 1H, $J$=4.8 Hz); $^{13}$C NMR (CDCl$_3$) δ: 11.96 (2xCH$_3$), 12.23 (2xCH$_3$), 12.25 (2xCH$_3$), 37.71 (CH$_2$), 38.52 (CH$_2$), 41.56 (CH$_2$), 47.10 (2xCH$_2$), 447.58 (2xCH$_2$), 47.66 (2xCH$_2$), 49.67 (CH$_2$), 49.85 (CH$_2$), 51.63 (CH$_2$), 99.88 (C), 119.33 (C), 120.26 (CH), 123.52 (C), 124.21 (CH), 126.01 (C), 127.63 (C), 129.52 (C), 131.04 (CH), 152.09 (C), 162.98 (C=O), 163.01 (C=O), 163.34 (C=O), 165.66 (C=O); HRMS (ES+) calcd. C$_{32}$H$_{46}$N$_6$O$_4$ [M+H]$^+$ 579.3659. Found: 579.3616.
AyV'-Bis(3-(pyrrolidin-1-yl)propylamino)-2,6-bis(3-(pyrrolidin-1-yl)propylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.9) and AyV'-Bis(3-(pyrrolidin-1-yl)propylamino)-2-(3-(pyrrolidin-1-yl)propylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.10):

Compound 5.0.2 (50 mg, 0.117 mmol) and 1-(3-aminopropyl)pyrrolidine (0.2 ml) were treated according to general procedure E. 5.9 and 5.10 were obtained as a blue and an orange solid respectively. Yield 5.9 (13.2 mg, 0.018 mmol, 15.2%), 5.10 (14.6 mg, 0.024 mmol, 20.3%). 5.9: \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 1.74 (m, 8H), 1.81 (m, 8H), 1.92-2.03 (m, 8H), 2.53 (m, 16H), 2.60 (t, 4H, J=7.4 Hz), 2.63 (t, 4H, J=1.4 Hz), 3.59 (m, 4H), 4.26 (t, 4H, J=7.4 Hz), 8.19 (s, 2H), 9.44 (t, 2H, J=5.4 Hz); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\): 23.42 (4xCH\(_2\)), 23.49 (4xCH\(_2\)), 26.71 (2xCH\(_2\)), 28.39 (2xCH\(_2\)), 38.54 (2xCH\(_2\)), 41.43 (2xCH\(_2\)), 53.58 (2xCH\(_2\)), 53.67 (2xCH\(_2\)), 53.71 (4xCH\(_2\)), 54.06 (4xCH\(_2\)), 101.88 (2xC), 118.46 (2xCH), 121.29 (2xC), 125.65 (2xC), 149.17 (2xC), 163.00 (2xC=O), 166.17 (2xC=O); HRMS (ES+) calcd. C\(_{42}\)H\(_{60}\)N\(_8\)O\(_4\) [M+H]^+ 741.4816. Found: 741.4779. 5.10: \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 1.70 (m, 4H), 1.75 (m, 4H), 1.82 (m, 4H), 1.93-2.04 (m, 6H), 2.55 (m, 12H), 2.59-2.65 (m, 6H), 3.69 (m, 2H), 4.27 (m, 4H), 8.29 (s, 1H), 8.33 (d, 1H, J=7.8 Hz), 8.64 (d, 1H, J=7.8 Hz), 10.22 (t, 1H, J=5.4 Hz); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\): 23.46 (2xCH\(_2\)), 23.48 (2xCH\(_2\)), 23.56 (2xCH\(_2\)), 27.17 (CH\(_2\)), 27.31 (CH\(_2\)), 28.78 (CH\(_2\)), 38.84 (CH\(_2\)), 39.40 (CH\(_2\)), 41.63 (CH\(_2\)), 53.52 (CH\(_2\)), 53.92 (CH\(_2\)), 54.00 (CH\(_2\)), 54.02 (2xCH\(_2\)), 54.07 (2xCH\(_2\)), 54.25 (2xCH\(_2\)), 99.86 (C), 119.43 (C), 120.00 (CH), 123.56 (C), 124.31 (CH), 126.23 (C), 127.94 (C), 129.56 (C), 131.20 (CH), 152.47 (C), 163.04 (C=O), 163.12 (C=O), 163.45 (C=O), 166.13 (C=O); HRMS (ES+) calcd. C\(_{35}\)H\(_{46}\)N\(_6\)O\(_4\) [M+H]^+ 615.3659. Found: 615.3663.
**N,N’-Bis(2-(pyrrolidin-1-yl)ethylamino)-2,6-bis(2-(pyrrolidin-1-yl)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.11)** and **N,N’-Bis(2-(pyrrolidin-1-yl)ethylamino)-2-(2-(pyrrolidin-1-yl)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.12):** Compound 5.0.2 (100 mg, 0.234 mmol) and 1-(2-aminoethyl)pyrrolidine (0.6 ml) were treated according to general procedure E. 5.11 and 5.12 were obtained as a blue and an orange solid respectively. Yield 5.11 (34.1 mg, 0.050 mmol, 21.3%), 5.12 (37.2 mg, 0.065 mmol, 27.7%).

**5.11:** 
\[\text{H NMR (CDCl}_3\text{)} \delta: 1.76-1.84 \text{ (m, 16H), 2.62-2.66 \text{ (m, 16H), 2.77 \text{ (t, 4H, J=7.3 Hz), 2.87 \text{ (t, 4H, J=6.6 Hz), 3.59 \text{ (m, 4H), 4.32 \text{ (t, 4H, J=7.4 Hz), 8.05 \text{ (s, 2H), 9.44 \text{ (t, 2H, J=5.1 Hz); \text{C NMR (CDCl}_3\text{)} \delta: 23.59 (4xCH}_2, 23.63 (4xCH}_2, 39.12 (2xCH}_2, 42.28 (2xCH}_2, 53.61 (2xCH}_2, 54.23 (4xCH}_2, 54.33 (4xCH}_2, 54.90 (2xCH}_2, 101.88 (2xC), 118.22 (2xCH), 121.05 (2xC), 125.58 (2xC), 148.91 (2xC), 162.92 (2xC=O), 165.77 (2xC=O); HRMS (ES+) calcd. C_{38}H_{52}N_8O_4 [M+H]^+ 685.4190. Found: 685.4207.} \]

**5.12:** 
\[\text{H NMR (CDCl}_3\text{)} \delta: 1.78-1.84 \text{ (m, 12H), 2.65-2.68 \text{ (m, 12H), 2.81 \text{ (m, 4H), 2.90 \text{ (t, 2H, J=6.5 Hz), 3.67 \text{ (m, 2H), 4.29-4.36 \text{ (m, 4H), 8.13 \text{ (s, 1H), 8.24 \text{ (d, 1H, J=7.8 Hz), 8.54 \text{ (d, 1H, J=5.0 Hz), \text{C NMR (CDCl}_3\text{)} \delta: 23.59 (2xCH}_2, 23.66 (2xCH}_2, 38.94 (CH}_2, 39.52 (CH}_2, 42.41 (CH}_2, 53.52 (CH}_2, 54.19 (2xCH}_2, 54.30 (2xCH}_2, 54.34 (2xCH}_2, 54.70 (CH}_2, 99.90 (C), 119.31 (C), 119.95 (CH), 123.44 (C), 124.29 (CH), 126.00 (C), 127.69 (C), 129.42 (C), 131.07 (CH), 152.10 (C), 162.86 (C=O), 162.90 (C=O), 163.22 (C=O), 165.75 (C=O); HRMS (ES+) calcd. C_{32}H_{40}N_6O_4 [M+H]^+ 573.3189. Found: 573.3185.} \]
$N,N'$-Bis(2-(piperidin-1-yl)ethylamino)-2,6-bis(2-(piperidin-1-yl)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.17) and $N,N'$-Bis(2-(piperidin-1-yl)ethylamino)-2-(2-(piperidin-1-yl)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.18): Compound 5.0.2 (100 mg, 0.234 mmol) and 1-(2-aminoethyl)piperidine (0.6 ml) were treated according to general procedure E. 5.17 and 5.18 were obtained as a blue and an orange solid respectively. Yield 5.17 (19.3 mg, 0.026 mmol, 11.1%), 5.18 (20.9 mg, 0.034 mmol, 14.5%). 5.17: $^1$H NMR (CDCl$_3$) $\delta$: 1.43-1.48 (m, 8H), 1.56-1.65 (m, 16H), 2.51 (m, 8H), 2.56 (m, 8H), 2.63 (m, 4H), 2.72 (t, 4H, $J$=6.5 Hz), 3.57 (m, 4H), 4.33 (m, 4H), 8.07 (s, 2H), 9.50 (t, 2H, $J$=5.3 Hz); $^{13}$C NMR (CDCl$_3$) $\delta$: 24.39 (2xCH$_2$), 24.45 (2xCH$_2$), 26.06 (4xCH$_2$, 4xCH$_2$), 37.48 (2xCH$_2$), 40.48 (2xCH$_2$), 54.56 (4xCH$_2$), 54.74 (4xCH$_2$), 56.36 (2xCH$_2$), 57.36 (2xCH$_2$), 101.97 (2xC), 118.38 (2xCH), 121.10 (2xC), 125.61 (2xC), 148.89 (2xC), 163.01 (2xC=O), 165.71 (2xC=O); HRMS (ES$^+$) calcd. C$_{42}$H$_{60}$N$_8$O$_4$ [M+H]$^+$ 741.4816. Found: 741.4855. 5.18: $^1$H NMR (CDCl$_3$) $\delta$: 1.42-1.50 (m, 6H), 1.53-1.59 (m, 8H), 1.61-1.67 (m, 4H), 2.52-2.56 (m, 12H), 2.64 (m, 4H), 2.74 (t, 2H, $J$=6.4 Hz), 3.64 (m, 2H), 4.30 (t, 2H, $J$=7.2 Hz), 4.35 (t, 2H, $J$=7.3 Hz), 8.16 (s, 1H), 8.27 (d, 1H, $J$=7.8 Hz), 8.58 (d, 1H, $J$=7.8 Hz), 10.28 (t, 1H, $J$=4.9 Hz); $^{13}$C NMR (CDCl$_3$) $\delta$: 24.33 (CH$_2$), 24.37 (CH$_2$), 24.39 (CH$_2$), 26.03 (2xCH$_2$, 2xCH$_2$), 26.05 (2xCH$_2$), 37.42 (CH$_2$), 38.02 (CH$_2$), 40.64 (CH$_2$), 54.56 (2xCH$_2$), 54.73 (2xCH$_2$), 54.76 (2xCH$_2$), 56.23 (CH$_2$), 56.32 (CH$_2$), 57.17 (CH$_2$), 99.98 (C), 119.36 (C), 120.20 (CH), 123.52 (C), 124.24 (CH), 126.03 (C), 127.66 (C), 129.52 (C), 131.06 (CH), 152.08 (C), 162.93 (C=O), 162.98 (C=O), 163.30 (C=O), 165.70 (C=O); HRMS (ES$^+$) calcd. C$_{35}$H$_{46}$N$_8$O$_4$ [M+H]$^+$ 615.3659. Found: 615.3669.
Chapter 9 - Experimental procedures

\[ \text{N,N'-Bis(3-(morpholine-4-yl)propylamino)-2,6-bis(3-(morpholine-4-yl)propylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.13) and N,N'-Bis(3-(morpholine-4-yl)propylamino)-2-(3-(morpholme-4-yl)propylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.14):} \]

Compound 5.0.2 (25 mg, 0.058 mmol) was suspended in 3-morpholine-1-propylamine (2 ml) in a microwave reaction vessel. The tube was flushed with nitrogen, sealed and treated at 150°C for 10 min in the microwave. The mixture was then diluted with water (50 ml) and extracted with chloroform (5 x 10 ml). The organics were treated in the usual manner to afford a dark brown solid. The crude mixture was purified by HPLC (as described in the general methods) to obtain 5.13 and 5.14 as a blue and an orange solid respectively. Yield 5.13 (4.97 mg, 0.006 mmol, 10.5%), 5.14 (3.19 mg, 0.005 mmol, 8.2%).

5.13: \[ ^1H \text{ NMR (CDCl}_3 \] δ: 1.89-1.99 (m, 8H), 2.44-2.53 (m, 24H), 3.59 (m, 4H), 3.62 (m, 8H), 3.75 (m, 8H), 4.25 (t, 4H, J=7.3 Hz), 8.16 (s, 2H), 9.43 (t, 2H, J=5.4 Hz); \[ ^{13}C \text{ NMR (CDCl}_3 \] δ: 24.68 (2xCH), 26.31 (2xCH), 38.82 (2xCH), 41.32 (2xCH), 53.57 (2xCH), 53.84 (2xCH), 56.26 (4xCH), 56.53 (4xCH), 66.91 (4xCH), 66.96 (4xCH), 101.98 (2xC), 118.32 (2xCH), 121.19 (2xC), 125.84 (2xC), 149.18 (2xC), 163.10 (2xC=O), 166.17 (2xC=O); HRMS (ES+) calcd. C_{42}H_{60}N_{8}O_{7} [M+H]^+ 805.4607. Found: 805.4607. 5.14: \[ ^1H \text{ NMR (CDCl}_3 \] δ: 1.92-1.96 (m, 6H), 2.43-2.54 (m, 18H), 3.56 (t, 4H, J=4.5 Hz), 3.62 (t, 4H, J=4.5 Hz), 3.70 (m, 2H), 3.76 (m, 4H), 4.27 (m, 4H), 8.27 (s, 1H), 8.34 (d, 1H, J=7.8 Hz), 8.65 (d, 1H, J=7.8 Hz), 10.21 (t, 1H, J=5.7 Hz); \[ ^{13}C \text{ NMR (CDCl}_3 \] δ: 24.42 (CH), 24.44 (CH), 26.31 (CH), 38.79 (CH), 39.35 (CH), 41.34 (CH), 53.65 (2xCH), 53.83 (2xCH), 55.94 (CH), 56.45 (CH), 56.53 (CH), 66.87 (2xCH), 66.92 (2xCH), 66.95 (2xCH), 98.84 (C), 116.27 (C), 119.82 (CH), 123.65 (C), 124.45 (CH), 126.28 (C), 127.20 (C), 129.61 (C), 132.75 (CH), 151.51 (C), 163.09 (C=O), 163.42 (C=O), 166.24 (C=O), 166.76 (C=O); HRMS (ES+) calcd. C_{35}H_{46}N_{6}O_{7} [M+H]^+ 663.3501. Found: 663.3524.
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$N,N'$-Bis(2-(morpholine-4-yl)ethylamino)-2,6-bis(2-(morpholine-4-yl)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.15) and $N,N'$-Bis(2-(morpholine-4-yl)ethylamino)-2-(2-(morpholine-4-yl)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.16): Compound 5.0.2 (25 mg, 0.058 mmol) was suspended in 2-morpholine-1-ethylamine (2 ml) in a microwave reaction vessel. The tube was flushed with nitrogen, sealed and treated at 150°C for 10 min in the microwave. The mixture was then diluted down with water (50 ml) and extracted with chloroform (5 x 10 ml). The organics were treated in the usual manner to afford a dark brown solid. The crude mixture was purified by HPLC (as described in the general methods) to obtain 5.15 and 5.16 as a blue and an orange solid respectively. Yield 5.15 (2.74 mg, 0.004 mmol, 6.3%), 5.16 (2.48 mg, 0.004 mmol, 6.8%). 5.15: $^1$H NMR (CDCl$_3$) $\delta$: 2.57-2.61 (m, 16H), 2.70 (t, 4H, $J=6.9$ Hz), 2.78 (t, 4H, $J=6.2$ Hz), 3.61 (m, 4H), 3.69 (m, 8H), 3.77 (m, 8H), 4.35 (t, 4H, $J=6.9$ Hz), 8.14 (s, 2H), 9.58 (t, 2H, $J=4.9$ Hz); $^{13}$C NMR (CDCl$_3$) $\delta$: 37.14 (2xCH$_3$), 39.99 (4xCH$_2$), 53.49 (4xCH$_2$), 53.84 (4xCH$_2$), 56.15 (2xCH$_2$), 56.93 (2xCH$_2$), 67.04 (4xCH$_2$, 4xCH$_2$), 102.17 (2xC), 118.59 (2xC), 121.33 (2xC), 130.00 (2xC), 148.95 (2xC), 163.09 (2xC=O), 165.88 (2xC=O); HRMS (ES+) calcd. C$_{38}$H$_{52}$N$_8$O$_{8}$ [M+2H]$^{2+}$ 375.2027. Found: 375.2008. 5.16: $^1$H NMR (CDCl$_3$) $\delta$: 2.60 (m, 12H), 2.70 (m, 4H), 2.81 (t, 2H, $J=6.2$ Hz), 3.65-3.70 (m, 10H), 3.78 (m, 4H), 4.33 (t, 2H, $J=6.7$ Hz), 4.38 (t, 2H, $J=6.9$ Hz), 8.23 (s, 1H), 8.35 (d, 1H, $J=7.8$ Hz), 8.66 (d, 1H, $J=7.8$ Hz), 10.35 (t, 1H, $J=4.9$ Hz); $^{13}$C NMR (CDCl$_3$) $\delta$: 37.11 (CH$_3$), 37.64 (CH$_3$), 40.13 (CH$_2$), 53.48 (2xCH$_2$), 53.84 (2xCH$_2$, 2xCH$_2$), 56.05 (CH$_2$), 56.14 (CH$_2$), 56.82 (CH$_2$), 67.01 (2xCH$_2$), 67.03 (2xCH$_2$), 67.05 (2xCH$_2$), 104.37 (C), 114.032 (C), 120.20 (CH), 123.69 (C), 124.59 (CH), 126.12 (C), 127.25 (C), 129.12 (C), 131.31 (CH), 149.11 (C), 163.05 (C=O), 163.39 (C=O), 164.52 (C=O), 165.91 (C=O); HRMS (ES+) calcd. C$_{32}$H$_{40}$N$_6$O$_7$ [M+H]$^+$ 621.3031. Found: 621.3049.
$N,N'$-Bis(5-hydroxypentanamino)-2,6-bis(5-hydroxypentanamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.19) and $N,N'$-Bis(5-hydroxypentanamino)-2-(5-hydroxypentanamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.20):  Compound 5.0.2 (50 mg, 0.117 mmol) was suspended in 5-amino-1-pentanol (0.5 ml) in a microwave reaction vessel. The tube was flushed with nitrogen, sealed and treated at 150°C for 10 min in the microwave. The mixture was then diluted with water (100 ml) and left at RT for 24 h. The sticky precipitate was taken by filtration, dissolved in DMF (5 ml) and evaporated in vacuo. The crude mixture was purified by HPLC (as described in the general methods) to obtain 5.19 and 5.20 as a blue and an orange solid respectively. Yield 5.19 (3.8 mg, 0.006 mmol, 5.1%), 5.20 (3.25 mg, 0.006 mmol 5.1%). 5.19 $^1$H NMR (DMSO-$d_6$) δ: 1.36 (m, 4H), 1.45-1.62 (m, 16H), 1.73 (5q, 4H, J=6.9 Hz), 3.36 (m, 4H), 3.41 (4q, 4H, J=5.2 Hz), 3.47 (4q, 4H, J=5.3 Hz), 3.90 (m, 4H), 4.36 (t, 2H, J=5.1 Hz), 4.42 (t, 2H, J=5.1 Hz), 7.63 (s, 2H), 9.07 (t, 2H, J=5.0 Hz); $^{13}$C NMR (DMSO-$d_6$) δ: 23.03 (2xCH$_2$), 23.09 (2xCH$_2$), 27.16 (2xCH$_2$), 28.53 (2xCH$_2$), 32.10 (2xCH$_2$), 32.11 (2xCH$_2$), 40.24 (2xCH$_2$), 42.24 (2xCH$_2$), 60.40 (2xCH$_2$), 60.50 (2xCH$_2$), 100.27 (2xC), 119.71 (2xCH), 147.83 (2xC), 155.42 (2xC), 155.44 (2xC), 161.45 (2xC=O), 164.91 (2xC=O); HRMS (ES+) calcd. C$_{34}$H$_{48}$N$_4$O$_8$ [M+H]$^+$ 641.3550. Found: 641.3563. 5.20: $^1$H NMR (DMSO-$d_6$) δ: 1.37 (m, 4H), 1.47 (m, 8H), 1.63 (m, 4H), 1.73 (5q, 2H, J=6.9 Hz), 3.38-3.42 (m, 4H), 3.45 (4q, 2H, J=5.3 Hz), 3.55 (4q, 2H, J=6.5 Hz), 3.98 (m, 4H), 4.35 (t, 1H, J=5.1 Hz), 4.35 (t, 1H, J=5.1 Hz), 4.41 (t, 1H, J=5.1 Hz), 7.93 (s, 1H), 8.09 (d, 1H, J=7.8 Hz), 8.38 (d, 1H, J=7.8 Hz), 9.94 (t, 1H, J=5.4 Hz); $^{13}$C NMR (DMSO-$d_6$) δ: 23.04 (CH$_2$), 23.12 (CH$_2$), 27.26 (CH$_2$), 27.29 (CH$_2$), 28.75 (CH$_2$), 32.10 (CH$_2$), 32.18 (CH$_2$), 32.20 (CH$_2$), 41.77 (CH$_2$), 41.84 (CH$_2$), 42.46 (CH$_2$), 60.46 (CH$_2$), 60.48 (CH$_2$), 60.55 (CH$_2$), 98.38 (C), 119.14 (CH), 121.26 (C), 122.41 (C), 122.82 (C), 212
123.53 (CH), 128.57 (C), 130.43 (C), 130.48 (CH), 141.29 (C), 162.09 (C=O), 162.63 (C=O), 165.19 (C=O), 165.43 (C=O); HRMS (ES+) calcd. C₂₉H₃₇N₃O₇ [M+H]+ 540.2710. Found: 540.2715.

\[ \text{N,N'-Bis(3-(dimethylamino)propylamino)-2,6-dichloro-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.0.21.1)} \]

Compound 5.0.1 (50 mg, 0.150 mmol) was suspended with sonication in glacial acetic acid (1.5 ml) in a microwave reaction vessel. \( \text{N,N-Dimethyl-1,3-propanediamine} \) (180 µL, 1.5 mmol, 10 eq) was added dropwise to the stirring mixture. The reaction tube was sealed and treated for 10 min at 120°C in the microwave. The solution was then basified with 2M sodium carbonate in water and extracted with chloroform (3 x 5 ml). The organics were treated in the usual manner to afford a red solid. Yield 5.0.21.1 (61 mg, 0.121 mmol, 80.5%): \(^1\)H NMR (CDCl₃) δ: 1.91 (s, 4H, J=7.4 Hz), 2.22 (s, 12H), 2.44 (t, 4H, J=7.0 Hz), 4.25 (t, 4H, J=7.5 Hz), 8.76 (s, 2H); \(^13\)C NMR (CDCl₃) δ: 25.54 (2xCH₂), 39.74 (2xCH₂), 45.18 (4xCH₃), 57.07 (2xCH₂), 122.32 (2xC), 125.94 (2xC), 127.08 (2xC), 135.82 (2xCH), 140.02 (2xC), 160.50 (2xC=O), 160.89 (2xC=O); HRMS (ES+) calcd. C₂₄H₂₆Cl₂N₄O₄ [M+H]+ 506.4016. Found: 506.4006.

\[ \text{N,N'-Bis(3-(dimethylamino)propylamino)-2,6-bis(2-(piperidin-1-yl)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.21)} \]

Compound 5.0.21.1 (45 mg, 0.089 mmol) was suspended in 1-(2-aminoethyl)piperidine (0.5 ml) in a microwave reaction vessel. The tube was flushed with nitrogen, sealed and treated at 150°C for 10 min in the microwave. The amine was then evaporated off in vacuo. The crude mixture was purified by HPLC (as described in the general methods) to obtain 5.21 as a blue solid. Yield 5.21 (6.62 mg, 0.0096 mmol, 10.8%): \(^1\)H NMR (CDCl₃) δ: 1.47 (m, 4H), 1.64 (m, 8H), 1.90 (s, 4H, J=7.3 Hz), 2.26 (s, 12H), 2.43 (t, 4H, J=7.3 Hz), 2.51 (m, 8H), 2.73 (t, 4H, J=6.5 Hz),
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3.58 (m, 4H), 4.22 (t, 4H, J=7.5 Hz), 8.07 (s, 2H), 9.50 (t, 2H, J=4.9 Hz); $^{13}$C NMR (CDCl$_3$) δ: 24.44 (2xCH$_2$), 26.06 (4xCH$_2$, 2xCH$_3$), 38.66 (2xCH$_2$), 40.49 (2xCH$_2$), 45.34 (4xCH$_2$), 54.57 (4xCH$_3$), 57.29 (2xCH$_2$), 57.38 (2xCH$_2$), 101.97 (2xC), 118.38 (2xCH), 121.07 (2xC), 125.62 (2xC), 148.90 (2xC), 163.05 (2xC=O), 165.79 (2xC=O); HRMS (ES+) calcd. C$_{38}$H$_{56}$N$_8$O$_4$ [M+2H]$^+$ 345.2285. Found: 345.2293.

$N,N'$-Bis(3-(dimethylamino)propylamino)-2,6-bis(3-hydroxypropylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide (5.22): Compound 5.0.21.1 (45 mg, 0.089 mmol) was suspended in 3-amino-propanol (0.5 ml) in a microwave reaction vessel. The tube was flushed with nitrogen, sealed and treated at 150°C for 10 min in the microwave. The mixture was then diluted with water (25 ml), basified with 2M sodium carbonate and extracted with chloroform (5 x 5 ml). The organics were treated in the usual manner to afford a blue solid. The crude product was purified by HPLC (as described in the general methods) to obtain 5.22 as a blue solid. Yield 5.22 (4.13 mg, 0.0071 mmol, 7.9%): $^1$H NMR (MeOD) δ: 1.98-2.07 (m, 8H), 2.61 (s, 12H), 2.84 (m, 4H), 3.53 (t, 4H, J=6.9 Hz), 3.84 (t, 4H, J=6.0 Hz), 4.09 (t, 4H, J=7.1 Hz), 7.68 (s, 2H); $^{13}$C NMR (MeOD) δ: 25.98 (2xCH$_2$), 33.15 (2xCH$_2$), 39.15 (2xCH$_2$), 41.29 (2xCH$_2$), 44.71 (4xCH$_3$), 57.66 (2xCH$_2$), 60.63 (2xCH$_2$), 102.06 (2xC), 118.41 (2xCH), 121.52 (2xC), 126.06 (2xC), 146.49 (2xC), 163.80 (2xC=O), 166.72 (2xC=O); HRMS (ES+) calcd. C$_{30}$H$_{42}$N$_6$O$_6$ [M+H]$^+$ 583.3239. Found: 583.3260.
**N,N'-Bis(3-(dimethylamino)propyl amino)-1,4,5,8-naphthalene tetracarboxylic acid diimide (5.23):** Naphthalene dianhydride (100 mg, 0.373 mmol) was suspended in N,N-dimethyl-1,3-propanediamine (2 ml) in a microwave reaction vessel. The tube was flushed with nitrogen, sealed and treated at 120°C for 10 min in the microwave. Water (20 ml) was added to the mixture. The crystalline solid was taken by filtration and washed with water (2 x 10 ml), ethanol (2 x 10 ml) and ether (2 x 10 ml). The solid was then dissolved in chloroform and treated in the usual manner to afford a yellow solid. Yield 5.23 (95 mg, 0.218 mmol, 58.3\%): $^1$H NMR (CDCl$_3$) $\delta$: 1.92 (m, 4H), 2.23 (s, 12H), 2.43 (m, 4H), 4.27 (m, 4H), 8.75 (s, 4H); $^{13}$C NMR (CDCl$_3$) $\delta$: 26.00 (2xCH$_2$), 39.38 (2xCH$_2$), 45.38 (4xCH$_3$), 57.24 (2xCH$_2$), 126.66 (4xC), 126.70 (2xC), 130.87 (4xC), 162.83 (4xC=O); HRMS (ES+) calcd. C$_{24}$H$_{28}$N$_4$O$_4$ [M+H]$^+$ 437.2183. Found: 437.2197.

**N,N'-Bis(3-(dimethylamino)ethyl amino)-1,4,5,8-naphthalene tetracarboxylic acid diimide (5.24):** Naphthalene dianhydride (100 mg, 0.373 mmol) was suspended in N,N-dimethyl-1,2-ethanediamine (2 ml) in a microwave reaction vessel. The tube was flushed with nitrogen, sealed and treated at 120°C for 10 min in the microwave. Water (20 ml) was added to the mixture. The crystalline solid was taken by filtration and washed with water (2 x 10 ml), ethanol (2 x 10 ml) and ether (2 x 10 ml). The solid was then dissolved in chloroform and treated in the usual manner to afford a yellow solid. Yield 5.24 (105 mg, 0.257 mmol, 68.9\%): $^1$H NMR (CDCl$_3$) $\delta$: 2.34 (s, 12H), 2.66 (t, 4H, $J$=6.8 Hz), 4.34 (t, 4H, $J$=6.8 Hz), 8.74 (s, 4H); $^{13}$C NMR (CDCl$_3$) $\delta$: 38.68 (2xCH$_2$), 45.78 (4xCH$_3$), 56.96 (2xCH$_2$), 126.63 (2xC), 126.76 (4xC), 130.95 (4xC), 162.88 (4xC=O); HRMS (ES+) calcd. C$_{22}$H$_{24}$N$_4$O$_4$ [M+H]$^+$ 409.1870. Found: 409.1861.
# 9.5 HPLC Analysis Data for the Naphthalene DHMIDE Final Compounds

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Table 9.2 HPLC analysis data for the naphthalene diimide final compounds 5.1-5.24.
BIBLIOGRAPHY
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