

THE IDENTIFICATION AND VALIDATION OF  
AUGER ELECTRON-EMITTING  
RADIOPHARMACEUTICALS TARGETING  
TELOMERASE FOR CANCER THERAPY



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## Abstract

### The identification and validation of Auger electron-emitting radiopharmaceuticals targeting telomerase for cancer therapy

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Telomerase is expressed in the majority (>85%) of tumours but not in differentiated normal tissue. This enzyme catalyses the elongation of telomeres – a process critical for continued cell proliferation. Telomerase is a potential novel target for molecularly-targeted radiotherapy (mRT), due to its nuclear localization and expression profile. The radiolabelling of telomerase inhibitors may accelerate and enhance the cytotoxicity of such molecules, as a result of irradiation of the DNA.

An oligonucleotide targeting telomerase RNA (hTR), shown to inhibit enzyme activity *in vitro*, was selected for study. Complementary and non-targeting control oligonucleotides were conjugated to a metal chelator (DTPA) to allow radiolabelling with indium-111. The radioiodination of MST-312, BIBR-1532 and flavonoid-derived small molecule inhibitors of telomerase was also pursued. The inhibitory activity of the candidate molecules was analysed using the telomeric repeat amplification protocol (TRAP). The internalization of inhibitors was assessed by gamma-counting following cell lysis. The clonogenic assay was employed to measure the effect of modified inhibitors on cell survival.

Small molecule telomerase inhibitors were modified for labelling with iodine-123, which led to a modest decrease in inhibitory potency, compared to the parent molecules. Radiolabelled small molecules exhibited poor stability and internalization into cancer cells, so were unsuitable for mRT. Modified oligonucleotides potently inhibited telomerase activity, whereas a non-targeting oligonucleotide exhibited no inhibitory activity. Indium-111 radiolabelled oligonucleotides decreased the clonogenic survival of telomerase-positive breast cancer cells but not telomerase-negative cells, in a sequence-specific manner. Accordingly, complementary radiolabelled oligonucleotides were found to induce the DNA damage marker  $\gamma$ H2AX. Oligonucleotides localized to nuclear Cajal bodies, the sites of telomerase assembly, in a proportion of cancer cells.

Telomerase inhibitors of different classes were radiolabelled with Auger electron-emitting radionuclides, and delivered to cells. Radiolabelled oligonucleotides targeting telomerase significantly reduced the clonogenicity of cancer cells *in vitro*. This study represents a novel approach for the mRT of telomerase-positive cancers.

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## List of abbreviations

2-5A – 2'-5' oligoadenylate	HPLC – high performance liquid chromatography
2'OMeRNA – 2'O-methyl modified RNA	HR – homologous recombination
ALDH+ – aldehyde dehydrogenase positive	hTERT – human telomerase reverse transcriptase
ALT – alternative lengthening of telomeres	hTR – human telomerase RNA component
APBs – ALT-associated promyelocytic leukaemia bodies	IC <sub>50</sub> – concentration required for 50 % of maximum inhibition
AUC – area under the curve	ID/g – injected dose per gram
Bp – base-pairs	IF – immunofluorescence
CBs – Cajal bodies	IGRT – image-guided radiotherapy
cDTPA – cyclic DTPA	IMRT – intensity-modulated radiotherapy
CEAs – carcinoembryonic antigens	IR – ionizing radiation
CST – CTC1/STN1/TEN1 complex	ITLC – instant thin-layer chromatography
CT – computed tomography	LET – linear energy transfer
DAPI – 4',6-diamidino-2-phenylindole	MeOH – methanol
DDR – DNA damage response	MIRD – medical internal radiation dose
DTPA – diethylenetriaminepentaacetic acid	m/rIgG – mouse/rabbit immunoglobulin type G
EGF – epidermal growth factor	mRNA – messenger RNA
EGFR – EGF receptor	mRT – molecularly-targeted radiotherapy
EGCG – epigallocatechingallate	mTERT – murine telomerase reverse transcriptase
EPR – enhanced perfusion and retention	NHEJ – non-homologous end-joining
ESI-MS – electrospray ionization mass spectrometry	
HMFG – human milk-fat globule	

NIS – sodium iodide symporter

NLS – nuclear localization sequence

NMR – nuclear magnetic resonance

OB – oligosaccharide binding

PAGE – polyacrylamide gel electrophoresis

PE – plating efficiency

PLGG – paediatric low-grade glioblastoma

PNA – peptide nucleic acid

pRB – retinoblastoma protein

PRIT – pre-targeted radioimmunotherapy

PS – phosphorothioate

RICs – radioimmunoconjugates

RIT – radioimmunotherapy

RP – reverse primer

RT – radiotherapy

SEC – size-exclusion chromatography

SF – surviving fraction

shRNA – short-hairpin RNA

SPECT – single photon-emission computed tomography

TFO – triplex-forming oligonucleotides

Tfx – transfection

TRAP – telomeric repeat amplification protocol

TRF – telomeric repeat-binding factor

tRT – targeted radiotherapy

TS – telomerase substrate

VEGF – vascular endothelial growth factor

XRT – external beam radiotherapy

# **Chapter 1: Introduction**

## Chapter 1: Introduction

A recent study estimated that in 2008, 28.8 million people worldwide were living with a cancer diagnosed in the preceding five year period (Bray *et al.*, 2013). In this year, there were 7.6 million global cancer deaths, highlighting the demand for novel and efficacious therapeutic strategies.

Cancer, representing a seemingly heterogeneous array of diseases, was eloquently defined using a series of hallmark characteristics (Hanahan and Weinberg, 2000, 2011). These essential characteristics, including management of genome-integrity; sufficiency in proliferative and resistance to anti-proliferative and death signalling; altered metabolism; induction of angiogenesis, invasion and metastasis; and acquisition of the capacity for unlimited replication, propound possible modes of therapeutic intervention, as disruption of such characteristics is expected to reduce cancer growth.

Anti-cancer therapy can be broadly divided into three strategies: surgical resection, radiotherapy and chemotherapy, which also encompasses molecularly targeted drugs. Indeed, the combination of these modalities is often associated with improved outcome and so forms the basis of most treatment plans currently in clinical use.

## 1.1. Radiation therapy for the treatment of cancer

The use of radiation to sterilize malignant cells, via the induction of genotoxic damage, has shown efficacy and has been used extensively in the clinical management of cancer. Radiation can be delivered in either a site-specific or systemic context.

### 1.1.1. Site-specific radiotherapy

External beam radiotherapy (XRT), typically employing linear accelerator-generated photons or electrons, is thought to be beneficial in around half of cancer presentations, for both radical and palliative purposes (Delaney *et al.*, 2005). In standard XRT therapeutic regimens, a treatment plan based around identification of the location of a tumour obtained by computed tomography (CT) scanning, is prepared. Radiation is then administered in a series of fractions, with the aspiration of maximizing the recovery of treated-normal tissue. Advances in the administration of XRT have aimed to reduce the dose of radiation delivered to normal tissues, particularly to sensitive organs at risk surrounding the tumour. This allows for the maximum tolerable dose to be applied to the tumour, as local tumour control probability is directly related to the dose administered (Hanks *et al.*, 1988; Zelefsky *et al.*, 2001).

Intensity-modulated radiotherapy (IMRT) employs multiple non-uniform radiation beams to generate steep dose gradients around the target volume. Thus, IMRT is capable of delivering the required dose to the tumour while optimizing the sparing of normal tissues. A phase III clinical trial compared parotid-sparing IMRT to conventional radiotherapy (RT) in head and neck cancer, and demonstrated a highly significant reduction in xerostomia after two years (Nutting *et al.*, 2011). Despite

reducing the radiation dose to normal tissue, IMRT increases the volume of tissue receiving a low-dose bath, and has therefore been linked to an increased risk of radiation-induced secondary cancer (Hall and Wu, 2003). Similarly, image-guided radiotherapy (IGRT) aims to ensure that the planned radiation dose is accurately spatially delivered to the tumour, compensating for motion during fraction administration and organ movement throughout the treatment (reviewed in Verellen *et al.*, 2007). Other advances potentiated by the development of IMRT and IGRT include the use of isotoxic hypofractionation protocols (Haviland *et al.*, 2013; Norkus *et al.*, 2013) and stereotactic RT, whereby high dose RT is administered rapidly to maximize the therapeutic ratio (Short and Tobias, 2010). In addition to photon XRT, the clinical application of charged particle RT is gaining momentum, at least for particular cancer indications, including central nervous system, ocular and prostate cancers, as well as in paediatric cases (reviewed in Schulz-Ertner and Tsujii, 2007). Heavier ions have a higher relative biological effectiveness when compared to photons and so may be particularly useful for treatment of more radioresistant tumours. Charged particle therapy offers the advantage of minimizing dose delivered to tissues beyond the target volume, as energy is deposited in the so-called Bragg peak. However, the requirement for generation of charged particles greatly increases the cost of therapy.

In contrast to XRT, brachytherapy involves the implantation of a sealed-source of radioactivity within the affected tissue, again requiring spatial location of the tumour by imaging. High and low dose-rate brachytherapy have been employed in the treatment of different cancers, with particular reference to prostate cancer (Pisansky *et al.*, 2008). Such a strategy allows for a treatment highly-conformal to the target, whilst also ensuring the target never leaves the radiation field. Brachytherapy has therefore been

shown to reduce off-target side-effects when compared to XRT (Ferrer *et al.*, 2008) and radical prostatectomy (Crook *et al.*, 2011), in this specialized situation. However, the occurrence of radiation-induced tissue necrosis in proximity to the implanted seeds is frequently limiting. Thus, an additional radiotherapeutic option has been developed to address such limitations.

### **1.1.2. Systemic radiotherapy**

As discussed, the irradiation of malignant cells represents an attractive therapeutic modality in cancer treatment. However, delivery of external or interstitial ionizing radiation (IR) requires the spatial identification of a target volume and so cannot be employed to treat micrometastases or single disseminated cancer cells, not yet discernable by imaging. Thus, the notion of systemic or molecularly-targeted radiotherapy (mRT) has been explored, in order to potentiate the irradiation of diffuse malignancies – the presence of which detrimentally affects morbidity and survival. In this strategy, a cancer-homing therapeutic radionuclide is administered systemically, allowing for the targeting and eradication of disseminated cells.

Radionuclides that decay by emission of alpha- and beta-particles or low energy electrons are particularly suitable for incorporation into an mRT strategy, owing to the respective linear energy transfer (LET) and distance of ionization associated with such modes of decay. Beta-electrons, emitted by radionuclides such as yttrium-90, iodine-131, lutetium-177, rhenium-186, rhenium-188 and gold-198, with LET of the order of  $\sim 0.2$  keV/ $\mu\text{m}$  are emitted following nucleon decay and are capable of traversing several millimetres in tissue (Nias, 1998). This characteristic allows for the irradiation of non-

targeted neighbouring cells, in a process known as cross-fire. The cross-fire effect is advantageous when targeting bulk tumours with heterogeneous target expression but is a disadvantage when directed to micrometastases of small diameter, surrounded by normal tissue (Odonoghue *et al.*, 1995). The range of an alpha-particle, composed of two protons and two neutrons, is equivalent to a few cell diameters (30 – 90  $\mu\text{m}$ ) (Nias, 1998), significantly lessening the cross-fire effect and making this mode of decay suitable for targeting metastases (Zalutsky, 2006). Alpha-particles, emitted by radionuclides including bismuth-213 and radium-223, have an LET of up to 300  $\text{keV}/\mu\text{m}$ , giving them a high relative biological effectiveness (Nias, 1998). The dense ionization associated with high LET radiation promotes the formation of clustered biological damage. The clustering or complexity of damage reduces the rate of DNA repair in most organisms (Malyarchuk *et al.*, 2003). Low energy electrons, for example Coster-Kronig and Auger electrons, are emitted from isotopes including indium-111, iodine-123 and iodine-125, following decay events such as electron capture and internal conversion, which yield an inner-shell electron vacancy. The occupancy of this vacancy by a higher energy electron leads to energy release in the form of an electron. In fact, such decay often initiates the emission of a cascade of electrons. Auger electrons have low energy, typically of a few electron volts to 100 keV, and so a highly restricted range, up to a maximum of a few micrometres in tissue, with most in the nanometre scale (Kassis, 2004). This characteristic essentially abrogates the cross-fire effect mediated by Auger electron-emitting radionuclides, eliminating the radiotoxic irradiation of adjacent normal tissue. Indeed, the therapeutic efficacy of Auger electron-emitting radionuclides is enhanced upon their delivery to the cell nucleus, as a result of increased proximity to the DNA target (Hofer *et al.*, 1975). Despite the absence of a direct cross-fire effect, mRT agents labelled with Auger electron-emitting

radionuclides, as well as alpha- and beta-particle emitters, have been shown to lead to radiation-induced biological bystander effects, where the survival of untreated neighbouring cells is reduced following exposure to irradiated cells or conditioned cell-culture medium (Boyd *et al.*, 2006; Kishikawa *et al.*, 2006). Low energy electrons are densely ionizing (LET 4-26 keV/ $\mu\text{m}$ ) making them an attractive choice for mRT (Wright *et al.*, 1990).

In addition to the informed selection of radionuclide, the choice of target is clearly a key consideration when designing an mRT agent. The choice of target often logically leads to selection of the class of targeting molecule, which usually falls into one of several discrete categories.

#### **1.1.2.1. Intrinsic targeting**

In specialized situations, administered radionuclides may have some intrinsic capacity to target tumours. For example, differentiated thyroid cancers frequently upregulate the sodium iodide-symporter essential for normal thyroid function. Thus, administration of beta-particle-emitting radioiodine (iodine-131) leads to transient accumulation of the radionuclide in, and so therapeutic irradiation of tumour tissue (Bal *et al.*, 2004; Beierwaltes, 1978). Beta-particle-emitting strontium-89 accumulates in bone and so facilitates the irradiation of osseous metastases (Porter and Davis, 1994; Robinson *et al.*, 1995). More recently, administration of bone-seeking radium-223 (Xofigo/Alpharadin, Algeta ASA) was shown to modestly improve overall survival and delay skeletal events in castration-resistant prostate cancer patients with bone

metastases, in phase III clinical trial (Parker *et al.*, 2012). Thus, molecular targeting to enhance delivery and retention may improve the potential of such strategies.

### 1.1.2.2. Small molecules

Small molecules, typically analogues of substrates, agonists or inhibitors of biomolecules, have been radiolabelled for mRT. The possibility of labelling nucleotide analogues, which are incorporated into DNA during replication, with Auger electron-emitting radionuclides has also been explored (Bloomer and Adelstein, 1977). Further experimental compounds, including Auger electron-emitting radionuclide-labelled tamoxifen (Bloomer *et al.*, 1980) and oestrogen (Yasui *et al.*, 2001), and chemotherapeutic agents have been developed (Areberg *et al.*, 2001; Jaaskela-Saari *et al.*, 2005). Astatine-211- and iodine-131-labelled benzylguanidine analogues of norepinephrine were developed as experimental radiopharmaceuticals (Vaidyanathan *et al.*, 1996). Subsequently, guidelines for the clinical use of meta-iodine-131-benzylguanidine ( $M^{131}IBG$ ) in norepinephrine-transporter overexpressing cancers have been published (Giammarile *et al.*, 2008). Auger electron-emitting variants have also been produced (Cunningham *et al.*, 1998; He *et al.*, 2004a). One such Auger electron-emitting derivative, labelled with iodine-125, showed some promise in a small phase I study in neuroblastoma patients (Sisson *et al.*, 1990).

### 1.1.2.3. Oligonucleotides

Radiolabelled oligonucleotides have been developed, but mostly for pre-clinical molecular imaging, so far. Antisense imaging of messenger RNAs (mRNA) including c-Myc (Dewanjee *et al.*, 1994), PKA-R1 $\alpha$  (Liu *et al.*, 2009; Zhang *et al.*, 2001); k-RAS (Amirkhanov *et al.*, 2010; Chakrabarti *et al.*, 2007); TGF $\alpha$  (Cammilleri *et al.*, 1996);

p21WAF-1/CIP-1 (Wang *et al.*, 2003) and RhoC (Wang *et al.*, 2009a) has been conducted using radionuclides such as indium-111, technetium-99m, copper-64 and iodine-125 for cancer imaging. The potential of therapeutically radiolabelled oligonucleotides has also been explored. Peptide nucleic acids labelled with indium-111 were shown to cleave single-stranded DNA in a sequence specific manner (He *et al.*, 2004b). An Auger electron-emitting radionuclide-conjugated DNA oligonucleotide was capable of inducing breaks in target mRNA *in vitro* (Gaidamakova *et al.*, 2004). Furthermore, an indium-111-labelled morpholino oligonucleotide targeting PKA-R1 $\alpha$  mRNA specifically reduced the colony forming ability of breast cancer cells *in vitro* (Liu *et al.*, 2009). Similarly, indium-111 labelled oligonucleotides complementary to n-Myc mRNA reduced neuroblastoma tumour formation in mice (Watanabe *et al.*, 2006). In an alternative strategy, known as antigene radiotherapy, iodine-125-labelled triplex forming oligonucleotides (TFO) were employed (Sedelnikova *et al.*, 2002; Sedelnikova *et al.*, 2000). This class of oligonucleotide bind to the major groove of duplex genomic DNA by Hoogsteen base-pairing, and were shown to produce site-specific breaks within the targeted human *mdr1* gene, leading to reduction in carcinoma cell survival after 60 days of decay accumulation *in vitro*. Such decay accumulation is clearly of little relevance for the therapeutic context, but serves to demonstrate the principle of the sequence-specific oligonucleotide-directed induction of DNA breaks. Analysis of the clonogenic survival of treated cells over a more clinically-relevant time-course would increase the significance of such a study. Another iodine-125-labelled TFO targeting the androgen receptor gene reduced expression and, critically, the growth of a prostate cancer xenograft (Zhang *et al.*, 2008).

#### 1.1.2.4. Peptides and proteins

Radiolabelled peptides, often mimicking endogenous peptide ligands of cell-surface receptors, have shown some promise as mRT agents. Peptides targeting the somatostatin receptor, frequently overexpressed in neuroendocrine tumours, have been successfully radiolabelled (reviewed in de Jong *et al.*, 2009). A somatostatin-targeting peptide, octreotide, labelled with Auger electron-emitting indium-111 was assessed in phase I trial, with some therapeutic effect being noted (Valkema *et al.*, 2002). Yttrium-90-labelled DOTATOC administered to patients with neuroendocrine tumours provided significant clinical gain (Waldherr *et al.*, 2002). Similarly, a second beta-particle-emitting peptide, lutetium-177-labelled octreotate, offered a survival benefit in neuroendocrine tumour patients (Kwekkeboom *et al.*, 2008). Following from these successful findings, a clinical guide for the use of this strategy, termed peptide receptor radionuclide therapy, in cases of neuroendocrine cancer has recently been published (Zaknun *et al.*, 2013). Interestingly, targeting of the somatostatin receptor with peptides labelled with different beta-particle-emitting radionuclides, improved tumour control in a rat model of cancer (de Jong *et al.*, 2005), suggesting combinations of radionuclides may be advantageous. Additionally, lutetium-177-labelled octreotate in combination with the chemotherapeutic agents capecitabine and temozolomide showed efficacy in advanced neuroendocrine tumours; after 24 months of follow-up, median survival had not been reached (Claringbold *et al.*, 2012). The incorporation of D-amino acids improves the stability of such peptides – a strategy often required with mRT agents. Recently, bombesin-antagonist peptides radiolabelled with indium-111 internalized to 6% *in vitro* and exhibited rapid blood clearance and accumulated in prostate cancer xenografts in mice (Marsouvanidis *et al.*, 2013). Cell-surface nucleolin, expressed in some cancer cells, has also been targeted with radiolabelled peptides in pre-clinical

studies. The nucleolar trafficking of the ligand has been reported, so potentiating radiolabelling with Auger electron-emitting indium-111 (Cornelissen *et al.*, 2012b). Internalization to 0.5 % of the administered radioactivity was reported, with 37 % of that activity being localized to the nucleus. In addition, alpha-emitting radionuclides were tested in this strategy (Vallon *et al.*, 2012). Another notable pre-clinical molecule under development is indium-111-labelled exendin, which targets the glucagon-like peptide-1 receptor overexpressed in insulinomas (Wicki *et al.*, 2007).

Cell-surface receptors have also been targeted using radiolabelled proteins. A minority pathway activated following the targeting of the epidermal growth factor cell-surface receptor (EGFR) with its natural ligand facilitates the receptor-mediated internalization and nuclear accumulation of the radiolabelled protein – so potentiating the use of short-range radionuclides. Thus, Auger electron-emitting radionuclides were considered for use in protein-mediated mRT. Indium-111-labelled epidermal growth factor (EGF) internalized to 80 % in 4 hours and was found to be selectively toxic to EGFR-overexpressing breast cancer cells (Reilly *et al.*, 2000). More recently, the identification of alternative pathways of EGF nuclear trafficking may be relevant for this strategy, as localization of EGF on the inner nuclear membrane has been reported – potentially limiting irradiation of the DNA (Han & Lo, 2012). Indium-111-labelled splice variants of vascular endothelial growth factor (VEGF), transported to the nucleus following internalization of the VEGF receptor, internalized to 3 % and reduced the clonogenic survival of endothelial cells *in vitro* (Chan *et al.*, 2010).

#### **1.1.2.5. Antibodies**

Therapeutically radiolabelled antibodies, also known as radioimmunoconjugates (RICs), have shown particular efficacy for the treatment of diffuse cancers in the clinic.

Anti-CD20 antibodies labelled with yttrium-90 (Zevalin, Spectrum Pharmaceuticals) or iodine-131 (Bexxar, GlaxoSmithKline) have shown efficacy above the unlabelled antibodies in B-cell non-Hodgkin lymphoma (Goldsmith, 2010). Following from this success, other cell-surface antigens, including CD33, CD45 and CD66, have been targeted with beta-particle-emitting RICs (Burke *et al.*, 2003; Lauter *et al.*, 2010; Matthews *et al.*, 1995). Furthermore, a humanized anti-CD33 antibody radiolabelled with alpha-particle-emitting bismuth-213, showed positive preliminary results in a phase I/II clinical trial in acute myeloid lymphoma patients (Rosenblat *et al.*, 2010). The use of RICs in solid malignancy has proved more challenging, although significant progress has been made. Targeting of tenascin with an iodine-131-labelled antibody in the post-surgical cavity, allowed the application of approximately a 44 Gy boost in local radiation dose in glioma patients (Reardon *et al.*, 2008). Several studies have investigated the targeting of carcinoembryonic antigens (CEAs) and associated cell-surface glycoproteins in multiple cancer indications (Behr *et al.*, 1997; Liersch *et al.*, 2005; Meredith *et al.*, 1996; Robert *et al.*, 2003). Despite this, a comparative phase III clinical trial of an yttrium-90 labelled anti-human milk fat globule 1 and 2 (HMFG1 and HMFG2) antibody showed no real improvement in overall survival of patients with ovarian cancer (Verheijen *et al.*, 2006). However, the RIC was administered intraperitoneally in this study and so may have failed to effectively target distant metastases. Carbonic anhydrase IX expression is induced by hypoxia and may therefore associate with a treatment-resistant population of cells within a tumour (Kaluz *et al.*, 2003). Iodine-131-labelled antibodies targeting this antigen, administered in a fractionated protocol, have been trialled in renal carcinoma (Divgi *et al.*, 2004). A humanized antibody targeting a mucin antigen, and so pancreatic ductal carcinoma, was labelled with yttrium-90 and administered to patients with stage III/IV disease, in

combination with gemcitabine (Ocean *et al.*, 2012). Interestingly, the fractionation protocol employed in this study allowed a much higher dose of radiation to be safely delivered when compared to traditional single-dose radioimmunotherapy (RIT), potentially facilitating a better outcome. Other experimental RICs are also under development. The anti-angiogenic antibody bevacizumab (Avastin, Genentech/Roche), which targets VEGF isoforms, has been radiolabelled with iodine-131 and shown to target ovarian cancer cells *in vitro* (Ashrafi *et al.*, 2012). In a strategy designed to potentiate the chemo- and radiation-therapy guided delivery of RICs, an indium-111-labelled antibody, modified with a cell penetrating peptide, targeting the DNA double strand marker  $\gamma$ H2AX showed therapeutic potential in xenograft models (Cornelissen *et al.*, 2012a). In addition the HER2/Neu cell-surface receptor, the overexpression of which may correlate with poor prognosis, has been targeted in the pre-clinical setting with an Auger electron-emitting RIC, following internalization to 12 % (Costantini *et al.*, 2007).

The use of radiolabelled antibody fragments, with the aim of improving pharmacokinetics, has also been pursued. However, such antibody fragments may increase the potential radiation dose received by the kidneys, which are implicated in their excretion (Behr *et al.*, 1998). Nanobodies (~15 kDa) targeting HER2/Neu labelled with lutetium-177 gave a favourable tumour to background ratio in a mouse model of cancer (D'Huyvetter *et al.*, 2012). A similar approach with iodine-131 labelled nanobodies was reported recently (Pruszynski *et al.*, 2013).

Pre-targeting strategies, whereby administration of a bispecific tumour-targeting antibody occurs prior to delivery of the radionuclide conjugated to a smaller species containing the second antibody epitope, may further spare normal tissue by allowing for faster clearance of radioactivity from non-targeted tissues. Therapeutically radiolabelled

haptens, which are recognized by antibody fragments already bound to tumour, are often cleared from the circulation within several hours, compared to days with traditional RICs (reviewed in Sharkey *et al.*, 2010). Intelligent engineering of the radiolabelled hapten has been shown to further manipulate *in vivo* characteristics including normal tissue accumulation (Sharkey *et al.*, 2003). Thus, off-target toxicity can be greatly reduced without compromising tumour dose, making pre-targeted RIT (PRIT) an encouraging new therapeutic platform. Administration of a trivalent bispecific antibody construct capable of binding TROP-2; a frequently cancer-upregulated membrane glycoprotein associated with poor prognosis, and an indium-111-labelled hapten, shows promise for imaging and therapy (Sharkey *et al.*, 2012). A similar approach employed a bispecific antibody targeting a CEA and the metal chelator DOTA (Yazaki *et al.*, 2013). Following unbound antibody clearance, 6 % injected dose (ID) /g was achieved in xenograft tumours, 24 hours subsequent to administration of <sup>177</sup>Lu-DOTA. Tumour to blood and tumour to liver ratios of 199:1 and 30:1 were recorded, respectively. Clinical studies with various strategies are on-going (Kraeber-Bodere *et al.*, 2006; Salaun *et al.*, 2012), although the magnitude of the benefit observed *in vivo* has not so far been translated in full.

#### **1.1.2.6. Nanoparticles**

Increasingly, the potential of nanoparticle-based systems for mRT is being explored. Such a system offers selective targeting whilst delivering a much higher payload of radionuclide than could be directly conjugated to a targeting agent, especially useful when the target is expressed at low density. However, the macromolecular nature of nanoparticle constructs often means nuclear localization is difficult to achieve, perhaps precluding the use of Auger electron-emitting radiopharmaceuticals. Addition of

functionality to enhance endosomal escape and nuclear localization may modestly ameliorate this limitation. The large size of the delivery agent confers an advantage with regards to targeting, as macromolecules tend to passively accumulate in tumours due to the enhanced perfusion and retention (EPR) effect, often found in tumours with disorganized vasculature (Muggia, 1999). A common nanoparticle delivery system employs the use of liposomes targeted to a cell-surface antigen with tumour-specific antibodies or antibody fragments. Binding of liposomes containing alpha-particle-emitting radionuclides (radium-223, radium-224, actinium-225) targeted with F(ab')<sub>2</sub> antibody fragments to the folate receptor of tumour cells was demonstrated *in vitro* (Henriksen *et al.*, 2004). A similar approach was taken to deliver actinium-225 to HER2/Neu overexpressing cells (Chang *et al.*, 2008b). Beta-emitting radionuclides have also been used in this context. Prostate specific membrane antigen targeted-nanoparticles labelled with yttrium-90, iodine-131 or rhenium-188 were delivered to tumour spheroids *in vitro* (Emfietzoglou *et al.*, 2005). Unilamellar liposomes were found to afford the greatest penetration into the three-dimensional tumour model, where a favourable absorbed dose profile was recorded. This study also considered Auger electron-emitting nuclides (iodine-123 and iodine-125), although a more heterogeneous dose distribution was noted in this case. The targeting of yttrium-90-labelled nanoparticles to markers of angiogenesis, utilizing either an antibody or small molecule, resulted in delayed growth of a mouse xenograft tumour, although no non-specific control was included in this study (Li *et al.*, 2004). A second study targeting integrins thought to be involved in the angiogenic response, with iodine-131, demonstrated reduced tumour blood flow and growth delay (Hallahan *et al.*, 2003).

Other nanoparticle formulations, capable of delivering beta-particle-irradiation, have also been considered. Gold-198 labelled dendrimers were able to reduce tumour volume

in a murine model of melanoma following intratumoural administration (Khan *et al.*, 2008). Such administration is often impractical in the clinical context, limiting the relevance of this work. Upon arterial injection, yttrium-90 embolized resin microspheres accumulated in the liver of patients and were shown to decrease tumour burden whilst increasing overall survival (Gray *et al.*, 2001). However, liposomes perhaps afford the greatest opportunity in nanoparticle-based mRT, due to their ability to encapsulate a second therapeutic agent and so facilitate a combinatorial therapeutic approach. The combination of rhenium-186 or -188 and doxorubicin-labelled liposomes has been investigated in the pre-clinical setting (Chang *et al.*, 2008a; Soundararajan *et al.*, 2009).

## **1.2. Telomerase as a target for the molecular radiotherapy of cancer cells**

The criteria for target selection in mRT strategies rely on the molecular characteristics of the putative target. Perhaps most importantly, the targeting agent must exhibit preference for malignant over normal tissue, in order that a toxic dose of radiation is selectively delivered to the desired sites. Ideally, target expression should be homogeneously abundant in cancer cells but absent in normal tissue, to allow for maximal accumulation and retention of the radiopharmaceutical in the tumour. The specificity of the targeting molecule must also be high, to maximally realize the differential in target expression. The subcellular distribution of target is another key consideration, and will likely influence the selection of therapeutic radionuclide. In

some cases the targeting agent may itself possess some anti-tumour activity that acts in isolation or in concert with IR.

One such novel candidate target for mRT is telomerase, an enzyme overexpressed in the majority of tumour samples (> 85 %) but absent from differentiated normal tissue (Kim *et al.*, 1994; Shay and Bacchetti, 1997). Telomerase exists as a nuclear-species in cancer cells (Etheridge *et al.*, 2002; Lee and Chung, 2010), permitting the use of genotoxic, high LET Auger electron-emitting radionuclides, whose effects will be limited to targeted cells. The availability of highly specific telomerase-binding inhibitors supplements this favourable expression profile, and furthermore, telomerase inhibition has been shown to reduce tumour cell proliferation in its own right (Damm *et al.*, 2001; Herbert *et al.*, 2005; Herbert *et al.*, 1999). Moreover, some evidence to suggest telomerase inhibition-mediated radiosensitization of cancer cells has been generated (Gomez-Millan *et al.*, 2007).

### **1.2.1. Telomerase maintains telomere length**

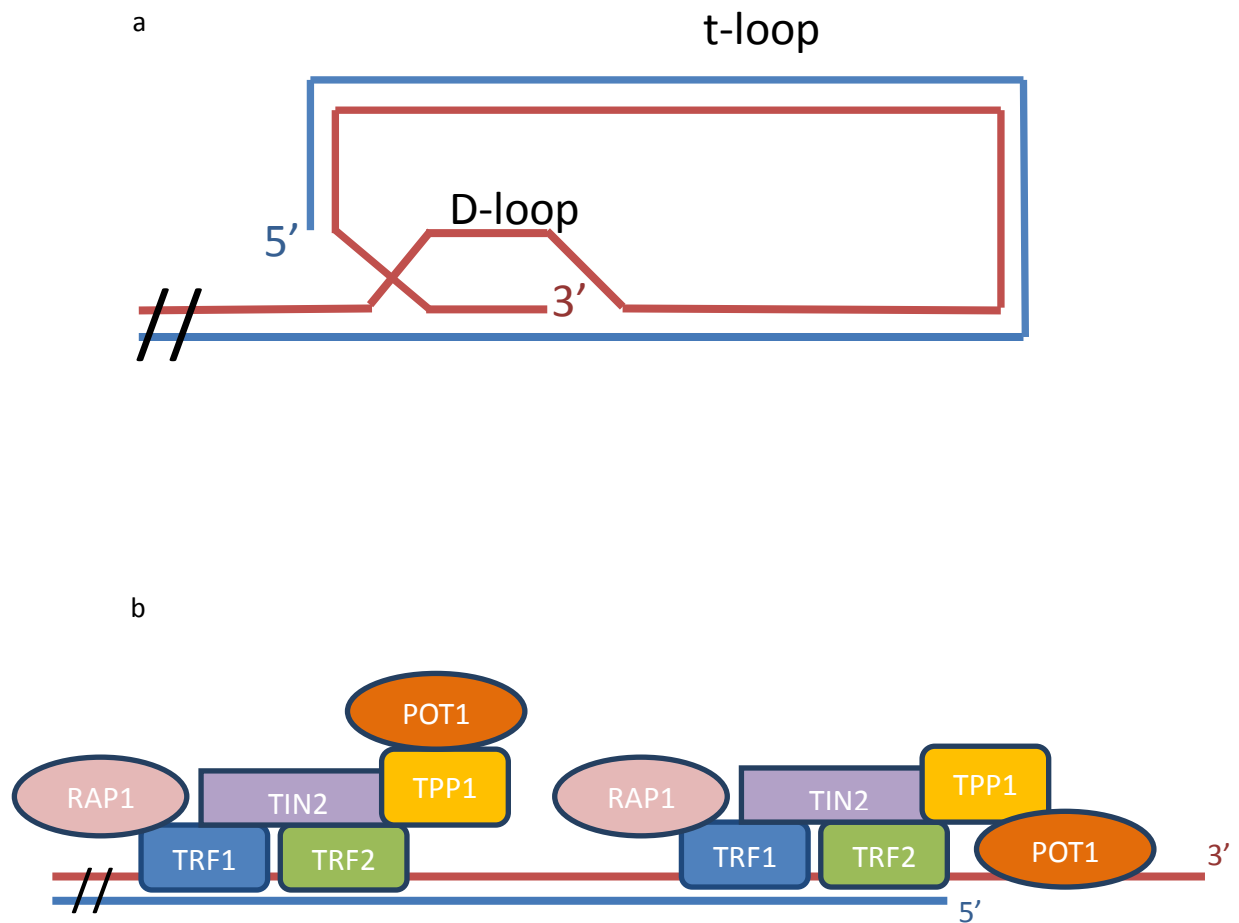
The canonical role of telomerase involves the *de novo* addition of hexanucleotide repeats to the telomeres, found at the chromosome termini, to facilitate continued cell proliferation (Cohen *et al.*, 2007). Telomeric DNA has critical functions in protecting the genome, rendering its maintenance a key consideration for cell survival.

#### **1.2.1.1. Telomeres protect the linear chromosomes**

The telomeres cap the ends of the linear eukaryotic chromosomes. In humans, the telomeres are composed of a tract of double-stranded DNA spanning 5 – 20 thousand

base-pairs (bp), with repetitive DNA sequence, 5'-GTTAGG-3' (Moyzis *et al.*, 1988). In addition to this double-stranded telomeric sequence, a single-stranded G-rich 3'-overhang of approximately 300 nucleotides is thought to be important for telomere function (Wright *et al.*, 1997; Zhao *et al.*, 2008). Processing of the telomere-termini was shown to commonly yield a C-strand ending in the sequence -CCAATC-5', whereas the G-strand terminus was less tightly regulated (Sfeir *et al.*, 2005). Analysis of telomere structure by electron microscopy suggested the presence of a large telomeric duplex lariat (Griffith *et al.*, 1999). Moreover, a similar structure was observed in native telomeric chromatin (Nikitina and Woodcock, 2004). This structure was termed the t-loop, and is thought to be generated following the invasion of the 3'-overhang into regions of duplex telomeric DNA (*figure 1.1 a*). It was proposed that the generation of a t-loop functions to conceal the free-DNA end, so preventing its recognition by the DNA damage repair machinery (Griffith *et al.*, 1999).

In addition to the DNA sequence, telomeres contain a characteristic six-protein complex, known as shelterin, that coats the telomere by contacting both the double- and single-stranded regions (*figure 1.1 b*) (Liu *et al.*, 2004). This protein complex associates in the absence of DNA but has specificity for telomeres, determined by the DNA-binding proteins. Telomeric repeat binding factor-1 and -2 (TRF1 and -2) bind specifically to double-stranded telomeric DNA as homodimers (Court *et al.*, 2005; Hanaoka *et al.*, 2005), but do not interact directly (Broccoli *et al.*, 1997; Fairall *et al.*, 2001). Addition of TRF2 to telomeric DNA *in vitro* was sufficient to promote t-loop formation (Griffith *et al.*, 1999). This shelterin protein was further implicated in the stabilization of the t-loop by its DNA junction-binding ability (Fouche *et al.*, 2006). The TRF proteins were identified as being involved in the localization of telomeric DNA to the nuclear matrix (Luderus *et al.*, 1996). The TPP1/POT1 heterodimer is capable of



**Figure 1.1: A schematic of the proposed structure of the human telomere.**

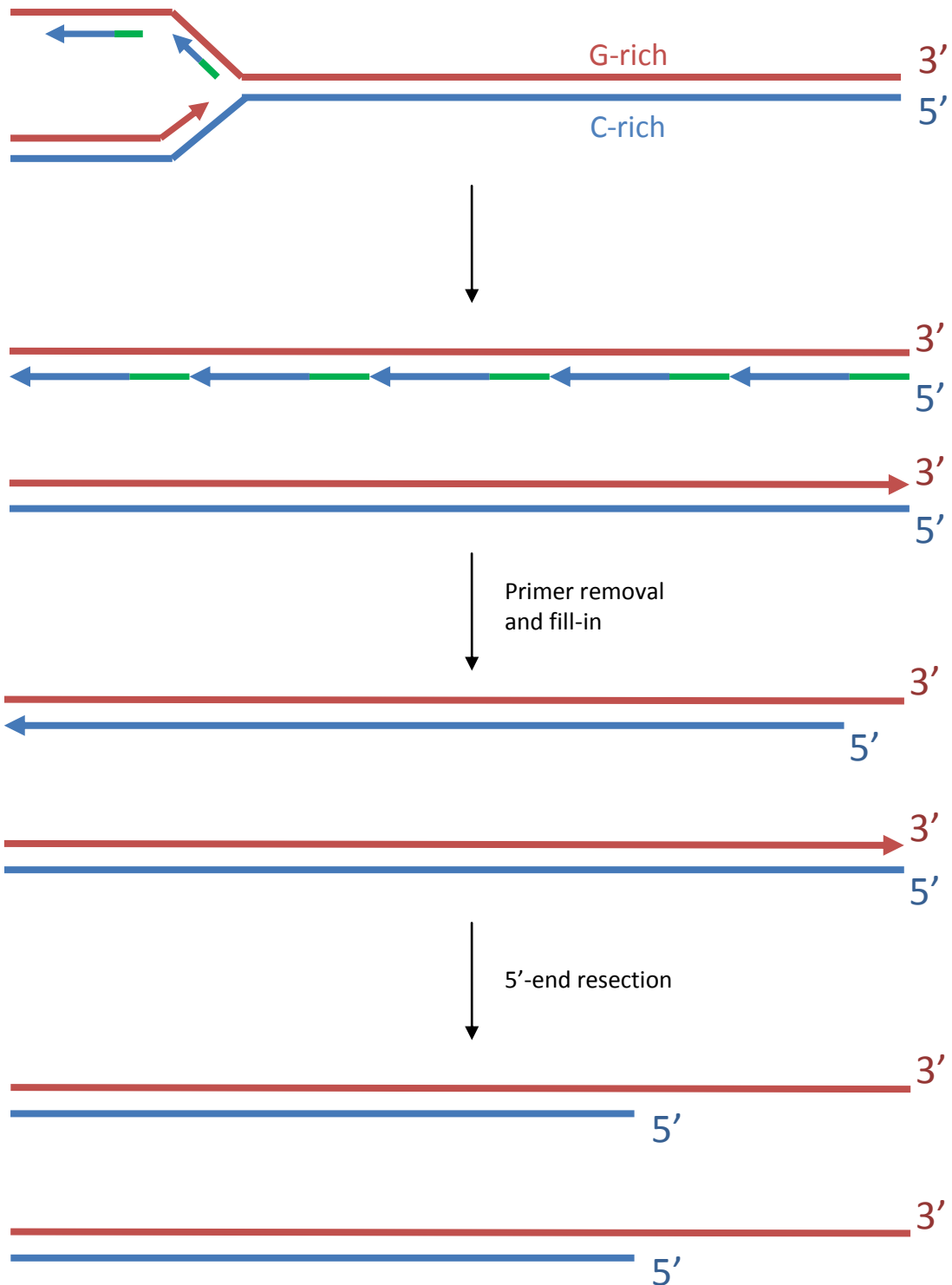
Telomeric DNA, composed of hexanucleotide repeats, of sequence 5'-GTTAGG-3', are hypothesised to form a t-loop lariat structure *in vivo* (a). The six member shelterin protein complex contacts telomeric DNA by binding to double- and single-stranded regions (b).

binding single-stranded telomeric DNA via oligosaccharide-binding (OB) domains (Lei *et al.*, 2004; Loayza *et al.*, 2004). Accordingly, TPP/POT1 proteins are less abundant at the telomere than the other shelterin components (Takai *et al.*, 2010). The other proteins constituting shelterin are RAP1, a TRF2-dependent component (Li *et al.*, 2000), and TIN2, a structural component capable of bridging TRF1, TRF2 and TPP1 (Houghtaling *et al.*, 2004; Ye *et al.*, 2004a; Ye *et al.*, 2004b). In addition to shelterin, other proteins, including DNA damage response (DDR) factors are transiently localized to the telomeres during the cell-cycle (reviewed in Palm and de Lange, 2008). For example, the Ku heterodimer was found to associate with telomeric DNA in the absence of DNA-PKcs (Hsu *et al.*, 1999). Conditional knockout of Ku86 in colon carcinoma cells led to a loss of viability associated with massive chromosomal telomere losses, implicating Ku in telomere function (Wang *et al.*, 2009b). The MRE11/RAD50/NBS1 complex has also been shown to associate with telomeric DNA, likely via interaction with TRF2 (Zhu *et al.*, 2000) and may have a role in enhancing telomerase activity via regulation of access and telomere dysfunction through the activation of ATM signalling (Lamarche *et al.*, 2010). Such findings further highlight the complex relationship between telomeres and the DDR.

As already alluded to, a major function of the telomeres is the prevention of recognition of the chromosome ends as damaged DNA. Loss of shelterin function, for example following POT1 inhibition was shown to elicit a telomeric DDR, in this case via ATR activation (Denchi and de Lange, 2007). Similarly, expression of dominant-negative TRF2, led to the activation of an ATM-initiated DDR (Karlseder *et al.*, 1999; Takai *et al.*, 2003), and promoted chromosomal end-to-end fusion (van Steensel *et al.*, 1998). Such fusion was found to be largely dependent on ligase IV expression, implicating the

non-homologous end-joining (NHEJ) DNA repair pathway (Smogorzewska *et al.*, 2002). The TRF2-binding protein RAP1 may also be involved in the inhibition of NHEJ at the telomere (Sarthy *et al.*, 2009). Truncation studies suggested that TRF2, in combination with Ku, may also play a role in the suppression of homologous recombination (HR) at the telomere (Celli *et al.*, 2006; Wang *et al.*, 2004). Recent work employing conditional knockout of both TRF1 and TRF2, and so complete loss of shelterin from telomeric DNA, reported the activation of multiple DDR pathways, including NHEJ and HR (Sfeir and de Lange, 2012). Further evidence for repression of repair at the telomere has also been obtained. Ultra-violet radiation-induced cyclobutane pyrimidine dimers were shown to accumulate in telomeric DNA, where nucleotide excision repair was largely absent (Rochette and Brash, 2010). Similarly, normal human fibroblasts treated with IR accumulated telomeric DNA damage that persisted and promoted adoption of a senescence-phenotype (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). In addition, shelterin-bound telomeric sequences were found to inhibit the repair of inducible-DNA breaks *in vitro*.

The second function of the telomeres involves the protection of the coding genome from incurring losses during replication. Linear chromosomes shorten with successive rounds of DNA replication, due to the so-called end-replication problem (*figure 1.2*) (Harley *et al.*, 1990; Lindsey *et al.*, 1991). The unidirectional nature of DNA replication coupled to the requirement for priming, means the lagging strand cannot be fully replicated. Additional processing of the replicated telomeres, for example during generation of the 3'-overhang, leads to losses of approximately 50 – 200 bp per round of replication. Thus, telomeres buffer the replication-associated loss of nucleotides, so protecting coding regions of DNA.



**Figure 1.2: The end-replication problem.**

Following removal of the primer (shown in green), required for the terminal Okazaki fragment synthesis, the lagging strand cannot be fully replicated. Additional 5'-end resection, necessary to generate the 3'-telomeric overhang, leads to telomere shortening with each round of replication.

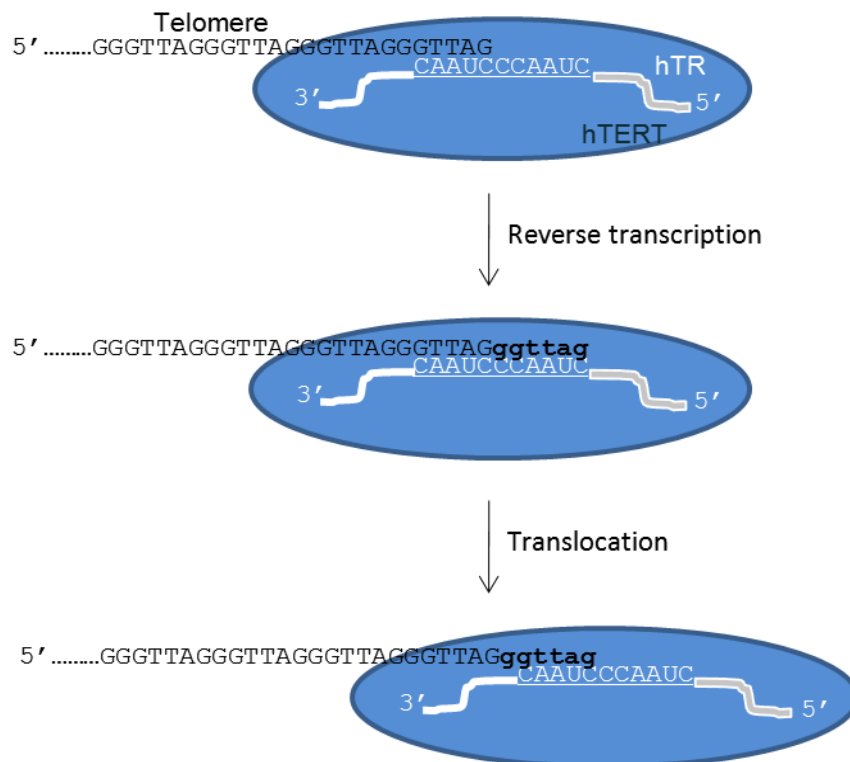
Further to this function, it has been hypothesised that telomere erosion acts as a tumour-suppressive mechanism. Primary cells cultured *in vitro* exhibit a finite capacity for DNA replication determined by telomere shortening, in a phenomenon known as the Hayflick limit to proliferation (Hayflick, 1965). Following continued replication, cells irreversibly enter senescence, a state characterized by altered morphology, chromatin organization and gene expression (Katakura *et al.*, 1999). Senescent fibroblasts were found to display DNA damage foci, particularly at the chromosome ends (di Fagagna *et al.*, 2003). Thus, it was proposed that telomere shortening leads to loss of shelterin and instigation of a telomeric DDR, and subsequent checkpoint activation directs cells to enter senescence. Telomere shortening to < 1 kbp was shown to be necessary (Damm *et al.*, 2001) and the presence of a single short telomere sufficient (Hemann *et al.*, 2001) for induction of senescence in cancer cells *in vitro*. In situations where checkpoint function has been lost, including during tumorigenesis, cells circumvent this limit to proliferation and continue to replicate their genomes, leading to further telomere attrition (Hara *et al.*, 1991). Thus, illegitimate-repair ensuing from a telomeric DDR is expected to promote genomic rearrangement and accordingly, critical telomere shortening was found to promote chromosome end-to-end fusion, in a Ku-dependent manner (Espejel *et al.*, 2002). Studies in human fibroblasts suggested as few as 13 telomeric repeats (78 bp) were sufficient to prohibit chromosome fusion events *in vitro* (Capper *et al.*, 2007). Cells incapable of reversing telomere shortening now enter crisis, characterized by gross genomic instability and cell death.

The ability to maintain telomere length is essential for acquisition of the potential for limitless proliferation, also referred to as cell immortalization – a hallmark of tumorigenesis (Hanahan and Weinberg, 2000, 2011). Under normal circumstances,

telomere shortening is counteracted by telomerase in normal progenitor cells and the overriding majority of cancer cells (Kim *et al.*, 1994; Shay and Bacchetti, 1997). However, a second, ill-defined pathway, termed alternative lengthening of telomeres (ALT), is also employed by some cancer cells (reviewed in Henson *et al.*, 2002). Remarkably, a recent report suggested that ALT may also occur in mitogen-stimulated somatic mouse cells, perhaps as an evolutionary relic (Neumann *et al.*, 2013). However, addition of the exogenous telomere-tag used in this study may have led to altered telomere regulation. A similar transfer of an exogenous sequence between telomeres was observed in immortalized fibroblasts (Dunham *et al.*, 2000). Thus, the predominating hypothesis suggests ALT operates by employing HR-like mechanisms. Depletion of the MRE11/RAD50/NBS1 complex, implicated in HR, reduced the capacity for cells to maintain their telomeres by ALT (Zhong *et al.*, 2007). Cells employing ALT are characterized by heterogeneous telomere lengths (Londono-Vallejo *et al.*, 2004), along with the presence of linear or circular extrachromosomal telomeric DNA (Wang *et al.*, 2004) and ALT-associated PML bodies (APBs) (Yeager *et al.*, 1999). The chromatin status of the genes encoding the components of telomerase has been implicated in the adoption of ALT, which is predominantly observed in cancers of mesenchymal origin (sarcomas) (Atkinson *et al.*, 2005). The exogenous introduction of telomerase expression into ALT cells *in vitro* did not eliminate ALT, suggesting the activity of this pathway does not depend on the presence of short telomeres (Cerone *et al.*, 2001).

#### **1.2.1.2. The telomerase-mediated maintenance of telomeres**

As described, the ribonucleoprotein telomerase catalyses the *de novo* addition of the characteristic hexanucleotide motif to the telomeres (*figure 1.3*), counteracting losses



**Figure 1.3: Telomerase catalyses the *de novo* addition of telomeric repeats to the chromosome ends.**

The human RNA component of telomerase (hTR) acts to align the telomeric substrate, prior to the reverse transcription of the hexanucleotide-telomeric repeat of sequence 5'-GGTTAG-3', mediated by the human telomerase reverse transcriptase (hTERT). Translocation of the enzyme facilitates the processive addition of multiple telomeric repeats during a single interaction of telomerase and telomere.

incurred owing to the end-replication problem and end-processing. Reconstitution of telomerase activity *in vitro* minimally requires the telomerase reverse transcriptase (TERT) protein and telomerase RNA subunit (TR) (Cohen *et al.*, 2007; Feng *et al.*, 1995; Nakamura *et al.*, 1997). However, endogenous telomerase is thought to have a molecular weight of around 650 kDa (Cohen *et al.*, 2007), consistent with a dimeric complex containing two copies of human TERT (hTERT), human TR (hTR), dyskerin and NOP10 (Beattie *et al.*, 2001). Recently, the elucidation of a low-resolution structure of human telomerase appeared to confirm this assumption (Sauerwald *et al.*, 2013). Mutation of the shelterin protein TIN2 reduced the binding of heterochromatin protein-1 and so reduced sister telomere cohesion (Houghtaling *et al.*, 2012). In this setting, telomere elongation was inhibited suggesting sister chromatid cohesion may be important for telomerase function, supporting the requirement for telomerase to act as a dimer. Dyskerin was identified as a component of telomerase following phenotypic comparison in patients and cells possessing a mutated *DKC* gene (Mitchell *et al.*, 1999). Despite the identification of the minimal components necessary for telomerase activity, many other associated factors have been described, often involved in the assembly, trafficking and regulation of the enzyme (Fu and Collins, 2007; Venteicher *et al.*, 2009; Venteicher *et al.*, 2008).

Human TERT is relatively well conserved and is composed of several functional domains: an N-terminal telomere-binding domain, an RNA-binding domain, the reverse transcriptase domain, and a poorly-conserved C-terminal domain (reviewed in Autexier and Lue, 2006). Binding of hTERT to the telomeric substrate was shown not to require hTR (Wyatt *et al.*, 2007), and indeed substrate primers of different upstream-sequence exhibited different binding-affinities (Wallweber *et al.*, 2003) – indicative of the

interaction with protein predominating. The RNA component of telomerase is less well conserved at the sequence-level, but structural similarities between the phyla have been reported (Chen *et al.*, 2000; Theimer and Feigon, 2006). Human TR contains an 11 nucleotide template region, acting to align the substrate and to code for the telomeric repeat sequence (*figure 1.3*), along with pseudoknot, transactivating and stability domains (Ly *et al.*, 2003; Tesmer *et al.*, 1999; Ueda and Roberts, 2004). Association of small nucleolar RNA-binding proteins is important in the stabilization of hTR (Pogacic *et al.*, 2000). In addition, hTR contains a 3' H/ACA motif required for dyskerin binding (Egan and Collins, 2012; Lukowiak *et al.*, 2001) and a CAB box necessary for the accumulation of hTR in Cajal bodies (CBs) (Cristofari *et al.*, 2007; Jady *et al.*, 2004) – a requirement of telomerase assembly.

The combination of purified hTERT and hTR requires the presence of eukaryotic cell lysate for the formation of active telomerase, suggesting holoenzyme assembly is a regulated process (Weinrich *et al.*, 1997). In ciliates, component-association during telomerase assembly was shown to occur in a step-wise manner (Stone *et al.*, 2007). Human TERT contains a nucleolar localization sequence, important for enzyme assembly (Etheridge *et al.*, 2002; Yang *et al.*, 2002). The telomerase holoenzyme, resident in CBs, is then recruited to the telomere during S-phase of the cell cycle (Jady *et al.*, 2006; Tomlinson *et al.*, 2006; Zhao *et al.*, 2009), with a substrate-preference for shorter telomeres (Britt-Compton *et al.*, 2009). Telomerase was shown to preferentially interact with substrates bound by TPP1/POT1 (Latrack and Cech, 2010), and indeed the presence of TPP1 was sufficient to recruit telomerase to an artificial, non-telomeric locus (Zhong *et al.*, 2012). Mutational studies allowed further refinement and identified the TEL-patch of amino acids as being the critical site for telomerase recruitment

(Nandakumar *et al.*, 2012). Indeed TPP1/POT1 was shown to be a processivity factor for telomerase, acting to reduce enzyme-substrate dissociation (Latrack and Cech, 2010; Nandakumar *et al.*, 2012). Recent work has raised the possibility that delivery of CB-localized telomerase to the telomere involves the protein HOP1 (Kappei *et al.*, 2013).

Zhao *et al.* (2011) suggested that a single telomerase complex was responsible for elongating telomeric G-strands by ~ 60 nucleotides in a processive manner, under homeostatic conditions. In contrast, critically short telomeres are thought to be preferentially elongated by multiple telomerase complexes, highlighting the need for careful interpretation of mechanistic studies performed using artificially shortened telomeres (Zhao *et al.*, 2011). A possible mechanism for this processive elongation was proposed following the modelling of experimental findings (Steczkiewicz *et al.*, 2011). The recently described CST (CTC1/STN1/TEN1) complex was identified as promoting the termination of elongation by substrate-sequestration (Chen *et al.*, 2012). POT1 may also be involved in the regulation of telomerase activity at the telomere (Lei *et al.*, 2005). Fill-in of the C-strand occurs during late S-phase (Zhao *et al.*, 2009). Under equilibrium-conditions, the majority of cancer cell telomeres are elongated each S-phase (Zhao *et al.*, 2009).

Regulation of telomerase activity appears to be a highly dynamic process. Telomerase activity is grossly regulated at the transcriptional level. Stimulation with EGF led to an ERK-mediated increase in hTERT expression and so telomerase activity in cancer cells (Maida *et al.*, 2002). A similar effect was observed downstream of HER2/Neu (Goueli and Janknecht, 2004). Additionally,  $\beta$ -catenin was shown to enhance expression through an interaction with Klf4 and binding to the *hTERT* promoter (Hoffmeyer *et al.*,

2012). The activation of TGF- $\beta$  signalling was found to repress hTERT expression in breast cancer cells (Li *et al.*, 2006). Telomerase has also been shown to be regulated by post-translation modification, for example, enzyme activity was abrogated following incubation with protein phosphatases *in vitro* (Li *et al.*, 1997). Moreover, telomerase activity was increased by addition of a phosphatase inhibitor to breast cancer cells. In agreement with this, consensus Akt phosphorylation sites were identified in hTERT, and addition of Akt kinase stimulated the telomerase activity of melanoma cells (Kang *et al.*, 1999). On the contrary, c-Abl-mediated tyrosine phosphorylation inhibits telomerase activity (Kharbanda *et al.*, 2000). An interaction between hTERT and NF $\kappa$ B p65 was postulated to mediate the observed stimulation of telomerase by TNF $\alpha$ ; by promoting the nuclear translocation of the enzyme (Akiyama *et al.*, 2003). Similarly, an association between hTERT and the 14-3-3 proteins was found to be necessary for the nuclear localization of telomerase (Seimiya *et al.*, 2000).

### **1.2.1.3. Telomerase expression promotes cell proliferation**

Paediatric low-grade glioblastomas (PLGG) lack telomere-maintenance pathways and exhibit spontaneous tumour regression (Tabori *et al.*, 2006). In contrast, high-grade gliomas frequently overexpress telomerase activity. Patients demonstrating more aggressive and recurrent PLGG frequently have longer initial telomere-length – highlighting the importance of this tumour suppressive mechanism.

Expression of hTERT has been shown to have prognostic significance in colorectal cancer, with patients expressing above median levels of hTERT mRNA exhibiting significantly worse overall survival (hazard ratio of 3.30) (Bertorelle *et al.*, 2013). The

forced overexpression of murine TERT (mTERT) in mouse skin increased chemically-induced and spontaneous tumorigenesis (Gonzalez-Suarez *et al.*, 2002). Analysis of tumour derived cells suggested gene amplification yielding greater than five copies of *hTERT* often occurred in breast, cervical and lung cancer (Saretzki *et al.*, 2002; Zhang *et al.*, 2002; Zhang *et al.*, 2000). Furthermore, mutations in the *hTERT* promoter associated with increased transcription were found in > 70 % of metastatic melanoma samples tested (Horn *et al.*, 2013; Huang *et al.*, 2013). Forced expression of wild-type p53 in cancer cells was shown to repress the transcription of *hTERT* (Shats *et al.*, 2004). In non-malignant cells, the *hTERT* gene resides in a DNase I-resistant region of condensed chromatin and is therefore silenced (Wang and Zhu, 2004). Relaxation of this chromatin domain, following inhibition of histone deacetylases, led to the transcription of *hTERT* in telomerase-negative cells. Antagonistic chromatin modification directed by c-Myc and Mad1 was shown to be involved in the control of hTERT expression (Xu *et al.*, 2001). Similarly, chromatin modification was implicated in mitogen-stimulation of hTERT expression in lymphocytes (Ge *et al.*, 2006)..

Human TR is expressed throughout development and associates with undifferentiated progenitor cells (Yashima *et al.*, 1998). In the adult, hTR expression comparable to that typically found in carcinomas, was only observed in the postpubertal testis. Differentiated normal cells lack robust hTR expression (Soder *et al.*, 1998). Expression of hTR is often upregulated in cancer, including carcinoma of the lung, breast, ovary, cervix and testis (Soder *et al.*, 1998). Indeed, amplification of the *TERC* gene, encoding hTR, had diagnostic potential in cases of suspected cervical cancer (Heselmeyer-Haddad *et al.*, 2005). Amplification of *TERC* has been observed in carcinoma cells, particularly those derived from non-small cell lung cancer patients (Soder *et al.*, 1997;

Yokoi *et al.*, 2003). Human TR was found to be haploinsufficient in stem-cells and its absence associated with telomere-defect disease, suggesting it may be limiting for telomerase activity (Marrone *et al.*, 2004). Genetic ablation of *mTERC* in mice led to a reduced incidence of cancer (Gonzalez-Suarez *et al.*, 2000). Cells derived from these knockout mice had severe genomic aberrations (Blasco *et al.*, 1997). The hammerhead ribozyme-mediated cleavage of hTR was sufficient to inhibit telomerase activity and limit the growth of cancer cells *in vitro* (Folini *et al.*, 2000; Yokoyama *et al.*, 1998). Accordingly, knockdown of hTR using short-hairpin RNA (shRNA) reduced the growth of cancer cells *in vitro* (Li *et al.*, 2005).

In addition to its canonical role in maintaining telomere length, telomerase has been implicated in the telomere-independent promotion of proliferation. Human TERT has been proposed to possess endonuclease (Huard and Autexier, 2004), terminal transferase (Lue *et al.*, 2005), RNA polymerase (Maida *et al.*, 2009) and telomere-independent reverse transcriptase (Sharma *et al.*, 2012) activities *in vitro*. Despite murine telomeres being longer than those of the human, and so rarely limiting for proliferation, telomerase upregulation is a characteristic of many mouse cancers (Broccoli *et al.*, 1996). Overexpression of a catalytically inactive mTERT mutant in keratinocytes was sufficient to promote proliferation and activate hair follicles in mouse skin (Choi *et al.*, 2008). The apoptotic response exhibited by breast cancer cells following knockdown of hTERT was rescued by the expression of a catalytically-dead mutant (Cao *et al.*, 2002). Similarly, overexpression of mutant hTERT reversed Bcl-2-inhibition-mediated cell death (Del Bufalo *et al.*, 2005). Ectopic expression of hTERT was found to inhibit p53-dependent apoptosis in colon carcinoma cells, following

treatment with mitomycin-C and 5-fluorouracil, irrespective of catalytic activity (Rahman *et al.*, 2005).

Such telomere-independent effects may indicate a role of hTERT in apoptosis signalling pathways. Interestingly, hTERT was identified as a substrate of caspase-6 and -7 (Soares *et al.*, 2011). Additionally, transcriptional analysis suggested hTERT may promote Myc- and Wnt-related modulation of gene expression (Choi *et al.*, 2008). Moreover, hTERT was found to interact with the  $\beta$ -catenin transcriptional complex and activate Wnt-response gene promoters (Park *et al.*, 2009). Chromatin immunoprecipitation experiments identified hTERT bound to Wnt-responsive promoters. Telomerase expression has also been correlated to activation of genes associated with proliferation and survival in lens epithelial cells (Xiang *et al.*, 2000; Xiang *et al.*, 2002), and angiogenesis in cancer cells (Zhou *et al.*, 2009). Depletion of hTR led to changes in the expression of genes involved in angiogenesis and metastasis in cancer cells (Li *et al.*, 2005).

Thus, telomerase may have additional pro-survival roles in cell signalling and transcriptional regulation. Indeed, in a recent review it was proposed that, through signalling, telomerase upregulation may assist in the maintenance of many of the hallmarks of cancer (Low and Tergaonkar, 2013).

### **1.3. Radiolabelled telomerase inhibitors for the molecular radiotherapy of cancer cells**

Telomerase activity has been shown to have prognostic significance in several cancer indications, including colorectal (Bautista *et al.*, 2007; Tatsumoto *et al.*, 2000), breast (Clark *et al.*, 1997) and lung (Marchetti *et al.*, 1999) cancer. This, coupled to its profile of expression, has led to development of clinically-relevant anti-telomerase-based therapies of different classes (recently reviewed in Ruden and Puri, 2013).

#### **1.3.1. Targeting telomerase for cancer therapy**

Therapies exploiting telomerase expression have been considered. Oncolytic viruses have been engineered to selectively replicate in telomerase-expressing cells and were therefore capable of inducing cytotoxicity in cancer cells (Sasaki *et al.*, 2011). An anti-cancer vaccine, based around an hTERT-derived peptide, has been developed in an attempt to direct an immune response towards telomerase-overexpressing cells (Kyte, 2009). This strategy has shown potential in phase I/II clinical trial in melanoma (Aamdal *et al.*, 2006), pancreatic cancer (Buanes *et al.*, 2008) and hepatocellular carcinoma (Greten *et al.*, 2010).

Inhibition of telomerase has also been pursued as a therapeutic strategy for cancer. Small molecule agents designed to stabilize G-quadruplexes, found in telomeric DNA, have been shown to inhibit telomere elongation and promote uncapping (Doi *et al.*, 2011; Shin-ya *et al.*, 2001). Perhaps the best characterized, small molecule (Chapter 3) and oligonucleotide (Chapters 4 and 5) inhibitors of telomerase have received much attention (Damm *et al.*, 2001; Herbert *et al.*, 2005). Indeed, an oligonucleotide-targeting

hTR has entered clinical trial for cancer indications including multiple myeloma (Chanan-Khan *et al.*, 2008) and breast cancer (Kozloff *et al.*, 2010).

### **1.3.2. Telomerase-dependent molecular radiotherapy of cancer cells**

As described in Chapter 1.2., the targeting of telomerase in an mRT strategy offers advantages. Telomerase expression in normal tissues is low compared to the majority of carcinomas (Kim *et al.*, 1994; Shay and Bacchetti, 1997). Excluding the germ line, telomerase expression in stem-cell compartments is rarely sufficient to maintain telomere length, as shortening is observed with increasing age (Harley *et al.*, 1990). Thus, targeting telomerase provides a high degree of selectivity for malignant over normal tissue, whilst also being potentially applicable to a wide range of clinical tumour types.

An experimental mRT strategy employed the exogenous expression of the sodium iodide-symporter (NIS), under the control of the *hTERT* promoter (Kim *et al.*, 2012). The symporter was expressed selectively in telomerase-positive cells, enhancing the uptake of beta-emitting iodine-131 by approximately five-fold, and was shown to reduce cancer cell survival *in vitro* and *in vivo*. The *TERC* promoter has also been used in a similar gene therapy strategy (Hingorani *et al.*, 2008). Such strategies offer the advantage of facilitating the internalization of the radionuclide – potentially conferring the advantages of the use of Auger electron-emitting iodine for the treatment of disseminated cells. However, the short radionuclide residency-time associated with NIS and necessity for viral transduction, mean alternative methodology may be more practical. In contrast to this, targeting telomerase with radiolabelled inhibitors would improve the cellular retention of the radionuclide in a telomerase-dependent manner

without the need for gene transduction. The existence and extensive validation of telomerase inhibitors, of high specificity and affinity, which possess anti-proliferative activity themselves, facilitates the development of novel radiopharmaceuticals. In addition, such enzymatic inhibition has been suggested to sensitize cancer cells to therapies of different classes, including IR (Gomez-Millan *et al.*, 2007; Marian *et al.*, 2010a). The nuclear distribution of telomerase in cancer cells potentiates the use of densely ionizing Auger electron-emitting radionuclides, restricting the off-target irradiation of adjacent normal tissue and so making such a therapy relevant to the targeting of micrometastatic disease. However, such an expressing profile necessitates the nuclear trafficking of the labelled targeting molecule.

Despite these advantages, one limitation of this strategy concerns the low abundance of telomerase in cells. This low abundance reduces the potential for the accumulation of radiolabelled inhibitors in malignant cells, so limiting the dose of radiation that can be delivered. Reports have suggested that there may be as few as 50 active telomerase molecules in cancer cells (Cohen *et al.*, 2007). However, particular components of the telomerase complex are expressed at higher levels. For example, it was suggested that HeLa cells contained ~ 50000 molecules of hTR, as determined by competitive RT-PCR (Yi *et al.*, 2001). In contrast, a later work employing Northern blotting and RT-PCR reported ~ 300 copies per cell (Cao *et al.*, 2008). Telomerase-deficient human disease, including dyskeratosis congenita and aplastic anaemia, have been attributed to the mutation of a single allele of *TERC*, the gene transcribed to produce hTR, indicating haploinsufficiency of *TERC* with respect to telomerase activity (Marrone *et al.*, 2004). Thus, the presence of a large excess of hTR compared to hTERT seems unlikely. In order to compensate for this low target abundance, radionuclides with a high Auger electron yield and deposited energy per decay are preferred (*table 1.1*). In this way,

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treated cells are exposed to the maximum number of Auger electrons despite the presence of fewer atoms of the radionuclide, resulting from low target abundance.

The prediction of likely off-target effects of this strategy, mediated following irradiation of telomerase-expressing normal tissue, is facilitated by analysis of human telomere-defect diseases, where the proliferation of progenitor cells is inhibited. Dyskeratosis congenita often results from mutations affecting telomerase function and is largely manifested as intestinal and bone marrow disorders, often leading to the onset of aplastic anaemia (reviewed in Hiyama and Hiyama, 2007).

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**Table 1.1: Physical characteristics of common Auger electron-emitting radionuclides.**

Adapted from Cornelissen and Vallis (2010).

<p>The table originally presented here cannot be made freely available via ORA because of copyright. The table was adapted from Table 1 in Cornelissen, B., and Vallis, K.A., 2010. Targeting the nucleus: an overview of Auger-electron radionuclide therapy. <i>Current Drug Discovery Technologies</i> 7, 263-279.</p>
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## 1.4. Aim of the thesis

The *in vitro* development and validation of Auger electron-emitting radiopharmaceuticals targeting telomerase is described here. Modification of existing small molecule and oligonucleotide inhibitors, interacting directly with telomerase, allowed for labelling with Auger electron-emitting radionuclides. The inhibitory potency, cellular uptake and effect on clonogenic cell-survival of the modified inhibitors are reported, in addition to further mechanistic studies.

This work aims to explore the potential of telomerase as a target for mRT and to identify suitable radiopharmaceuticals for further pre-clinical investigation, so expanding the range of mRT agents under development, designed to target micrometastatic cancer.

## **Chapter 2: Materials and methods**

## Chapter 2: Materials and methods

### 2.1. Cell culture

The cells lines employed in this work include breast (MDA-MB-435, MDA-MB-231/H2N, SKBR3), pancreatic (BxPC-3) and ovarian (HeLa 1.211) carcinoma, and osteosarcoma (U2OS) derived cells. Cells were obtained from the American Type Tissue Culture Collection, with the exception of MDA-MB-231/H2N cells that were stably transfected with *HER2/NEU* courtesy of Dr Robert Kerbel. Cells were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich #D5796) supplemented with 10 % fetal bovine serum (Gibco #10270) and 1 % penicillin/streptomycin/glutamate (Sigma-Aldrich #G1146). Cells were maintained below confluency by regular passaging using 0.05 % trypsin-EDTA (Gibco #25300-054) and were replaced upon reaching passage 25. Cells were regularly checked for Mycoplasma infection using a MycoAlert testing kit (Lonza #LT07), according to the manufacturer's instructions.

### 2.2. Cell lysate extraction

Materials: 1% CHAPS lysis buffer (EMD-Millipore #S7707); BCA Assay kit (Thermoscientific #23225), Bovine serum albumin (Sigma-Aldrich #A7906)

Cells were grown to 80 % confluency, harvested with trypsin and counted using a Neubauer haemocytometer. Cells were pelleted (160 x g; 10 minutes; 4 °C), washed with PBS and re-pelleted. CHAPS lysis buffer was added (200  $\mu$ L/ $10^6$  cells) and cell pellets incubated on ice for 30 minutes. Lysates were then centrifuged (12000 x g; 20 minutes; 4 °C) to remove insoluble material. Lysate protein concentration was

determined using the bicinchoninic acid (BCA) mediated reduction of copper, following the manufacturer's guidelines. The optical density of lysate titrations and a standard series of bovine serum albumin was analysed at 562 nm following incubation with BCA reagents A (bicinchoninic acid) and B (cupric sulphate). Lysates were aliquoted and snap frozen on dry-ice, before storage at -80 °C.

### 2.3. Telomeric repeat amplification protocol

Materials: TRAPeze XL Telomerase Detection kit (EMD-Millipore #S7707); 96-well flat black plate (Costar #3915); Tecan Infinite Fluorescence plate reader (M2000); Read buffer: 10mM Tris-HCl pH 7.4 (Sigma-Aldrich #T5941), 50mM NaCl (Sigma-Aldrich #S7653); 2mM MgCl<sub>2</sub> (Sigma-Aldrich #M1028); Jumpstart-*Taq* polymerase (Sigma-Aldrich #D9307-50UN)

The TRAP assay was set-up as recommended, with the following modifications: the PCR cycle was optimized to 30 °C, 30 minutes; followed by 36 cycles of 94 °C / 30 seconds; 53.5 °C / 30 seconds; 72 °C / 60 seconds; and a final extension step of 72 °C / 3 minutes. PCR reactions contained two units of Jumpstart-*Taq*, 2 µL of CHAPS-extracted cell lysate containing 200 ng of protein (unless otherwise indicated), and 5x Reaction buffer; and were made-up to 50 µL with nuclease-free water. TSR8 template positive (0.2 amoles) and heat-inactivated-lysate (80 °C, 10 minutes) negative controls were included in each experiment. For inhibition experiments, inhibitor or control was added to a total volume of 55.5 µL, to the telomerase extension step (30 °C, 30 minutes). Samples were analysed by addition of 40 µL of each reaction to 160 µL of read buffer in 96-well format using a fluorescence plate reader. Signals attributed to the addition of telomeric repeats to a substrate primer (TS), and so telomerase activity, are represented by fluorescein emission following excitation (485/535 nm). The assay also

includes an internal PCR control template and primer labelled with sulforhodamine (585/620 nm). Data processing involves subtraction of background signals, obtained from extract-free and *Taq* polymerase-free controls, producing telomerase ( $\Delta\text{Fl}$ ) and internal control ( $\Delta\text{Sulf}$ ) signals respectively. Telomerase signals are then normalized to internal PCR control ( $\Delta\text{Fl}/\Delta\text{Sulf}$ ) to allow direct comparison of relative activities. The determined telomerase activity was normalized to untreated control. Data were fitted using fixed-slope non-linear regression and compared using an exact-sum-of-squares F-test in GraphPad Prism 5.0.

## 2.4. Statistical analyses

Data were plotted as the mean  $\pm$  standard deviation of the stated number of replicates, unless otherwise indicated. Data were fitted as appropriate using GraphPad Prism 5.0. The statistical significance achieved is indicated as follows: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

## **Chapter 3: Radiolabelled small molecule inhibitors of telomerase**

## Chapter 3: Radiolabelled small molecule inhibitors of telomerase

### 3.1. Introduction

This chapter describes the establishment of the telomeric repeat amplification protocol (TRAP) for detection of telomerase activity, and the subsequent identification and validation of small molecule inhibitors of telomerase, suitable for labelling with Auger electron-emitting radionuclides. Identification of candidate inhibitors involves the analysis of inhibitory potency and potential to reduce the clonogenic survival of cancer cells *in vitro*.

#### 3.1.1. Small molecule inhibitors of telomerase suitable for radiolabelling

The development of small molecules designed to inhibit telomerase has been extensively pursued. Small molecule inhibitors of telomerase may show activity through several mechanisms of action, including stabilization of telomeric G-quadruplex DNA (Fujimori *et al.*, 2011; Shin-ya *et al.*, 2001; Wei *et al.*, 2013); regulation of telomerase activity through modulation of expression (Chen *et al.*, 2013; Liu *et al.*, 2013), biogenesis (Kim *et al.*, 2008), recruitment (Lee and Chung, 2010) and cell-signalling (Jagadeesh *et al.*, 2006; Ouchi *et al.*, 2005); and direct catalytic inhibition (Damm *et al.*, 2001; Luo *et al.*, 2013; Menichincheri *et al.*, 2004; Naasani *et al.*, 1999; Seimiya *et al.*, 2002). Owing to their direct binding to the telomerase reverse transcriptase protein component (TERT), this latter group of compounds is most

suitable for the development of anti-telomerase radiopharmaceuticals. As the specificity of action of such molecularly-targeted radiotherapy (mRT) agents is derived from the contrast between target presence in disease and absence in normal tissue, compounds directed to ubiquitous structures, such as the telomeres themselves or signalling proteins, are less likely to induce telomerase-specific cytotoxicity. Thus, small molecule inhibitors targeting human TERT (hTERT) directly were selected for study. Small molecule inhibitors of telomerase offer no selectivity of internalization, as lipophilic compounds are expected to non-specifically traverse cell membranes upon administration *in vivo*, but crucially are retained only in cells expressing the target.

In the absence of a high resolution crystal structure of hTERT, direct telomerase inhibitors were largely identified through screening and optimization approaches, particularly focusing on naturally occurring compounds (reviewed in Chen *et al.*, 2011). Nucleosidic inhibitors, such as azidothymidine, show anti-telomerase activity, although such molecules often fail to achieve cancer-specificity due to broad-range polymerase inhibition (Melana *et al.*, 1998). Non-nucleosidic and epigallocatechingallate (EGCG) inhibitors of telomerase were selected for radiolabelling in this strategy, as independent validation of inhibition was available in the literature.

### **3.1.2. Epigallocatechingallate-based telomerase inhibitors**

One such screening approach employed EGCG as an initial compound for the identification of novel telomerase inhibitors (Seimiya *et al.*, 2002). Structure-activity relationship studies improved on the stability of the parent EGCG and yielded MST-312, a telomerase inhibitor with sub-micromolar potency. Long-term administration of MST-312 induced telomere shortening in leukaemia (Seimiya *et al.*, 2002), astrocytoma

(Wong *et al.*, 2009) and fibrosarcoma (Seimiya *et al.*, 2005) cells *in vitro*. Furthermore, treatment of mice bearing non-small cell lung cancer xenograft tumours led to an increase in apoptosis and a decrease in growth (Serrano *et al.*, 2011).

Structural modification of EGCG has also led to the development of flavonoid-based telomerase inhibitors (Menichincheri *et al.*, 2004). A biscatecholic-substituted tetrahydroxyflavanone exhibited sub-micromolar potency *in vitro* and was therefore identified as a promising telomerase inhibitor. Mechanistic analyses suggested EGCG-related compounds exhibit mixed-competitive type inhibition with respect to the TRAP substrate (Cohn *et al.*, 2012).

### **3.1.3. Non-nucleosidic telomerase inhibitors**

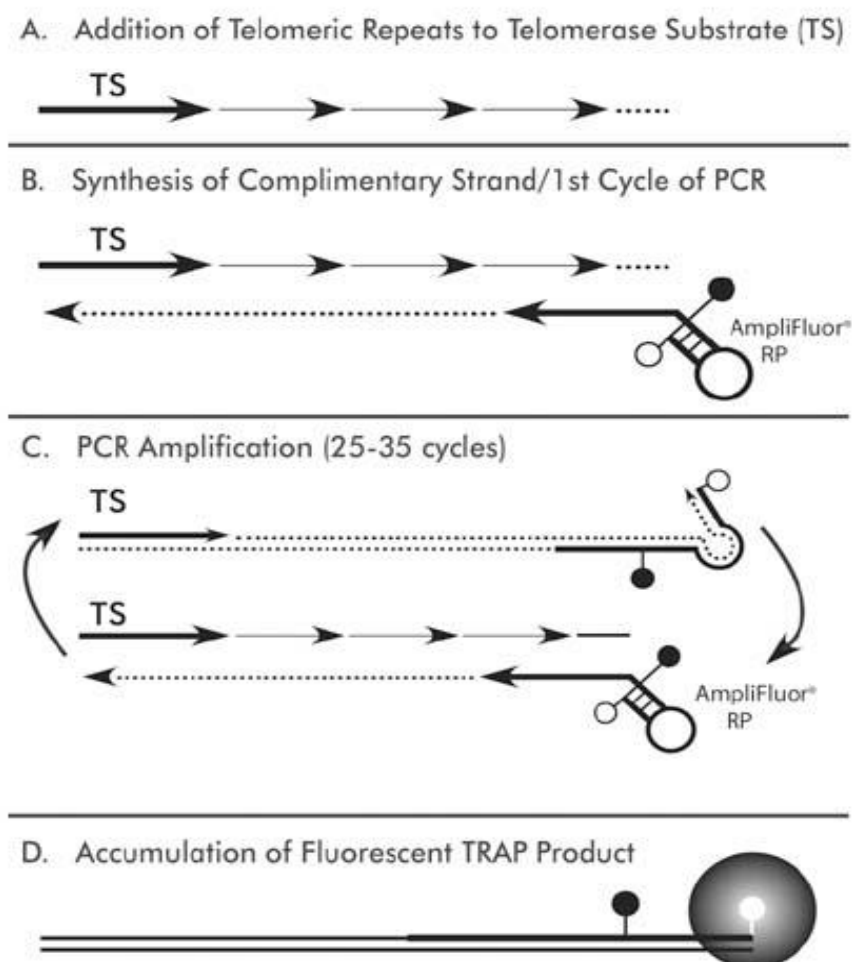
Non-nucleosidic molecules have also been developed (Damm *et al.*, 2001), and are perhaps the best characterized small molecule inhibitors of telomerase. One such compound, BIBR-1532, was found to potently inhibit recombinant telomerase activity *in vitro*. BIBR-1532-treatment induced telomere shortening in fibrosarcoma, and lung, breast and prostate carcinoma cells. Prolonged treatment eventually led to a marked reduction in cell proliferation, inhibition of colony formation and adoption of a senescence-like phenotype, following a lag period of around 130 population doublings necessary for critical telomere erosion. Such an effect was found to be reversible and limited to telomerase-positive cancer cells. BIBR-1532 was separately shown to elicit mixed type inhibition and may reduce telomerase processivity (Cohn *et al.*, 2012; Pascolo *et al.*, 2002). Oral administration of BIBR-1532 at a dose of 100 mg/kg/day delayed tumour growth in telomere-shortened fibrosarcoma xenograft-bearing nude mice (Damm *et al.*, 2001).

#### **3.1.4. Modification of small molecule inhibitors to facilitate radiolabelling**

As discussed in Chapter 1.3.2., the use of a radionuclide with a good yield of Auger electrons of suitable energy is favourable for this strategy. The introduction of a carbon-iodine bond was selected to facilitate radiolabelling of small molecule inhibitors, as metallic Auger electron-emitting radionuclides require the addition of a bulky chelating agent, which may perturb the pharmacophore, and so target binding. Despite iodine-125 being the best characterized experimental Auger electron-emitting radionuclide, with a high yield of Auger electrons per decay (24.9; *table 1.1*), the 59.4 day half-life and lack of imageable  $\gamma$ -photon have limited the clinical applicability of this nuclide. Therefore, iodine-123, emitting 7.5 Auger electrons per decay, was selected for incorporation owing to its 13 hour half-life and emission of a 200 keV photon. The site selected for radiohalogenation was informed by published structure-activity relationship studies. The position of the iodine was optimized to best preserve inhibitory activity. The exchange of non-radioactive iodine for iodine-123 proceeded via a stannane-precursor molecule, facilitating in-house radiolabelling to high specific activity and radiochemical yield (Oliveira *et al.*, 2012; Tavares *et al.*, 2011).

#### **3.1.5. The telomeric repeat amplification protocol detection of telomerase activity**

The telomeric repeat amplification protocol (TRAP) is the most commonly used assay for the detection of telomerase activity (reviewed in Fajkus, 2006). In this assay, a telomerase substrate (TS) primer is incubated with cell lysates, and, in the presence of active telomerase, extended with hexanucleotide telomeric repeats (*figure 3.1*)



**Figure 3.1: The telomeric repeat amplification protocol.**

The telomerase-mediated extension of a substrate primer (TS) facilitates association of a reverse primer (RP) and so formation of a PCR product. PCR amplification allows the detection of the telomerase-elongated substrate. The RP is labelled with a fluorophore and quenching moiety flanking a stem-loop structure, meaning fluorescence is only recorded following incorporation of the primer into a PCR product. (TRAPeZe XL Telomerase Detection kit, EMD-Millipore). Image provided courtesy of EMD Millipore Corporation. TRAPeZe is a registered trademark of Merck KGaA.

(Kim *et al.*, 1994). Extended substrate primers now associate with a reverse primer, facilitating the formation of a PCR product. This product is amplified by conventional PCR. Utilizing the energy transfer/molecular beacon approach, the reverse primer is labelled with a fluorophore and quenching moiety flanking a stem-loop structure (Tyagi and Kramer, 1996). Thus, fluorescence signals are only recorded following loss of primer secondary structure upon incorporation into a PCR product, providing a measure of the number of extended substrate primers and so telomerase activity (Uehara *et al.*, 1999). A telomerase-independent template and primers are included in each sample and therefore act as a control for the relative efficiency of PCR amplification. The telomerase-associated signal is normalized to the internal PCR amplification control, to give a relative measure of telomerase activity. The non-isotopic fluorescent TRAP assay has been shown to be equally quantitative as the more traditional polyacrylamide gel electrophoresis TRAP (Uehara *et al.*, 1999).

### **3.1.6. Aim and outline**

This chapter aims to describe the identification of small molecule telomerase inhibitors, based around existing non-nucleosidic and EGCG-related compounds, suitable for radiolabelling with Auger electron-emitting iodine-123. To do this, the establishment of a robust telomerase activity detection assay was necessary. The ability of the modified compounds to inhibit telomerase activity was assessed, in order to identify lead candidates for cell studies. The uptake of radiolabelled compounds into cancer cells was monitored over time.

The overarching aim was to assess the potential of radiolabelled small molecule inhibitors to reduce the clonogenic survival of cancer cells *in vitro*, which would act as an indicator of their therapeutic potential in the clinical context.

## 3.2. Materials and methods

### 3.2.1. Small molecules

Small molecule inhibitors were synthesised in-house by Dr Philip Waghorn. All structures were confirmed by  $^1\text{H}$  and  $^{13}\text{C}$  NMR, ESI-MS, HPLC and elemental analyses. Where available, the inhibitory activity of in-house parent compounds was compared to commercial molecules.

### 3.2.2. DNA polyacrylamide gel electrophoresis

Materials: 1x TBE buffer (100mM Tris, 100mM Boric acid; 2mM EDTA); Acrylamide/Bis-acrylamide, 30% solution (19:1) (Sigma-Aldrich #A3449); 10% Ammonium persulfate (Sigma-Aldrich #A3678); 99% N,N,N,N-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich #T9281); 10mg/mL Ethidium bromide (Sigma-Aldrich #E1510); TrackIt 10bp ladder (Invitrogen #10488); TrackIt Cyan/orange loading dye (Invitrogen #10482)

Polyacrylamide gels were poured to a final concentration of 10 % using the following: 1.67 mL Acrylamide/Bis-acrylamide; 50  $\mu\text{L}$  10 % ammonium persulfate; 5  $\mu\text{L}$  TEMED; and made to 5 mL with 1x TBE buffer. Gels were loaded with 6  $\mu\text{L}$  of TRAP products and run at 140 V for 40 minutes; stained with ethidium bromide (1  $\mu\text{g}/\text{mL}$ ) for 20 minutes and destained with water for 20 minutes; before visualization with a UV transilluminator.

### 3.2.3. Internalization of radiolabelled compounds

Materials: 24-well plate (Greiner bio-one #662160); 0.1M Glycine, pH 2.5 (Sigma-Aldrich #G8898); 0.1M Sodium hydroxide (Sigma-Aldrich #72068); Wizard<sup>2</sup> 3” Automatic gamma-counter (Perkin-Elmer 2480)

Cells were harvested into an eppendorf at a density of  $10^5$  cells in 250  $\mu$ L of medium. Cells were treated for the indicated time in a total volume of 500  $\mu$ L of medium, with compound at a concentration of 1.8 and 4.2 nM, at specific activities of 225 and 96 GBq/ $\mu$ mole, for B-5I-A and I-FLAV, respectively. Cell treatment was staggered to allow time-points to be processed together. Following incubation, cells were pelleted by centrifugation (300 x g, 5 minutes) and the medium aspirated and retained. Cells were then washed twice with 500  $\mu$ L and 250  $\mu$ L PBS, and the washes combined with the medium to constitute the free-fraction. Free-fractions were diluted 10-fold to facilitate counting in a  $\gamma$ -counter. Cells were re-pelleted and cell membranes washed using 500  $\mu$ L glycine (pH 2.5) and incubated at 4 °C for 6 minutes before washing with 500  $\mu$ L PBS. Following re-pelleting, 250  $\mu$ L of sodium hydroxide (0.1 M) was added and plates incubated for 20 minutes at room temperature, to lyse the cells. The internalized fraction was then collected and combined with two PBS washes of 500  $\mu$ L and 250  $\mu$ L. Fractions were counted in a Wizard  $\gamma$ -counter. Where appropriate, data were fitted with a two-phase association model and compared using an exact-sum-of-squares F-test in GraphPad Prism 5.0.

### 3.2.4. High performance liquid chromatography

Materials: Acetonitrile (Sigma-Aldrich #34851); Trifluoroacetic acid (TFA) (Sigma-Aldrich #T6508)

HPLC studies of compounds were performed by Dr Philip Waghorn on a Waters 2695 Separations Module with a Waters 2489 UV/Vis detector and Ludlum 2200 radio-detector in series. A Waters C-18 column (4.6 x 250 mm) with UV/Vis detection at  $\lambda_{\text{obs}} = 254$  nm with a 1.0 mL/min gradient elution method was used with the following methods; Method BIBR (Solvent A: acetonitrile with 0.1 % TFA v/v, Solvent B: water with 0.1 % TFA v/v): start 50 % A, gradient over 7 minutes reaching 80 % A, then gradient until 13 minutes reaching 95 % A, reverse gradient until 14 minutes reaching 50 % A, then hold to 15 minutes at 50 % A. Method FLAV (Solvent A: acetonitrile with 0.1 % TFA v/v, Solvent B: water with 0.1 % TFA v/v): start 5 % A, gradient over 15 minutes reaching 95 % A, hold to 16 minutes at 95 % A, reverse gradient until 18 minutes reaching 5 % A, then hold to 20 minutes at 5 % A.

### 3.2.5. Clonogenic assay

Materials: 6-well plate (Greiner bio-one #657160); Methylene blue (Alfa Aesar #A18174); Methanol (Fisher Scientific #M/4000/PC17)

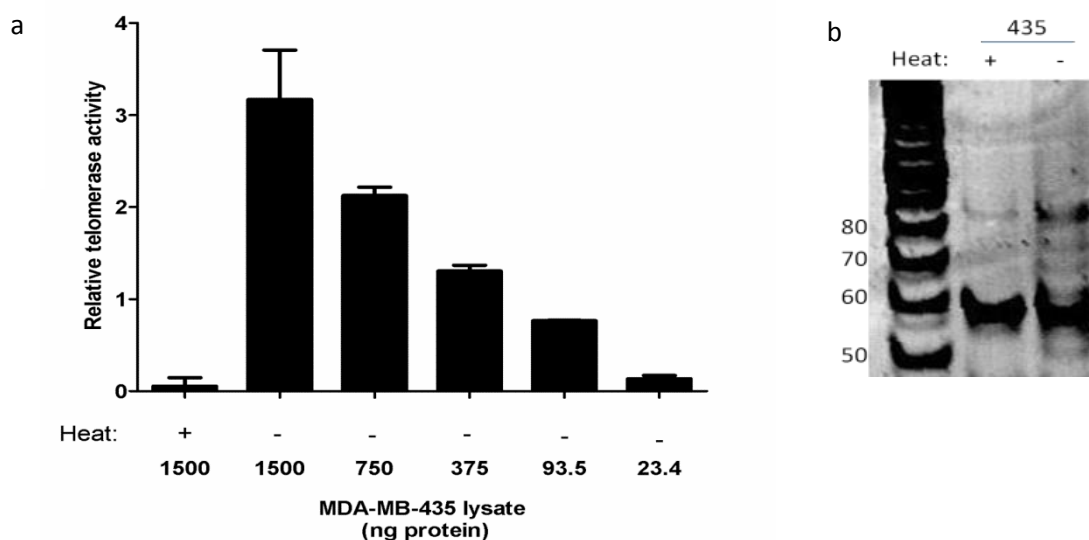
Cells were harvested and added to an eppendorf at a density of  $10^5$  cells in 250  $\mu\text{L}$  of medium. Compound was added to the desired activity concentration (0 – 16 MBq/mL) to a total volume of 500  $\mu\text{L}$  of medium. Solvent or cold compound (to a concentration equivalent to the highest activity) -treated cells were included as a control. Sodium [ $^{123}\text{I}$ ] iodide was used as a control for non-specific uptake of radioactivity. Cells were then incubated for 24 hours before plating in six-well plates at a density sufficient to give > 100 colonies for counting. Untreated cells were typically seeded at 750 cells/well. Colonies were grown for > 7 days, washed in PBS and stained with 1 % methylene blue (in 50 % methanol). Excess stain was washed-off with water. Colonies

containing > 50 cells were counted. The surviving fraction (SF) was calculated using the plating efficiency (PE) of untreated cells. Data were fitted with a one-phase decay model and subjected to a sum-of-squares F-test in GraphPad Prism 5.0.

### **3.3. Results**

#### **3.3.1. Optimization of the telomeric repeat amplification protocol**

Detection of telomerase activity was performed using a modified TRAP assay (Kim *et al.*, 1994). To ascertain the optimal lysate protein content for inclusion in the TRAP assay, a titration of MDA-MB-435 cell lysate was included (*figure 3.2 a*). A linear response to increasing lysate protein content was observed between 93.5 and 1500 ng of protein input. Future TRAP experiments employed 200 ng of MDA-MB-435 lysate. Heat treatment of the lysate is expected to be sufficient to denature heat-labile telomerase and abrogated the signal in the assay. To confirm the specificity of the telomerase signal, the TRAP assay products were examined by polyacrylamide gel electrophoresis (PAGE) (*figure 3.2 b*). In the absence of heat treatment, a characteristic hexanucleotide TRAP ladder was observed. The internal PCR control product was visualized at 56 bp, as expected.

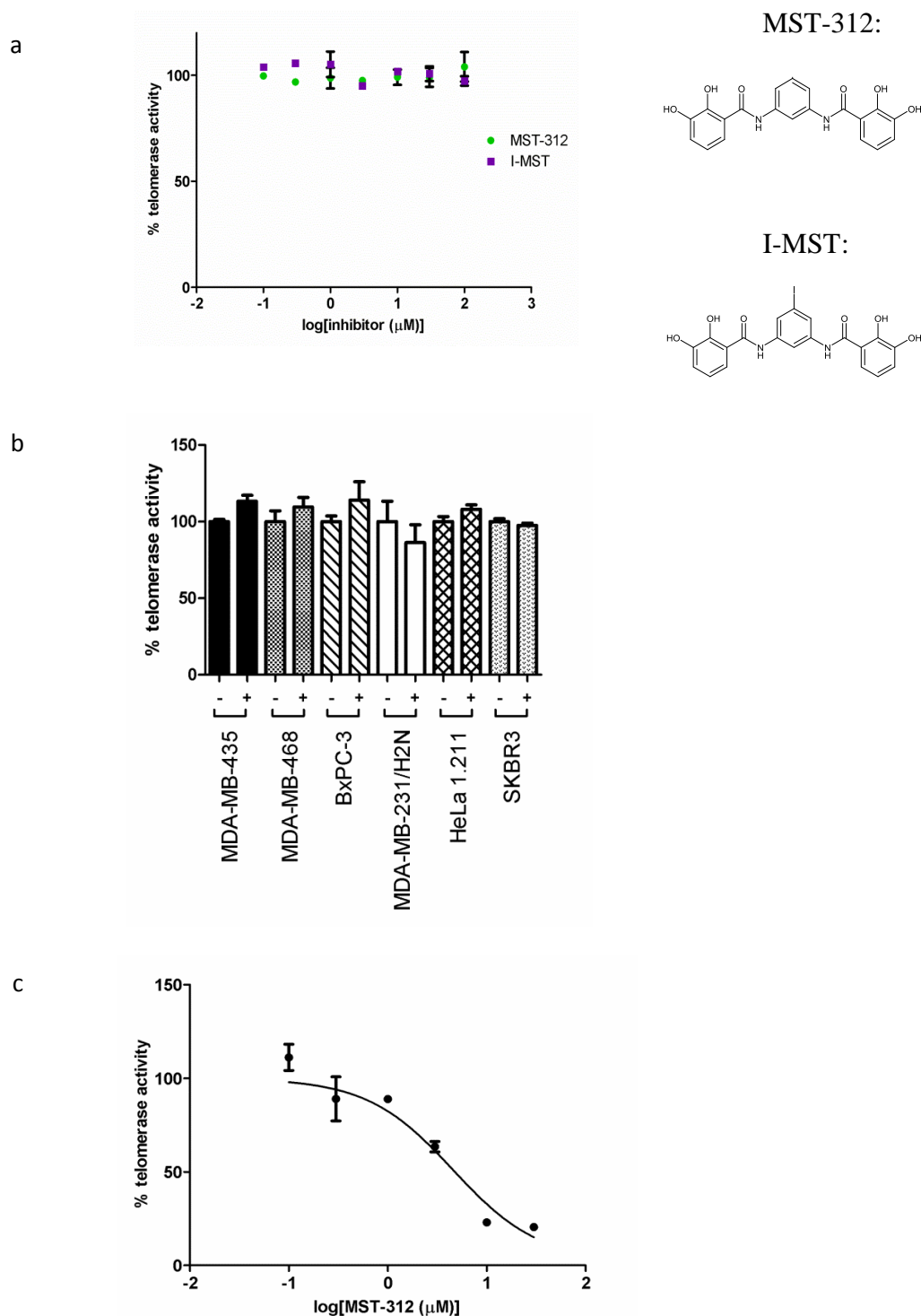


**Figure 3.2: Optimization of the telomeric repeat amplification protocol.**

The optimal lysate protein content acting as the source of telomerase activity in the TRAP assay was established (a). The products generated in the TRAP assay following the inclusion of MDA-MB-435 lysate at 1500 ng were analysed by PAGE (b). The internal PCR control band was visualized at 56 bp. Heat treatment abrogates the positive signal in both cases.  $n=2$ , data represents two independent experiments.

### 3.3.2. MST-312-based telomerase inhibitors

A synthetic small molecule telomerase inhibitor (MST-312), based around functional groups present in the tea catechin EGCG, was identified for labelling with radioiodine. However, both parental and iodine-modified MST-312 molecules were shown to have no inhibitory activity in the direct telomerase detection assay, up to a concentration of 100  $\mu\text{M}$  (figure 3.3 a). In addition, the same concentration of compound was insufficient to inhibit the telomerase activity prepared from several cancer cell lines



**Figure 3.3: The effect of MST-312 on telomerase activity *in vitro*.**

Variants of MST-312 were included in the TRAP assay, using MDA-MB-435 cell lysate as the source of telomerase activity (a). Cancer cell lysates (200 ng) exposed to vehicle (-) or MST-312 at a concentration of 100  $\mu\text{M}$  (+) were included in the TRAP assay (b). MST-312 was incubated with whole MDA-MB-435 cells for three days before lysate production and TRAP assay (c).  $n=2$ , data is representative of at least two independent repeat experiments.

(MDA-MB-435, MDA-MB-468, BxPC-3, MDA-MB-213/H2N, HeLa 1.211, and SKBR3) in the cell-free TRAP assay (*figure 3.3 b*).

Inhibition of telomerase activity was recorded following incubation of MST-312 with cells for three days prior to lysate production (*figure 3.3 c*). A three day incubation was necessary to achieve a concentration of lysate suitable for inclusion in the TRAP assay. In this modified assay the concentration required to elicit half-maximal inhibition ( $IC_{50}$ ) of telomerase activity was found to be  $4.56 \pm 1.40 \mu\text{M}$ .

### **3.3.3. BIBR-1532-based telomerase inhibitors**

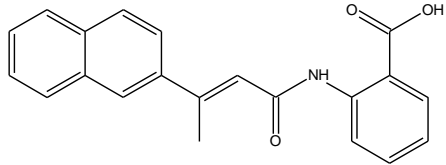
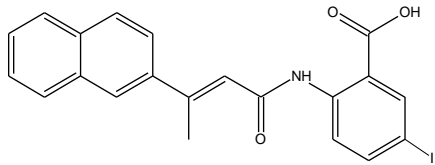
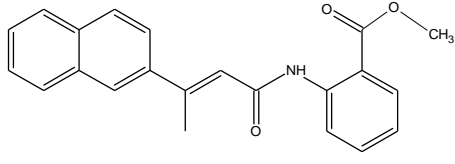
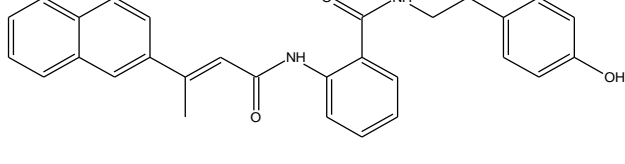
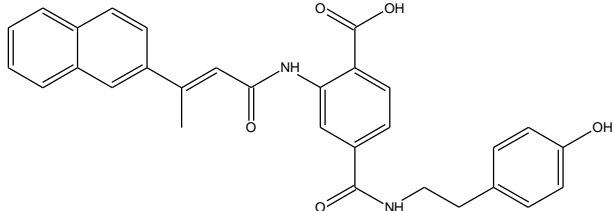
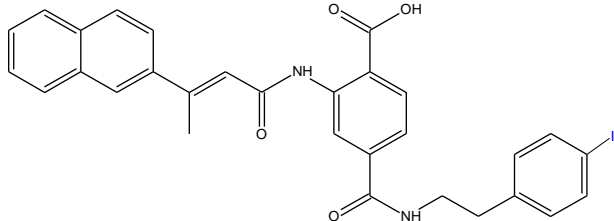
The non-nucleosidic small molecule BIBR-1532 has shown potent and specific inhibition of telomerase *in vitro* and *in vivo* (Damm *et al.*, 2001). As such, this molecule was selected for modification to facilitate radiolabelling. The  $IC_{50}$  of parental BIBR-1532 was found to be  $13.6 \pm 2.3 \mu\text{M}$  in the TRAP assay (*table 3.1; figure 3.4*). Several strategies to incorporate an Auger electron-emitting radionuclide into a BIBR-1532 derived molecule, without significant perturbation of inhibitory activity, were pursued. To assess the effect of modification upon target binding, BIBR-1532-related compounds containing non-radioactive iodine were synthesised, in the first instance. Previous structure-activity relationship studies suggested modification of the naphthyl moiety should be avoided (Barma *et al.*, 2003). Compounds modified at the phenyl ring were found to retain some inhibitory activity (Damm *et al.*, 2001), and so this group was chosen for functionalization. A single carbon-iodine aromatic bond was selected to provide minimal alteration to the parent structure (B-5I-A; *table 3.1*). In addition, compounds with a spacer-arm containing functionality for subsequent labelling with

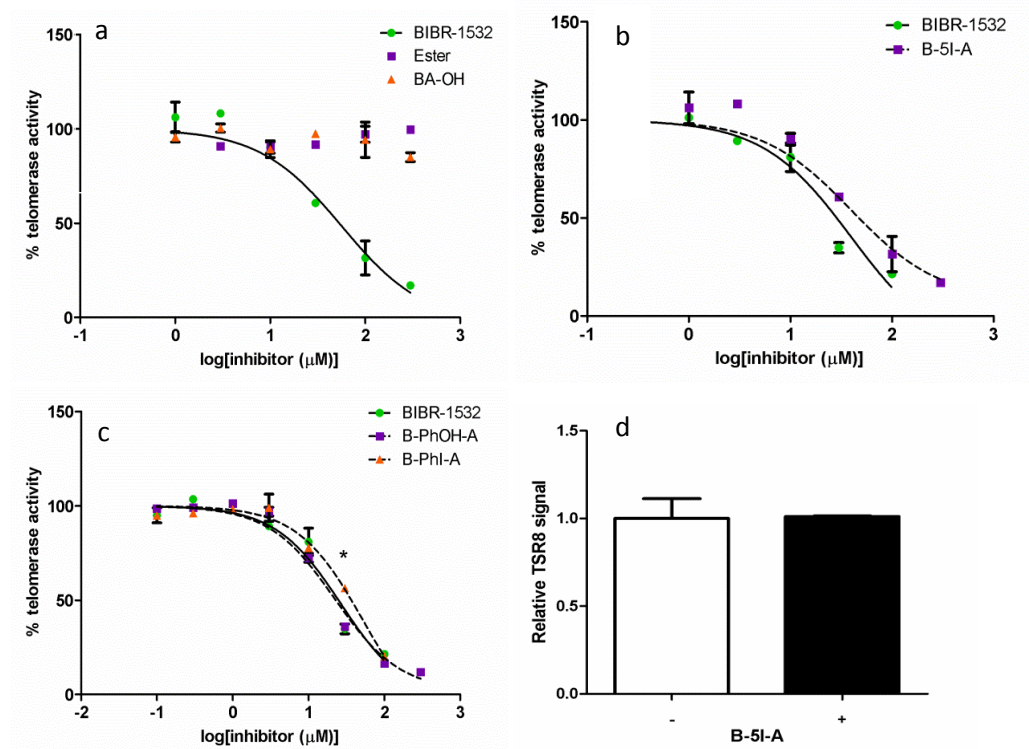
radioiodine were synthesised (B-PhOH-A, B-PhI-A; *table 3.1*). Functionalization of the free-acid group (Ester, BA-OH; *table 3.1*) abrogated inhibitory activity (*figure 3.4 a*). Inhibition of telomerase following addition of an aromatic carbon-iodine bond at position 5 was maintained, albeit with a 2-3 fold reduction in potency ( $IC_{50} 28.0 \pm 3.7$ , *figure 3.4 b*). Addition of the linker present in the compound B-PhOH-A did not affect the inhibitory potency of the molecule ( $IC_{50} 13.1 \pm 3.2$ , *figure 3.4 c*). The iodinated version of this molecule, B-PhI-A showed a loss of inhibitory activity ( $IC_{50} 25.7 \pm 3.7$ , *figure 3.4 c*) similar to that of the simpler B-5I-A molecule. Thus, B-5I-A was selected as the lead candidate for radiolabelling with Auger electron-emitting iodine-123.

To confirm that the compound acted at the telomerase-dependent substrate elongation step of the TRAP assay and not by inhibiting PCR amplification, a pre-elongated substrate primer (TSR8) was employed. Incubation with B-5I-A at a concentration of 300  $\mu$ M did not affect the amplification of this control primer (*figure 3.4 d*).

**Table 3.1: BIBR-1532-derived small molecule inhibitors of telomerase.**

IC<sub>50</sub> values were determined using a cell-free TRAP assay. ND – inhibitory activity not detected. *n*=2, data represents three independent repeat experiments.

Compound	Structure	IC <sub>50</sub> (μM)
BIBR-1532		13.6 ± 2.3
B-5I-A		28.0 ± 3.7
Ester		ND
BA-OH		ND
B-PhOH-A		13.1 ± 3.2
B-PhI-A		25.7 ± 3.7



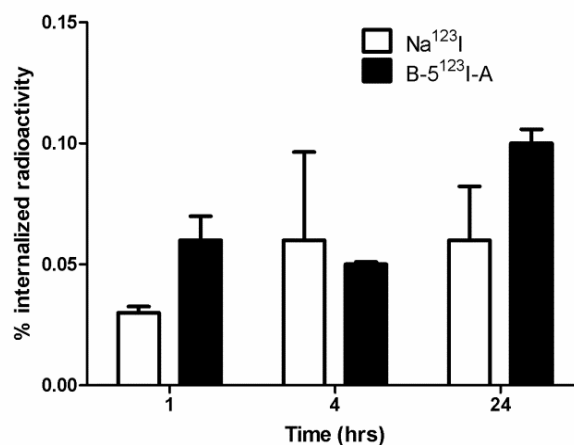
**Figure 3.4: Inhibition of telomerase activity mediated by BIBR-1532 related compounds.**

The inhibitory potency of modified BIBR-1532 compounds was assessed in the TRAP assay (a-c). A pre-elongated substrate primer (TSR8) was incubated with control (-) or B-5I-A at a concentration of 300  $\mu\text{M}$  (+) (d). \*  $P < 0.05$ .  $n=2$ , data represents three independent repeat experiments.

This compound was labelled with iodine-123 with radiochemical yield of  $76 \pm 6 \%$ , at a specific activity of 225 GBq/ $\mu$ mole. The final radiochemical purity was  $> 97 \%$ . The uptake of B-5<sup>123</sup>I-A into MDA-MB-435 cells was monitored over a 24 hour time-course (*figure 3.5*). Maximum cell-internalization, 0.1 % of the administered radioactivity, was recorded at 24 hours. Similar internalization of the radioiodide (Na<sup>123</sup>I) control was observed ( $P > 0.05$ ).

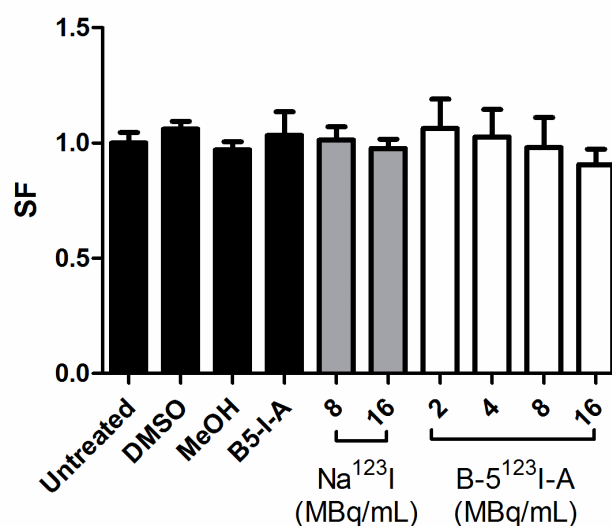
The clonogenic assay was utilized to determine the effect of B-5<sup>123</sup>I-A on cell survival. Following treatment with compound for 24 hours, cells were seeded and allowed to form colonies of more than 50 cells. No significant effect on clonogenic cell survival was observed in this assay (*figure 3.6*).

The stability of the radiolabelled compound incubated in PBS was followed over 24 hours by HPLC (*figure 3.7*). A single radiopeak was observed at all time-points.



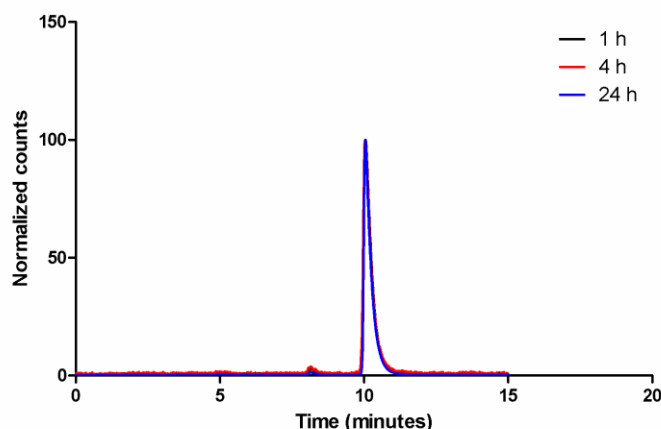
**Figure 3.5: The internalization of B-5<sup>123</sup>I-A into MDA-MB-435 cells.**

The internalization of radioactivity into MDA-MB-435 cells was determined by  $\gamma$ -counting. B-5<sup>123</sup>I-A, or an equivalent activity of Na<sup>123</sup>I, was added to 10<sup>5</sup> cells in 500  $\mu$ L of medium to a final concentration of 1.8 nM per sample.  $n=6$ , from three independent repeat experiments.



**Figure 3.6: The clonogenic survival of MDA-MB-435 cells treated with B-5<sup>123</sup>I-A.**

The clonogenic survival of MDA-MB-435 cells was determined following the treatment of 10<sup>5</sup> cells with solvent control (DMSO, methanol), non-radiolabelled B-5I-A, Na<sup>123</sup>I or B-5<sup>123</sup>I-A (225 GBq/ $\mu$ mole) for 24 hours in 500  $\mu$ L. Cells were plated to allow for colony formation. Colonies of > 50 cells were counted and the surviving fraction (SF) calculated.  $n=6$ , from two independent experiments.

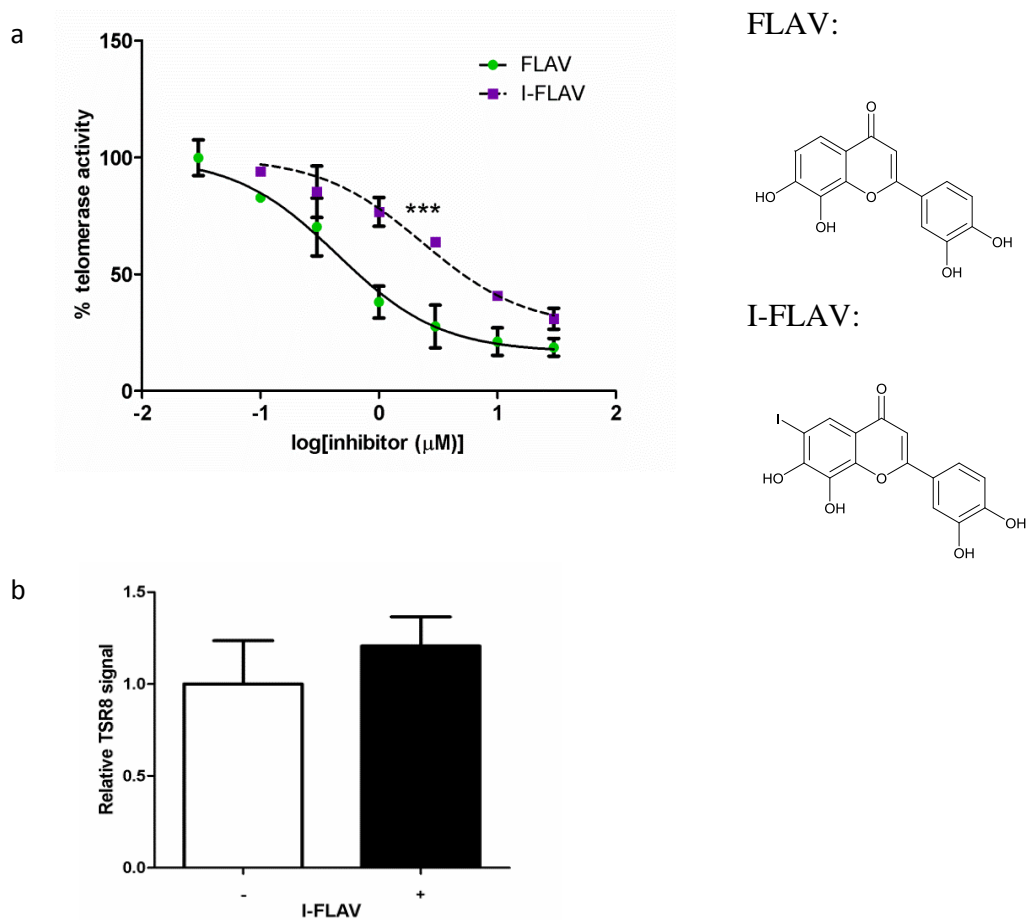


**Figure 3.7: The stability of B-5<sup>123</sup>I-A over 24 hours.**

The radiolabelled compound B-5<sup>123</sup>I-A was incubated in PBS over time. High performance liquid chromatography was employed to separate the radiospecies present in the samples. Experiment performed by Dr P. Waghorn.  $n=1$ , data represents at least two independent experiments.

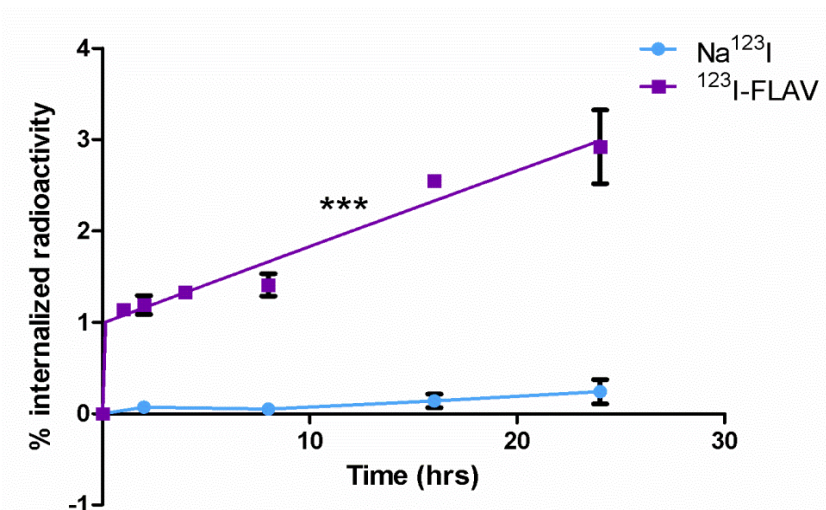
### 3.3.4. Flavonoid-based telomerase inhibitors

A parental and iodine-modified catecholic flavonoid inhibitor of telomerase were synthesised and tested in the telomerase inhibition assay. The parent flavonoid inhibited telomerase with an  $IC_{50}$  of  $0.45 \pm 0.3 \mu\text{M}$  (*figure 3.8 a*). Halogenated variants of the parent structure had been reported previously and such modifications were found to be compatible with maintenance of inhibitory activity (Menichincheri *et al.*, 2004). Thus, an analogous position was selected for iodination. Inclusion of iodine led to a decrease in inhibitory potency, with the iodinated inhibitor having an  $IC_{50}$  of  $2.33 \pm 0.7 \mu\text{M}$  (*figure 3.8 a*). The amplification of the TSR8 substrate primer was unaffected by the presence of compound at a concentration of  $30 \mu\text{M}$  (*figure 3.8 b*).



**Figure 3.8: Inhibition of telomerase activity mediated by flavonoid-based compounds.**

The effect of flavonoid compounds on telomerase activity was established using the TRAP assay (a). A pre-elongated substrate primer (TSR8) was incubated with control (-) or I-FLAV at a concentration of 30  $\mu\text{M}$  (+) (b). \*\*\*  $P < 0.001$ .  $n=4$ , from two independent experiments.

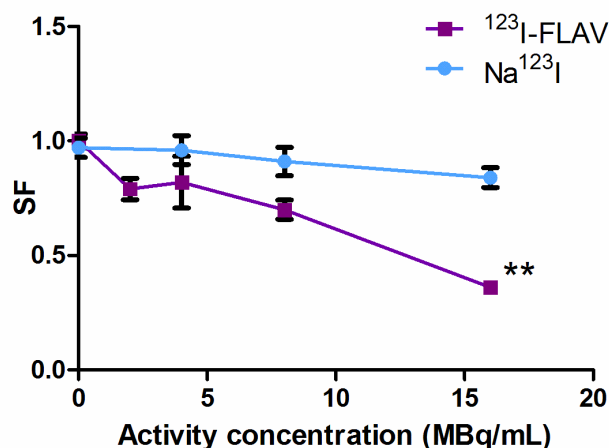


**Figure 3.9: The internalization of <sup>123</sup>I-FLAV into MDA-MB-435 cells.**

The uptake of radioactivity was measured over time by  $\gamma$ -counting. <sup>123</sup>I-FLAV, or an equivalent activity of Na<sup>123</sup>I, was added to 10<sup>5</sup> cells in 500  $\mu$ L of medium to a concentration of 4.2 nM per sample. \*\*\* P < 0.001. n=4, from two independent experiments.

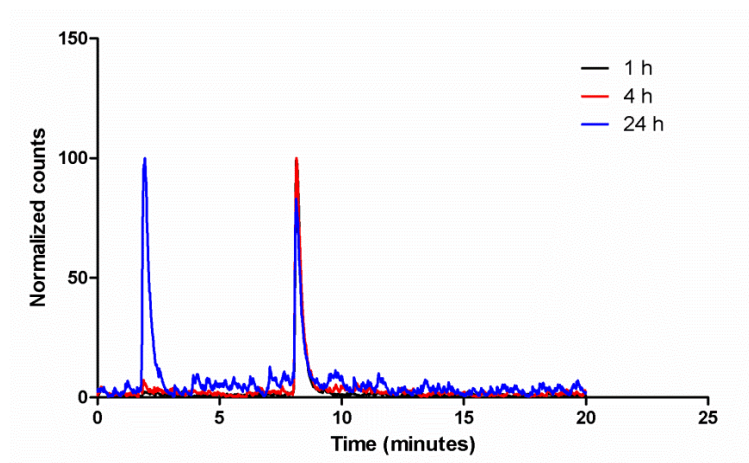
The flavonoid compound was labelled with iodine-123 with a radiochemical yield of 64  $\pm$  4 %, to a specific activity of 96.2 GBq/ $\mu$ mole. The final radiochemical purity of this compound was > 95 %. Following radiolabelling, 2.9  $\pm$  0.4 % of the administered radioactivity was internalized into MDA-MB-435 cells over 24 hours (*figure 3.9*). This internalization was significantly greater than that of the radioiodide (Na<sup>123</sup>I) control.

To determine the effect of radiolabelled flavonoids on clonogenic survival, cells were treated for 24 hours before plating to allow for colony formation. Increasing the concentration of the radioactive compound led to a significant activity-dependent decrease in clonogenic cell survival of MDA-MB-435 cells (*figure 3.10*). Cell survival



**Figure 3.10: Clonogenic assay of  $^{123}\text{I}$ -FLAV in MDA-MB-435 cells.**

The ability of  $^{123}\text{I}$ -FLAV to reduce the clonogenic survival of MDA-MB-435 cells was determined using the clonogenic assay. After harvesting,  $10^5$  cells were treated with  $\text{Na}^{123}\text{I}$  or  $^{123}\text{I}$ -FLAV (96 GBq/ $\mu\text{mole}$ ) for 24 hours in 500  $\mu\text{L}$ , before plating for colony formation. Colonies of > 50 cells were counted and the surviving fraction (SF) calculated. \*\*  $P < 0.01$ .  $n=6$ , from two independent experiments.



**Figure 3.11: The stability of  $^{123}\text{I}$ -FLAV over time.**

The compound  $^{123}\text{I}$ -FLAV was incubated in PBS for the indicated time. The presence of radiospecies was assayed using HPLC. Experiment performed by Dr P. Waghorn.  $n=1$ , data represents at least two independent experiments.

was reduced to  $36 \pm 2$  % following incubation with of  $^{123}\text{I}$ -FLAV at an activity concentration of 16 MBq/mL, compared to  $84 \pm 4$  % with the  $\text{Na}^{123}\text{I}$  control. As with the B-5 $^{123}\text{I}$ -A, the stability of compound was analysed. After 24 hours incubation in PBS, a second radiopeak, eluting at the solvent front, was observed in the HPLC-chromatograph (*figure 3.11*). Thus, the radioiodinated flavonoid compound was found to be unstable, with loss of approximately half of the original material over 24 hours.

## 3.4. Discussion

### 3.4.1. The TRAP assay provides a measure of relative telomerase activity

The establishment of a reliable, cell-free TRAP assay platform was critical to the identification of potential radiolabelled inhibitors of telomerase. The assay was optimized to give a linear response to lysate concentration, and so telomerase activity. The signal was found to be abrogated by heat treatment of the lysate (*figure 3.2*), suggesting a telomerase-dependent effect. Analysis of TRAP products by PAGE (*figure 3.2 b*) confirmed the presence of the characteristic hexanucleotide DNA ladder, indicative of detection of telomerase activity (Uehara *et al.*, 1999).

### 3.4.2. MST-312 may inhibit telomerase through an indirect mechanism

In the direct TRAP assay, MST-312 and an iodinated derivative were found to lack inhibitory activity up to a concentration of 100  $\mu$ M (*figure 3.3 a*). MST-312 also failed to inhibit the telomerase activity in several cell lines (MDA-MB-435, MDA-MB-468, BxPC-3, MDA-MB-231/H2N, HeLa 1.211, SKBR3) in this assay (*figure 3.3 b*), indicating an effect-independent of cell context. This lack of inhibitory activity is in contradiction to previously reported data, where MST-312 was described as a direct telomerase inhibitor (Cohn *et al.*, 2012; Seimiya *et al.*, 2005; Seimiya *et al.*, 2002). Such dissimilarities may reflect differences between the experimental systems employed, including the cell line origin and concentration of lysate used as a telomerase-source, and particularly the TRAP assay substrate. However, MST-312-mediated inhibition of recombinant telomerase, in isolation, has not been reported.

Following incubation with cells and subsequent lysate production, MST-312-mediated telomerase inhibition was recorded (*figure 3.3 c*). Several studies have demonstrated MST-312-mediated inhibition of telomerase activity in whole cells (Gurung *et al.*, 2010; Seimiya *et al.*, 2002; Serrano *et al.*, 2011; Wong *et al.*, 2009). The requirement for incubation of the compound with intact cells here, raises the possibility of an indirect effect upon telomerase activity, for example mediated via modulation of telomerase expression (Greenberg *et al.*, 1999; Maida *et al.*, 2002), or activation of regulatory signalling leading to post-translational modification (Kang *et al.*, 1999; Kharbanda *et al.*, 2000; Li *et al.*, 1997) and cellular relocalization (Akiyama *et al.*, 2003; Seimiya *et al.*, 2000), as shown previously for another small molecule (Jagadeesh *et al.*, 2006; Ouchi *et al.*, 2005). Wong *et al.* (2009) demonstrated an MST-312-mediated acute effect, before the telomeres had measurably shortened in astrocytoma

cells, which involved the instigation of a DNA damage response including ATM activation and H2AX phosphorylation. A similar effect was shown in non-small cell lung cancer cells *in vitro* (Serrano *et al.*, 2011). As a lag-period is anticipated before cellular effect with simple telomerase inhibition, such a finding may suggest an alternative telomere-independent mechanism of action of the compound.

As the direct inhibition, and so interaction of compound with the telomerase complex, was not confirmed, the radiolabelling of MST-312 was not pursued – as direct and specific telomerase binding is critical for the selective irradiation of telomerase-overexpressing tumour cells, in this strategy.

### **3.4.3. Iodinated BIBR-1532 variants inhibit telomerase *in vitro***

Parental BIBR-1532 was demonstrated to inhibit telomerase activity in the TRAP assay with  $IC_{50}$  of 13.6  $\mu$ M (*figure 3.4*). This inhibitory potency was found to be similar to that reported in some previous studies (Barma *et al.*, 2003), but was higher than others (Damm *et al.*, 2001; Pascolo *et al.*, 2002) – again probably representing differences between the experimental systems used. Modification of the parental structure, to facilitate inclusion of iodine-123, was performed and functionalization of the free-acid group was shown, for the first time, to abrogate inhibitory activity (*figure 3.4 a*). Modification of the phenyl ring was found to be compatible with inhibition, as reported previously (Damm *et al.*, 2001). Iodinated compounds did not inhibit the amplification of a pre-elongated control primer (TSR8), suggesting inhibition was occurring at the telomerase-dependent substrate elongation step of the TRAP assay (*figure 3.4 d*). Thus, the lead compound was found to be a telomerase inhibitor. Addition of the iodine atom, either through a direct carbon-iodine bond or longer linker, resulted in an approximately

three-fold decrease in inhibitory activity (*figure 3.4 b & c*), likely due to steric interference although the precise binding orientation is not known. The potency of BIBR-1532-related molecules was relatively poor. However, the structure-activity relationship studies described here, have successfully identified candidate BIBR-1532-derived small molecule inhibitors suitable for proof-of-principle radiolabelling with Auger electron-emitting iodine-123.

#### **3.4.4. Radiolabelled BIBR-1532 is not internalized into breast cancer cells *in vitro***

In further experimentation, B-5<sup>123</sup>I-A failed to significantly internalize into MDA-MB-435 cells *in vitro* (*figure 3.5*). Accordingly, little effect on clonogenic cell survival was noted following treatment with this molecule (*figure 3.6*) – as internalization, and preferably nuclear localization, of Auger electron-emitting radionuclides is necessary for therapeutic effect (Hofer *et al.*, 1975). The lack of uptake of the modified BIBR-1532-compound is seemingly attributed to the addition of iodine, although concentrations greatly above the IC<sub>50</sub> of parental BIBR-1532 (> 10 µM) were required to elicit a cellular response in other studies (Bashash *et al.*, 2013), potentially indicating poor internalization of the parent. The addition of iodine in this strategy, increased the lipophilicity (logP = 1.84) of the compound, but this did not lead to sufficient uptake.

#### **3.4.5. Iodinated flavonoids inhibit telomerase *in vitro***

A flavonoid-based small molecule was found to inhibit telomerase activity with an IC<sub>50</sub> of 0.45 µM (*figure 3.8 a*), making this class of molecule more potent than the BIBR-1532-derived compounds. This inhibitory potency was in agreement with that reported

during the original synthesis of the compound, in a flash-plate telomerase activity assay (Menichincheri *et al.*, 2004). Addition of an iodine atom caused a five-fold reduction in inhibitory activity (*figure 3.8 a*), presumably following a modest perturbation of the pharmacophore and so affinity for target. Incorporation of other halogen atoms, including chlorine, in an analogous position also increased the IC<sub>50</sub> by approximately four-fold (Menichincheri *et al.*, 2004). Using the TSR8 control primer, it was ascertained that the compound specifically disrupted the telomerase-dependent elongation step of the TRAP assay (*figure 3.8 b*).

#### **3.4.6. Radiolabelled flavonoids are readily internalized into and reduce the clonogenic survival of breast cancer cells *in vitro***

In contrast to the BIBR-1532-related molecules, <sup>123</sup>I-FLAV was internalized into breast cancer cells to a maximum of around 3 % of the administered radioactivity (*figure 3.9*). This internalization was significantly greater than that of the radioiodide control, indicating compound-dependent uptake of radioactivity.

The internalization of compound over 24 hours was sufficient to elicit a radioactivity-dependent loss of clonogenic survival in breast cancer cells, significantly above that mediated by the non-specific radioiodide control (*figure 3.10*). As expected, non-radiolabelled compound failed to affect cell survival in this short-term experiment, suggesting the reduction in survival was mediated by the presence of radioiodine. Thus, Auger electron-emitting iodine-labelled flavonoid inhibitors of telomerase were shown to reduce the clonogenic potential of cancer cells *in vitro*.

### 3.4.7. Flavonoid inhibitors of telomerase are unstable

Stability studies suggested that only around 50 % of the radiolabelled flavonoid remained intact after 24 hours, somewhat confounding the interpretation of the cell-based experiments (*figure 3.11*). The appearance of a radiopeak at the solvent front likely represents loss of radioiodide from the compound. The polyhydroxylated flavonoid compounds have been previously reported as being unstable (Menichincheri *et al.*, 2004). However, the presence of two such free hydroxyl groups was found to be necessary for inhibitory activity, with potency increasing with the inclusion of additional hydroxyls. As a result of the lack of stability, investigation into the potential of radiolabelled flavonoid inhibitors was ceased.

### 3.4.8. Conclusions and summary

The aim of this chapter was to identify small molecule inhibitors of telomerase suitable for radiolabelling with iodine-123, as potential therapeutic radiopharmaceuticals. The establishment of the TRAP assay facilitated the assessment of the inhibitory activity of the compounds. Using this assay, small molecule inhibitors of telomerase were identified. Modifications to allow radioiodination were explored and alternative structures retaining inhibitory-capacity were synthesised, albeit with a reduction in inhibitory potency. A small molecule previously identified as a telomerase inhibitor, MST-312, was found to lack activity in our assay. Radiolabelled BIBR-1532-related compounds did not readily internalize into cancer cells *in vitro*, and therefore had no therapeutic effect in a short-term clonogenic assay. Radioiodinated flavonoids internalized into cancer cells and mediated a reduction in clonogenic cell survival. Unfortunately, stability studies suggested that radiolabelled flavonoid inhibitors were

intrinsically labile, leading to a loss of radioiodide. As this loss of compound integrity likely impacts the effect on cells, further studies into this class of compound were terminated.

Overall, potential small molecule telomerase inhibitors were identified but ultimately proved to be unsuitable for future development.

**Chapter 4: The synthesis and validation of  
radiolabelled oligonucleotide inhibitors of telomerase**

## Chapter 4: The synthesis and validation of radiolabelled oligonucleotide inhibitors of telomerase

### 4.1. Introduction

This chapter describes the synthesis and *in vitro* validation of radio- and fluorlabelled oligonucleotide inhibitors of telomerase in cell-free systems. Such validation requires the labelling and purification of oligonucleotides and confirmation that modification does not abrogate binding to target.

Oligonucleotide-based approaches have perhaps been the mostly intensively studied modality for eliciting telomerase inhibition *in vitro* and *in vivo*. Indeed, one such oligonucleotide inhibitor of telomerase (GRN163L – Imetelstat, Geron) is currently undergoing clinical trial in several cancer indications, including multiple myeloma (Chanan-Khan *et al.*, 2008), and locally recurrent and metastatic breast cancer (Kozloff *et al.*, 2010), amongst others (recently reviewed in Ruden and Puri, 2013).

#### 4.1.1. Oligonucleotides as therapeutic agents

Oligonucleotide inhibitors offer exquisite specificity of action and high binding affinity, owing to the necessary formation of specific base-pairing during hybridization. The majority of experimental oligonucleotides function via an RNA interference mechanism, whereby oligonucleotide-binding leads to degradation of target mRNAs and reduced translation (reviewed in Kurreck, 2003). This oligonucleotide-mediated antisense effect has been used as an anti-cancer therapeutic strategy. Antisense against proteins including the Bcl-2 family (Jansen *et al.*, 2000; Miyake *et al.*, 2000); protein

kinase A (PKA) (Tortora *et al.*, 2000); protein kinase C (PKC) (Villalona-Calero *et al.*, 2004); clusterin (Chi *et al.*, 2008); X-linked inhibitor of apoptosis protein (Hu *et al.*, 2003); h-RAS (Cunningham *et al.*, 2001); p53 (Bishop *et al.*, 1996); and Mdm2 (Zhang *et al.*, 2003); amongst others (reviewed in Gleave and Monia, 2005), has been analysed in pre-clinical and clinical investigations with varying success. The potential of oligonucleotides to limit protein function, especially in the absence of suitable small molecule inhibitors, renders this class of molecule a promising therapeutic tool. In addition, oligonucleotides have also been developed as molecularly-targeted radiotherapy (mRT) agents, as discussed in Chapter 1.1.2.3. (Liu *et al.*, 2009; Sedelnikova *et al.*, 2002; Sedelnikova *et al.*, 2000). However, several disadvantages are associated with the use of oligonucleotides. Phosphodiester oligonucleotides are extremely susceptible to degradation upon entry into the organism and cell. In addition, the polyanionic nature of such molecules provides a barrier to cell internalization. Thus, strategies to improve oligonucleotide stability and cellular uptake are critical in the design of potential therapeutic oligonucleotides.

#### **4.1.2. Oligonucleotide inhibitors of telomerase**

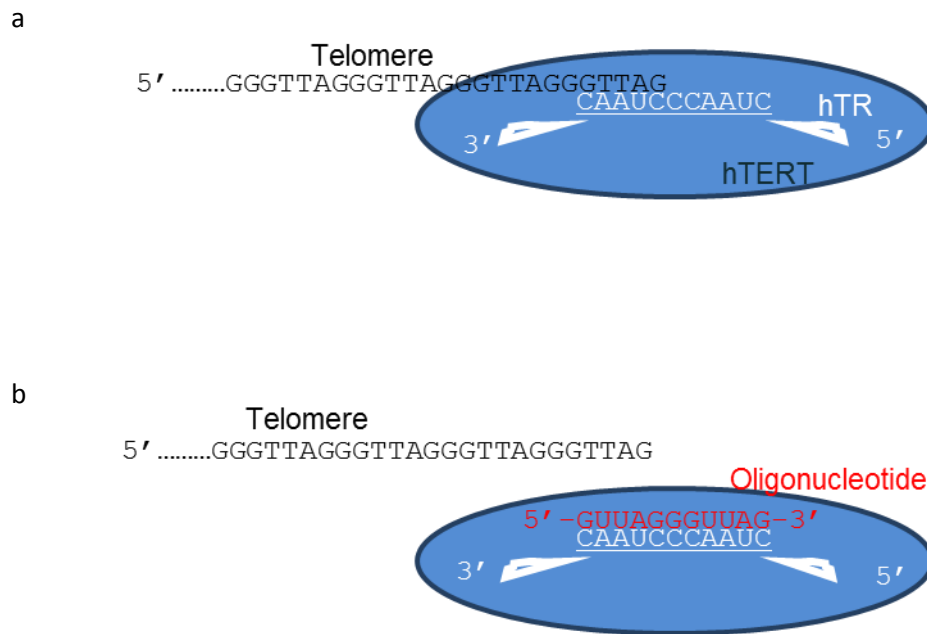
Oligonucleotide inhibitors of telomerase usually target human TR (hTR), the RNA component of telomerase (Feng *et al.*, 1995), although RNA interference against human TERT (hTERT) has also been reported (Chen *et al.*, 2009). As such, RNA degradation, and not inhibition of translation, is most relevant in the context of oligonucleotide-mediated telomerase-antisense. Hammerhead ribozyme-induced degradation of hTR inhibited telomerase activity and slowed the growth of melanoma and endometrial carcinoma cells *in vitro* (Folini *et al.*, 2000; Yokoyama *et al.*, 1998). Similarly,

oligonucleotides modified with 2'-5' oligoadenylate (2-5A) moieties efficiently target complementary RNA for degradation, in this case by RNase L (Zhou *et al.*, 1993). Ovarian carcinoma cells treated with 2-5A oligonucleotides targeting hTR exhibited decreased telomerase activity and cell survival (Kushner *et al.*, 2000).

In addition to degradative mechanisms, template-directed inhibitory oligonucleotides also function as catalytic inhibitors, occupying the telomere-binding region of hTR (*figure 4.1*) (Feng *et al.*, 1995). Such inhibitory oligonucleotides have undergone extensive validation *in vitro*, for example following expression from exogenously inserted DNA. The retroviral expression of a hexanucleotide motif complementary to the template region of hTR inhibited telomerase activity and promoted adoption of a senescence-like phenotype in a proportion of ovarian carcinoma cells *in vitro* (Bisoffi *et al.*, 1998). The delivery of exogenous oligonucleotides has also been pursued. Phosphorothioate (Mata *et al.*, 1997), 2'-O-alkylated RNA (Beisner *et al.*, 2010; Herbert *et al.*, 1999; Pitts and Corey, 1998), thio- N3'-P5' phosphoramidate (Herbert *et al.*, 2005), and peptide nucleic acid (PNA) (Herbert *et al.*, 1999; Norton *et al.*, 1996; Shamma *et al.*, 1999; Villa *et al.*, 2000) oligonucleotides have been demonstrated to inhibit telomerase activity with high potency in cancer cells *in vitro*.

#### **4.1.3. Radiolabelled oligonucleotide inhibitors of telomerase**

The use of oligonucleotides as therapeutic agents has been potentiated by the modulation of nuclease susceptibility, cell permeability and target affinity, following the manipulation of oligonucleotide chemistry (reviewed in Kurreck, 2003). Phosphorothioate (PS) oligonucleotides are commonly employed, as exchange of a non-bridging phosphate oxygen atom for sulphur confers improved stability



**Figure 4.1: Oligonucleotides targeted to the template region of hTR occupy the telomere binding site of telomerase.**

The RNA component of telomerase (hTR) aligns the telomeric substrate and acts as the template for reverse transcription of the six nucleotide telomeric sequence (a). Inhibitory oligonucleotides complementary to the template sequence of hTR compete with telomeric DNA, leading to telomerase inhibition (b).

(Campbell *et al.*, 1990; Koziolkiewicz *et al.*, 1997). Other beneficial modifications to oligonucleotide backbone and pentose-sugar have been proposed, including 2'-O-alkyl-RNA, which exhibits enhanced stability in serum (Pitts and Corey, 1998). However, 2'-O-methyl modified RNA (2'-OMeRNA) oligonucleotides are impotent in the activation of RNase H-mediated RNA interference (Monia *et al.*, 1993), narrowing their potential range of mechanism of action. The successful delivery of oligonucleotides to target cells is perhaps currently the greatest limitation to their clinical use. Despite this, delivery strategies, including nanoparticles (Beisner *et al.*, 2010), internalizing antibody- (Wang *et al.*, 2009a) and peptide-based constructs (Villa *et al.*, 2000), and cationic porphyrins (Benimetskaya *et al.*, 1998), have shown promise.

Oligonucleotides targeting hTR were selected for radiolabelling. One such molecule had been shown to elicit potent inhibition of telomerase activity *in vitro* (Herbert *et al.*, 1999). Following transfection of the oligonucleotide, telomerase was inhibited by 95 %. Prolonged treatment of prostate cancer cells led to telomere shortening and reduced proliferative rate. Cessation of treatment allowed the return to the original telomere length and growth rate, suggesting a reversible effect. Similar results were obtained with PNA oligonucleotides of identical sequence, supporting the specificity of the effect.

Accordingly, the 13-nucleotide sequence of these oligonucleotides, complementary to the template region of hTR (Herbert *et al.*, 1999), was chosen for study (*figure 4.2*). Phosphorothioate and 2'-OMeRNA oligonucleotides of identical sequence were obtained, facilitating the disambiguation of specific and non-specific effects. As the bases of an oligonucleotide are responsible for target binding and to avoid problems

associated with introduction of a weak carbon-iodine bond, the decision was taken to conjugate a chelating agent to the oligonucleotide terminus, to facilitate labelling with Auger electron-emitting indium-111. This radionuclide offers a good yield of Auger electrons per decay (6.8) and imageable photon emissions, and has a clinically suitable half-life of 2.8 days (*table 1.1*). Oligonucleotides with a 5'-amino linker were employed to facilitate the conjugation of the metal chelator diethylenetriaminepentaacetic acid (DTPA), necessary for labelling with indium-111.

#### **4.1.4. Aim and outline**

This chapter aims to identify and validate radiolabelled oligonucleotide inhibitors of telomerase. Initially, the inhibitory activity of a panel of oligonucleotides was demonstrated using the TRAP telomerase-activity assay, established as described in Chapter 3. Oligonucleotides were then conjugated to DTPA, radiolabelled and analysed. The effect of such modification on inhibitory activity was assayed. Stability studies were also performed. Finally, fluorlabelling of oligonucleotides was conducted, to facilitate future investigations.

The overall aim of this work was to identify suitable radiolabelled oligonucleotide candidates for investigation in cellular studies *in vitro*.

hTR	3' -GUCAAU <u>CCCAAUC</u> -5'	
Match	5' -CAGTTAGGGTTAG-3'	PS
Mismatch	5' -CAGTTAGAAATTAG-3'	PS
Match	5' -CAGUUAGGGUUAG-3'	2'OMeRNA
Scramble	5' -GCAGUGUGAUGAU-3'	2'OMeRNA

**Figure 4.2: Sequences of oligonucleotide inhibitors of telomerase.**

Phosphorothioate (PS) and 2'-O-methyl modified RNA (2'OMeRNA) sequences complementary to the template region of the RNA component of telomerase (hTR) were obtained. Mismatched and scrambled controls were also employed. Mismatched bases are shown in red. The bases in hTR prescribing the hexanucleotide telomeric repeat (GGTTAG) are underlined.

## 4.2. Materials and methods

### 4.2.1. Oligonucleotides

Phosphorothioate and 2'OMeRNA oligonucleotides, with or without 5'-amino linkers, were synthesised and HPLC-purified by Sigma-Aldrich (*figure 4.2*).

### 4.2.2. Radiolabelling of oligonucleotides

Materials: Diethylenetriaminepentaacetic dianhydride (cDTPA) Sigma-Aldrich #284025-1G); Sodium bicarbonate (0.1M pH8.3, Sigma-Aldrich #S5761); Sodium citrate (0.1M pH5, Sigma-Aldrich #71498); P4 Bio-gel (BioRad #150-4120); Indium-111 chloride (Perkin-Elmer NEZ304A000MC); Glass microfiber chromatography

paper (Varian #A120B12); DMSO (Sigma-Aldrich #276855); Spectrometer (NanoDrop #ND1000)

Oligonucleotides with 5'-amino linkers were reconstituted in sodium bicarbonate buffer (0.1 M, pH 8.3) to 1 mg/mL. Cyclic DTPA (cDTPA) was made to 10 mM in DMSO. Cyclic DTPA was combined with oligonucleotides to a twenty-fold molar excess, to minimize cross-linking, and incubated at room temperature for 60 minutes. Size-exclusion chromatography (SEC) columns were packed with glass wool and PBS-hydrated P4-biogel beads added to sodium citrate buffer. Reaction products were applied to the column and eluted into 50  $\mu$ L fractions with sodium citrate (0.1 M, pH 5). Fractions were analysed for absorbance at 260 nm using a NanoDrop spectrometer. Analytical radiolabelling was performed in sodium citrate using 0.2 MBq of indium-111 following incubation at room temperature for 60 minutes. Fractions were separated on a P4-column and  $\gamma$ -counted for 60 seconds using a Wizard scintillation counter. Once prepared, DTPA-conjugated oligonucleotides were stored at -20 °C. Oligonucleotides were radiolabelled to the desired specific activity and the radiolabelling efficiency determined by instant thin layer chromatography in sodium citrate (0.1 M, pH 5). The chromatography paper was counted in a Wizard scintillation counter and the proportion of bound radiolabel determined.

#### **4.2.3. Telomeric repeat amplification protocol modified to incorporate radiolabelled oligonucleotides**

Materials: TRAPeze XL Telomerase Detection kit (EMD-Millipore #S7707); 96-well flat black plate (Costar #3915); Tecan Infinite Fluorescence plate reader (M2000); Read buffer: 10mM Tris-HCl pH 7.4 (Sigma-Aldrich #T5941), 50mM NaCl (Sigma-Aldrich #S7653); 2mM MgCl<sub>2</sub> (Sigma-Aldrich #M1028); Jumpstart-*Taq* polymerase (Sigma-Aldrich #D9307-50UN)

In contrast to the standard TRAP assay, radiolabelled inhibitors were combined with MDA-MB-435 lysate at 26.64 ng/ $\mu$ L protein concentration in a total volume of 60  $\mu$ L. Following incubation for 24 hours to allow for radioactive decay, 7.5  $\mu$ L of inhibitor-enzyme complexes was added to 48  $\mu$ L of TRAP PCR mix, as before, to give 200 ng of lysate protein and the final concentration of inhibitor indicated, in the 55.5  $\mu$ L TRAP reaction. The TRAP assay amplification protocol was identical to that described in Chapter 2.3.. TSR8 template positive (0.2 amoles) and heat-inactivated-lysate (80 °C, 10 minutes) negative controls were included in each experiment. Data were fitted using fixed slope non-linear regression and compared using an exact-sum-of-squares F-test in GraphPad Prism 5.0.

#### **4.2.4. Fluorlabelling of oligonucleotides**

Materials: Cy3-Monoreactive dye (GE Healthcare #PA23001); Sodium bicarbonate (0.1M pH8.3, Sigma-Aldrich #S5761); DMSO (Sigma-Aldrich #276855); P4 Bio-gel (BioRad #150-4120), Spectrometer (NanoDrop #ND1000)

Oligonucleotides with 5'-amino linkers were reconstituted in sodium bicarbonate buffer (0.1 M, pH 8.3) to 1 mg/mL. Monoreactive functional Cy3 dye was reconstituted in DMSO and added to the oligonucleotide, before incubation for 60 minutes at room temperature. Reaction products were separated into 50  $\mu$ L PBS fractions using P4 SEC, as before. Fractions were analysed using a NanoDrop spectrometer at 260 and 550 nm. Once prepared, Cy3-conjugated oligonucleotides were stored at -20 °C.

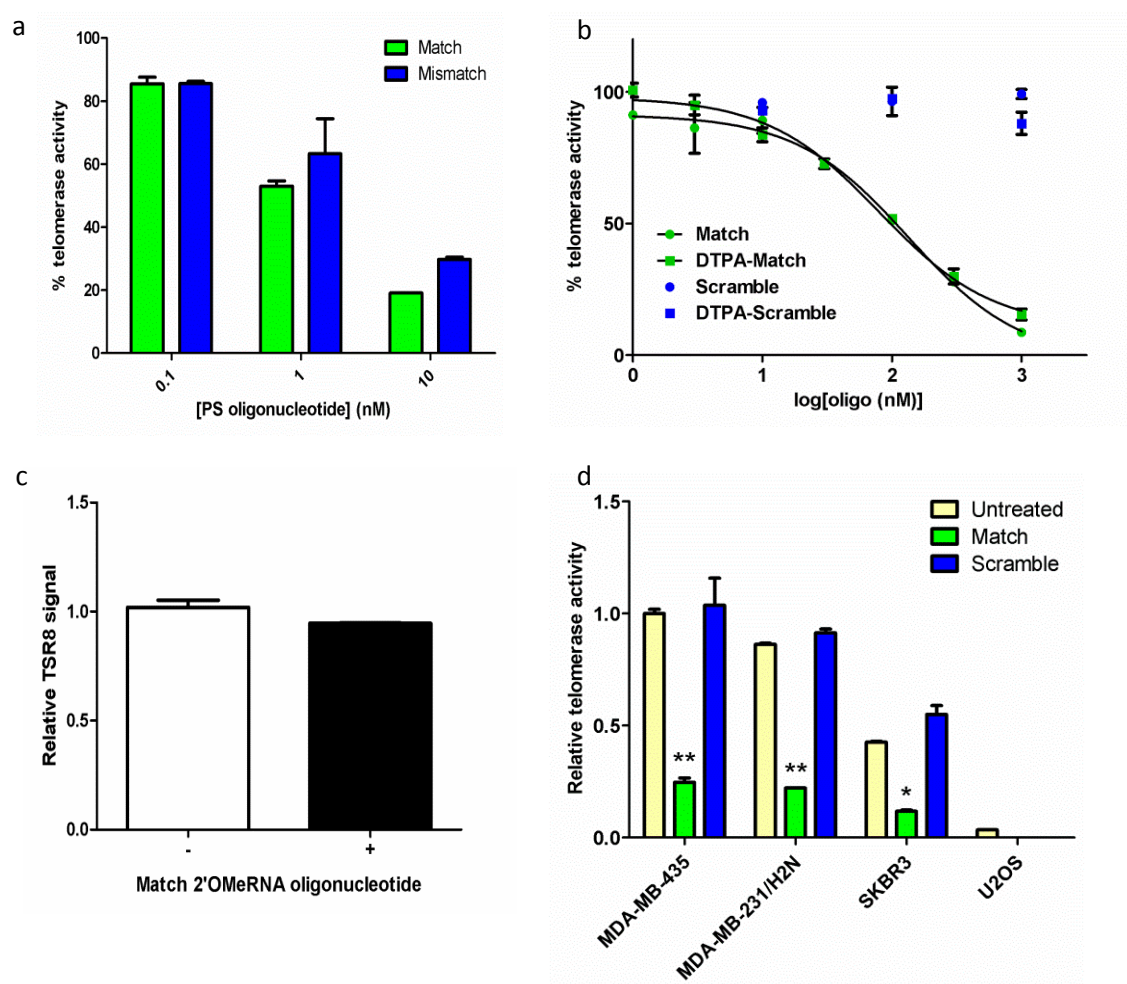
## 4.3. Results

### 4.3.1. Oligonucleotide-mediated inhibition of telomerase activity *in vitro*

The use of modified oligonucleotides is necessary to improve resistance to nucleases, and so the stability of the nucleic acids. Phosphorothioate oligonucleotides exhibited potent inhibition of telomerase activity in the TRAP assay, inhibiting the enzyme by 80 % at a concentration of 10 nM (*figure 4.3 a*). However, a mismatch control oligonucleotide elicited similar inhibition to the targeting molecule, suggesting inhibition was sequence-independent.

As a result, an alternative oligonucleotide chemistry was employed. A concentration-dependent inhibition of telomerase activity was recorded following addition of 2'OMeRNA oligonucleotides to the TRAP assay, with maximum inhibition of 91 % occurring at a concentration of 1  $\mu$ M with the match construct (*figure 4.3 b*). The  $IC_{50}$  of this inhibitor was found to be  $85.9 \pm 1.2$  nM. In contrast to the PS molecules, 2'OMeRNA oligonucleotides of non-complementary sequence showed no inhibitory activity. In addition, conjugation of the metal chelator DTPA, necessary for subsequent radiolabelling with indium-111, to the oligonucleotides did not significantly alter the inhibitory-potency of the match ( $IC_{50}$   $72.3 \pm 1.1$  nM) ( $P > 0.05$ ) or scrambled oligonucleotides.

The match oligonucleotide was introduced into a sample containing a substrate primer pre-elongated with telomeric repeats (TSR8), to ensure the inhibitor was acting specifically at the telomerase-dependent step of the TRAP assay (*figure 4.3 c*). The amplification of the TSR8 substrate was not affected by the inhibitory oligonucleotide at a concentration of 1  $\mu$ M.



**Figure 4.3: Oligonucleotide-mediated inhibition of telomerase activity.**

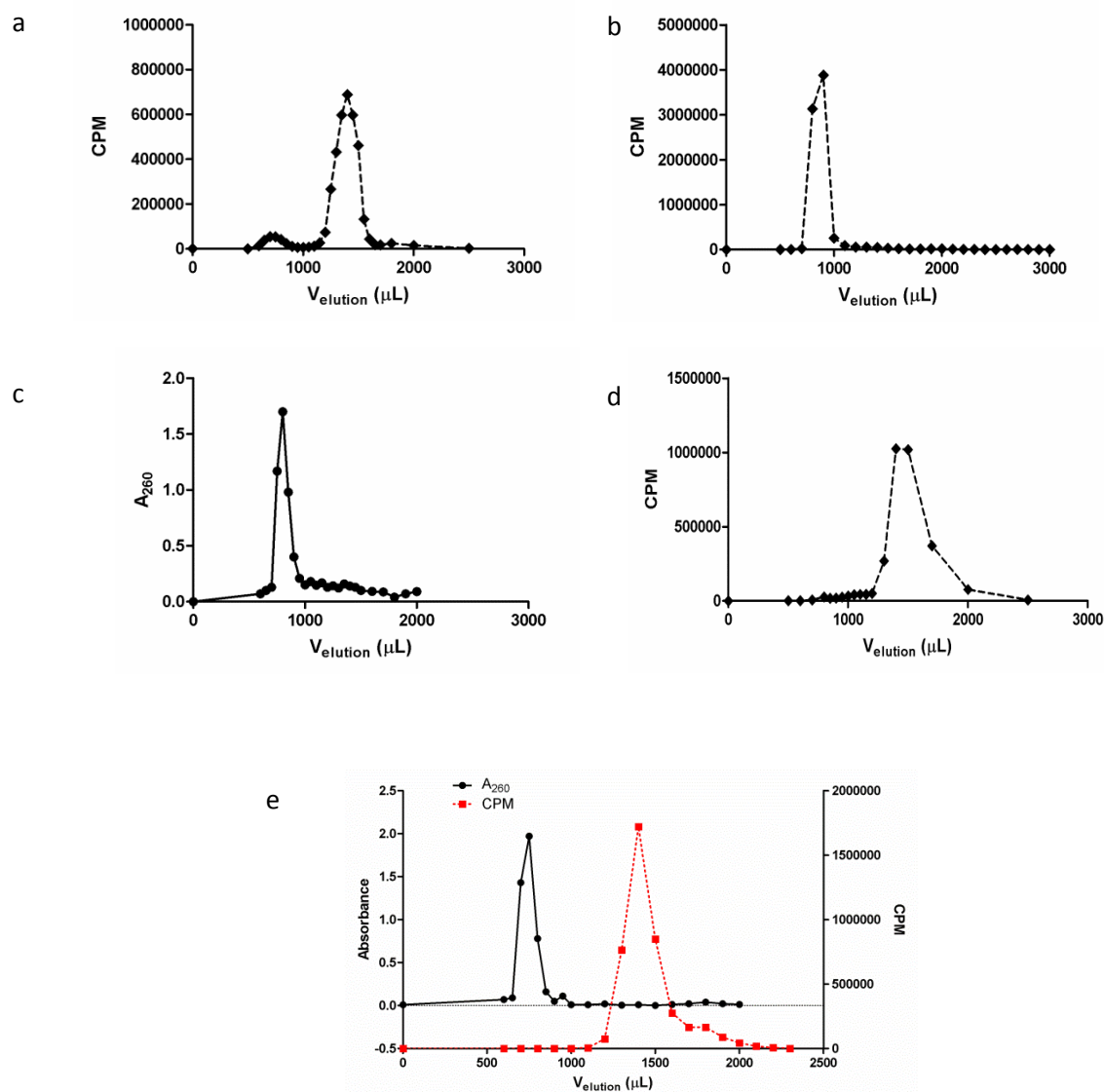
Phosphorothioate (PS) oligonucleotides of complementary and mismatch sequence were included in the TRAP assay (a). Likewise, 2'OMeRNA oligonucleotides were assayed for inhibitory activity, before and after the conjugation of the metal chelator DTPA (b). A pre-elongated substrate primer (TSR8) was incubated with control (-) or match oligonucleotide at a concentration of 1  $\mu$ M (+) (c). Match and scrambled 2'OMeRNA oligonucleotides were incubated with cell lysate derived from breast cancer or osteosarcoma (U2OS) cells at a concentration of 300 nM (d). \*\*  $P < 0.01$ , \*  $P < 0.05$ .  $n=2$ , data represents three independent repeat experiments.

The relative telomerase activity expressed in a variety of breast cancer cell lines was assayed (*figure 4.3 d*). MDA-MB-435 cells were found to have the highest telomerase activity, with MDA-MB-231/H2N and SKBR3 cells containing  $86.3 \pm 0.5$  and  $42.6 \pm 0.4$  % of that activity, respectively. An osteosarcoma cell line, U2OS, was found to lack detectable telomerase activity in the TRAP assay. In each cell system, the oligonucleotide-mediated sequence-dependent inhibition of telomerase was confirmed. Inhibition of 75, 74 and 72 % was recorded in MDA-MB-435, MDA-MB-231/H2N and SKBR3 lysates incubated with 300 nM of complementary oligonucleotide, respectively.

### **4.3.2. Radiolabelling of oligonucleotide telomerase inhibitors**

Oligonucleotides with a 5'-amino-linker were conjugated to DTPA to potentiate subsequent radiolabelling with indium-111. The conjugation reaction products were diagnostically radiolabelled (0.2 MBq) before separation by size-exclusion chromatography (SEC). Analysis of the reaction products by  $\gamma$ -counting demonstrated the elution of two distinct radiopeaks, at 700 and 1400  $\mu$ L, (*figure 4.4 a*). The ratio of the areas under the curve (AUC) defined by the peaks was determined to be 0.06 (AUC:  $9.57 \times 10^6$  and  $1.70 \times 10^8$ ). Unreacted oligonucleotide was found to elute at 700  $\mu$ L by absorbance at 260 nm (*figure 4.4 c*), and radiolabelled, unreacted DTPA at 1400  $\mu$ L (*figure 4.4 d*).

Collection of the fractions constituting the first peak and reinjection onto the SEC column verified the presence of a single radiospecies (*figure 4.4 b*). Radiolabelling to a specific activity of 6 MBq/ $\mu$ g was achieved, with radiolabelling efficiencies routinely > 90 % as assessed by instant thin layer chromatography (ITLC). As a control experiment, oligonucleotides lacking the 5'-amino-modification were incubated with



**Figure 4.4: Radiolabelling of oligonucleotide inhibitors.**

Oligonucleotides modified with a 5'-amino-linker were reacted with cyclic DTPA and radiolabelled with 0.2 MBq of indium-111, before being characterized by size-exclusion chromatography (SEC) (a). The fractions constituting the early eluting peak were radiolabelled to 0.8 MBq/ $\mu\text{g}$  before reinjection onto the SEC column (b). The elution volumes of unreacted oligonucleotide, measured by absorbance at 260 nm ( $A_{260}$ ) (c) and DTPA, measured by  $\gamma$ -counting following radiolabelling with 0.2 MBq indium-111 (d) were recorded. Oligonucleotides lacking the amino-modification were reacted with cyclic DTPA and radiolabelled before SEC fractions were analysed by  $A_{260}$  and  $\gamma$ -counting (e).  $n=1$ , data represents at least three independent repeat experiments.

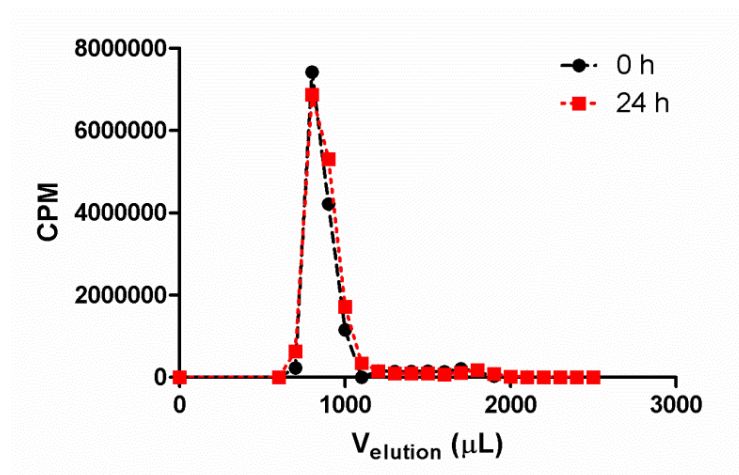
cyclic DTPA as before. The reaction products were separated by SEC and fractions analysed by absorbance at 260 nm and  $\gamma$ -counting following diagnostic radiolabelling. A peak eluting at 750  $\mu$ L exhibited absorbance at 260 nm and a single peak eluting at 1400  $\mu$ L was recorded in the radiotrace (*figure 4.4 e*).

### **4.3.3. Stability of radiolabelled oligonucleotides**

To assess the stability of the radiolabelled oligonucleotides, constructs were radiolabelled to a specific activity of 6 MBq/ $\mu$ g before incubation in 0.1 M sodium citrate (pH 5) for 24 hours and subsequent analysis by SEC. In both the case of oligonucleotides analysed immediately or after 24 hours, a single radiopeak eluting at 800  $\mu$ L was observed (*figure 4.5*). This peak accounted for 92 and 94 % of the radioactivity at 0 and 24 hours respectively, determined by area under the curve analysis.

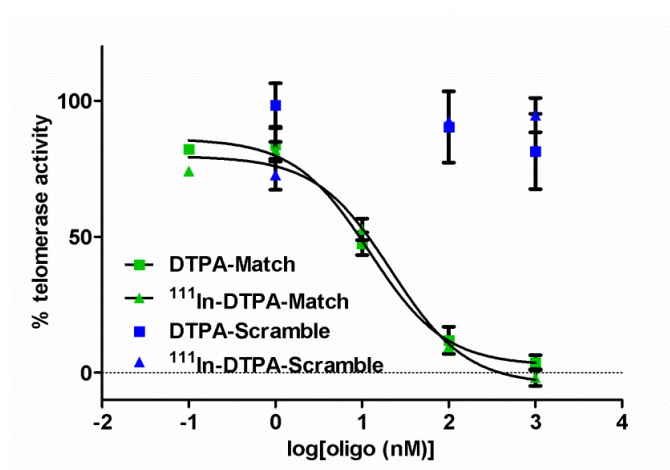
### **4.3.4. Inhibition of telomerase activity by radiolabelled oligonucleotides**

The effect on target binding and inhibitory activity of the addition of the radiolabel to DTPA-conjugated oligonucleotides was assessed using a TRAP assay modified to accommodate radioactive samples. In this system, radiolabelling to 6 MBq/ $\mu$ g and incubation with lysate for 24 hours did not confer inhibitory activity on the scramble control oligonucleotides (*figure 4.6*). The IC<sub>50</sub> of the match oligonucleotides in this modified TRAP assay were found to be  $12.2 \pm 1.2$  and  $21.7 \pm 1.3$  nM for DTPA-Match and <sup>111</sup>In-DTPA-Match, respectively ( $P > 0.05$ ). Inhibition of > 96 % was achieved with 1  $\mu$ M of match oligonucleotide.



**Figure 4.5: Stability of radiolabelled oligonucleotide inhibitors.**

Oligonucleotides were radiolabelled to 6 MBq/ $\mu$ g and subjected to size-exclusion chromatography immediately or after 24 hours incubation in sodium citrate (0.1 M, pH 5).  $n=1$ , data represents at least three independent repeat experiments.

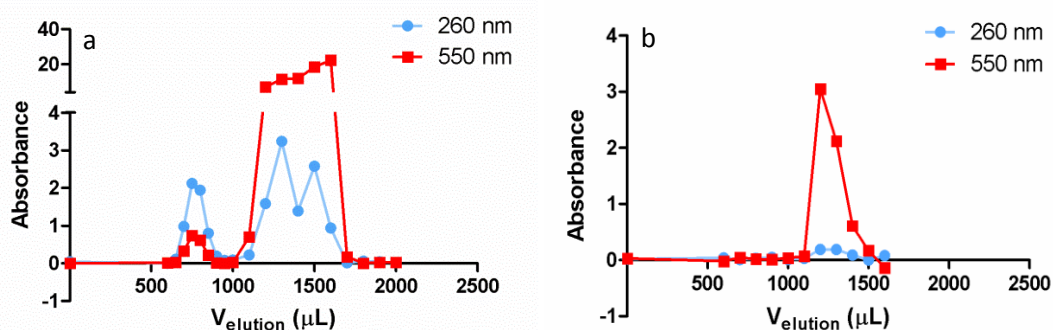


**Figure 4.6: Inhibition of telomerase activity by radiolabelled oligonucleotides.**

Oligonucleotides were radiolabelled to 6 MBq/ $\mu$ g and incubated with MDA-MB-435 lysate for 24 hours. Lysates were then assessed for relative telomerase activity in the TRAP assay.  $n=6$ , data from at least two independent repeat experiments.

### 4.3.5. Fluorlabelling of oligonucleotide inhibitors of telomerase

As successful transfection required adhered cells, the analysis of the subcellular distribution of radioactivity could not be performed using the standard cellular fractionation technique. Thus, targeting and scramble control oligonucleotides were conjugated to a cyanine (Cy3) fluorophore, for the future evaluation of their subcellular distribution by confocal microscopy. Following incubation of the amino-modified oligonucleotides with monoreactive-functional Cy3 dye, the reaction products were separated using SEC and the fractions examined by absorbance at 260 and 550 nm, to allow detection of the nucleic acid and fluorlabel, respectively. An initial peak eluted around 750  $\mu\text{L}$  and corresponded with absorbance measured at 260 and 550 nm (*figure 4.7 a*). A later peak, eluting beyond 1200  $\mu\text{L}$ , exhibited strong absorbance at 550 nm. The fluorlabel alone was found to elute at 1200  $\mu\text{L}$ , and also exhibited some absorbance at 260 nm (*figure 4.7 b*).



**Figure 4.7: Fluorlabelling of oligonucleotide inhibitors of telomerase.**

Oligonucleotides were conjugated with a Cy3 fluorophore and subjected to size-exclusion chromatography (a). Fractions were analysed using absorbance at 260 and 550 nm. Unreacted Cy3 fluorophore was employed as a control (b).  $n=1$ , data from at least two independent repeat experiments.

## 4.4. Discussion

Oligonucleotide inhibitors of telomerase offer exquisite specificity coupled to high binding affinity (Pitts and Corey, 1998). As endogenous nucleic acids are relatively labile, owing to their susceptibility to hydrolysis and nuclease degradation, alternative nucleic acid chemistries are routinely employed in the context of experimental oligonucleotides, including those that target telomerase (Herbert *et al.*, 2005; Herbert *et al.*, 1999; Kondo *et al.*, 1998a; Mata *et al.*, 1997; Norton *et al.*, 1996).

### 4.4.1. Phosphorothioate oligonucleotides inhibit telomerase in a sequence-independent manner

One such modified oligonucleotide chemistry involves the use of PS linkages between nucleotides. Phosphorothioate oligonucleotides exhibited potent inhibition of telomerase activity, but this was found to be largely independent of sequence (*figure 4.3 a*). Such sequence-independent inhibition indicates an alternative mechanism of action, likely involving non-specific interaction of oligonucleotide and hTERT. Non-specific interactions between PS oligonucleotides and proteins have been reported previously (Bock *et al.*, 1992; Guvakova *et al.*, 1995; Rockwell *et al.*, 1997). Moreover, sequence-independent PS oligonucleotide-mediated inhibition of telomerase has been demonstrated. A mismatched PS oligonucleotide inhibited telomerase with potency only 10-fold lower than that of the complementary sequence (Pitts and Corey, 1998). Indeed, an oligonucleotide with little complementarity to hTR inhibited telomerase with an  $IC_{50}$  of 100 nM. Similar findings with non-complementary PS oligonucleotides were reported elsewhere (Norton *et al.*, 1996). A putative non-specific interaction between PS oligonucleotides and a telomeric-substrate binding domain of hTERT was first

proposed by Matthes and Lehmann (1999). Phosphorothioate oligonucleotides lacking complementarity to hTR were found to compete with the telomerase-substrate primer, indicating a shared binding site. The existence of such a protein-substrate binding site, with low sequence-stringency, had been previously predicted (Harrington and Greider, 1991; Morin, 1991). Subsequently, it was discovered that hybridization with the template region of hTR, although important for alignment, contributed minimally to telomerase-substrate stability (Wallweber *et al.*, 2003). As the specificity of action of PS oligonucleotides was not confirmed, an alternative oligonucleotide chemistry was favoured.

#### **4.4.2. 2'OMeRNA oligonucleotides inhibit telomerase activity in a sequence-dependent manner**

In contrast to PS oligonucleotides, 2'OMeRNA oligonucleotides exhibited sequence-dependent inhibition of telomerase activity (*figure 4.3 b*), as shown previously (Beisner *et al.*, 2010; Herbert *et al.*, 1999; Piotrowska *et al.*, 2005; Pitts and Corey, 1998). This class of oligonucleotide inhibited telomerase in a concentration-dependent manner, with high potency ( $IC_{50}$  85.9 nM). This  $IC_{50}$  was higher than those reported previously (Beisner *et al.*, 2010; Pitts and Corey, 1998), likely due to use of alternative TRAP methodology, including a much higher concentration of cell lysate used as the source of telomerase activity. The presence of match oligonucleotide did not interfere with the amplification of the pre-elongated TRAP assay substrate primer (TSR8) (*figure 4.3 c*), suggesting that inhibition occurred during the telomerase-dependent elongation of substrate. The confirmation that PCR amplification was not inhibited during the TRAP assay was particularly important as the targeting oligonucleotide necessarily has a degree of complementarity to the reverse primer used for amplification (Piotrowska *et*

*al.*, 2005). The TRAP assay also indicated that addition of the metal chelator DTPA, did not significantly perturb inhibition and so the interaction of oligonucleotide with telomerase (*figure 4.3 b*). This finding was in agreement with a previous study where the binding of DTPA-modified oligonucleotides to target RNA was demonstrated (Dewanjee *et al.*, 1994).

The relative telomerase activity expressed in breast cancer cell lines was assayed. Robust activity was measured in MDA-MB-435 and MDA-MB-231/H2N cells, with lower levels in SKBR3 cells (*figure 4.3 d*). The majority of clinical breast cancer samples were found to exhibit upregulated telomerase activity (Murillo-Ortiz *et al.*, 2006). A negative signal was recorded following analysis of U2OS, a telomerase-negative osteosarcoma cell line (Scheel *et al.*, 2001), in the assay – further validating the TRAP platform. The potent and sequence-dependent inhibition of telomerase activity in these different cellular contexts provided additional evidence of the specificity of action of the match oligonucleotide. Furthermore, the relative magnitude of the inhibition achieved was consistent between the breast cancer cell systems, suggesting the determined inhibitory potency was largely independent of cell context.

#### **4.4.3. Modified oligonucleotide inhibitors can be radiolabelled to high specific activity with high radiolabelling efficiency**

The metal chelator DTPA has been shown to facilitate the therapeutic labelling of biomolecules with Auger electron-emitting indium-111 to high specific activity (6 MBq/ $\mu\text{g}$ ) with high radiolabelling efficiency (> 90 %) (Cornelissen *et al.*, 2012a; Cornelissen *et al.*, 2012b), and so was selected for use in this study. Analysis of the reaction products following conjugation of DTPA to the oligonucleotides and

subsequent radiolabelling, demonstrated that SEC was capable of resolving two distinct species (*figure 4.4 a*). The peaks seen at 700 and 1400  $\mu\text{L}$  co-eluted with unreacted oligonucleotide and DTPA, respectively (*figure 4.4 c & d*). The two radiospecies separated by SEC were therefore identified as radiolabelled oligonucleotide and excess DTPA. Moreover, calculation of the ratio of the area under the peaks and multiplication by the molar excess of DTPA used (20-fold), confirmed the expected conjugation of approximately one DTPA moiety per oligonucleotide. The presence of a single radiospecies following isolation of the radiolabelled oligonucleotide was indicative of a successful purification (*figure 4.4 b*). Thus, radiolabelled oligonucleotides were successfully synthesised and purified. Instant thin layer chromatography confirmed high radiolabelling efficiency was routinely achieved, highlighting the reproducibility of the method.

To confirm the site-specificity and necessity for radiolabelling of DTPA conjugation, oligonucleotides lacking the amino-modification were incubated with DTPA. Size-exclusion chromatography suggested that oligonucleotide, exhibiting absorbance at 260 nm, eluted at 750  $\mu\text{L}$  (*figure 4.4 e*). Radiolabelling of the same reaction products followed by SEC, demonstrated the absence of radioactivity associated with the oligonucleotide peak, with radiolabelled DTPA eluting at 1400  $\mu\text{L}$ , as before (*figure 4.4 e*). The lack of radiolabelling of the non-amino-modified oligonucleotides in this experiment was indicative of a lack of conjugation to DTPA. These data implied that indium-111 was unable to directly interact with oligonucleotide and that DTPA-conjugation requires the presence of the 5'-amino-linker. Thus, DTPA conjugation was found to be site-specific and radiolabelling dependent on the presence of DTPA.

As Auger electrons act over a limited range (Kassis, 2004), and the metal chelator was separated from the targeting oligonucleotide only by a hexyl-linker, the occurrence of autoradiolysis, either *in cis* or *in trans*, was of concern. Despite this, stability studies showed there was no loss of oligonucleotide-associated radioactivity in the early peak, eluting at 800  $\mu\text{L}$ , following labelling to high specific activity (6 MBq/ $\mu\text{g}$ ) and incubation in sodium citrate (pH 5) for 24 hours (*figure 4.5*). Moreover, no emergence of a second peak, representing free radiolabel or radiolabelled fragments, was observed. This investigation demonstrated that radioactivity is not significantly lost from the oligonucleotide over 24 hours, and therefore autoradiolysis of the 5'-linker is minimal.

The lack of a second peak also suggests that cleavage of the oligonucleotide was not observed. However, SEC provides only a simple separation of molecules based on a threshold size (in this case 4 kDa), and so is incapable of detecting subtle changes in molecular weight. High performance liquid chromatography would provide greater separation of species of similar molecular weight and so could be employed to address this limitation. Alternatively, mass spectrometry may also be useful for analysis of oligonucleotide stability.

#### **4.4.4. Radiolabelled oligonucleotides inhibit telomerase activity *in vitro***

Oligonucleotides radiolabelled to 6 MBq/ $\mu\text{g}$  were incorporated into the TRAP assay to determine whether the presence of indium-111 had an effect on telomerase inhibition. To incorporate radioactive samples, a modified TRAP assay was employed. In this assay, radiolabelled oligonucleotides were incubated with telomerase-containing MDA-MB-435 cell lysate for 24 hours, to allow for any accumulation of radioactive decay-

mediated damage of telomerase and to reduce the absolute radioactivity prior to PCR amplification. The presence of indium-111 did not significantly affect the sequence-dependency or inhibitory potency of the oligonucleotides (*figure 4.6*). These data suggest that the presence of radiolabel does not perturb target binding, compared to non-labelled control. The preservation of inhibitory activity following a 24 hour incubation provides supplementary evidence that the autoradiolytic cleavage of nucleotides within the radiolabelled oligonucleotides did not significantly occur.

It was previously reported that indium-111-labelled oligonucleotides exhibited greater antisense activity compared to non-labelled control, presumably following radiolytic cleavage of the target mRNA (Watanabe *et al.*, 2006). However, no enhancement of inactivation of telomerase, for example as a result of radiolysis of enzyme components, was observed. The induction of indium-111-labelled PNA- or DNA oligonucleotide-mediated cleavage of targeted DNA yielded breakage of < 7 % and < 14 % of molecules after 10 or 12 days, respectively (He *et al.*, 2004b; Karamychev *et al.*, 2000). An equivalent cleavage of telomerase RNA, and so reduction in telomerase activity, may fall below the detection limit of the TRAP assay.

The inhibitory potency of the oligonucleotide in this assay was greater than that reported in the unmodified TRAP assay. This difference is likely due to the pre-incubation of inhibitor with cell lysate, which may provide sufficient time for the establishment of inhibitor-enzyme equilibrium. Accordingly, a higher degree of maximum inhibition (96 %) was achieved compared to the direct TRAP assay (91 %). The IC<sub>50</sub> of complementary oligonucleotides recorded in the modified TRAP was in agreement with previously published data (Beisner *et al.*, 2010), particularly where pre-

incubation was explicitly employed (Pitts and Corey, 1998). Moreover, pre-incubation for several minutes was found to be necessary for maximal inhibition to be elicited by 2'OMeRNA (Pitts and Corey, 1998) and PNA oligonucleotides (Norton *et al.*, 1996). Our preference for instantaneous addition of inhibitor during the unmodified assay discounts the promotion of inhibitor-enzyme complexes prior to the *in vitro* addition of substrate, providing a more physiologically-relevant measure of inhibitory potency, that is readily comparable between inhibitors of different classes.

#### **4.4.5. Oligonucleotide inhibitors were fluorlabelled**

Fluorlabelling of the oligonucleotides was performed to facilitate their detection following administration to cells. An early SEC peak, co-eluting with oligonucleotide alone, exhibited absorbance at both 260 and 550 nm (*figure 4.7 a*), suggesting this peak represented fluorlabelled oligonucleotide. Thus, fluorlabelled oligonucleotides had been synthesised. The late peak was assigned as excess Cy3, as it co-eluted with the dye alone (*figure 4.7 b*). This secondary peak exhibited absorbance at 260 nm, a feature that was also apparent in the Cy3-only control. The absorbance spectrum of Cy3 supported the assumption that the observed absorbance at 260 nm was attributed to the fluorophore. This feature limited the accuracy of determination of the fluorlabelled-oligonucleotide concentration, which relies upon absorbance at 260 nm.

#### **4.4.6. Conclusions and summary**

This chapter aimed to synthesise and validate radiolabelled inhibitory-oligonucleotides targeting telomerase. Oligonucleotides modified to increase their stability were shown

to inhibit telomerase in a potent and sequence-dependent manner, in several cell lysate systems. The metal chelator DTPA was site-specifically conjugated to oligonucleotides and this facilitated radiolabelling to high specific activity. Radiolabelling did not significantly impinge upon oligonucleotide-mediated telomerase inhibition. Radiolabelled oligonucleotides were found to be resistant to autoradiolysis over 24 hours.

Thus, the radiolabelled oligonucleotides were validated *in vitro* as a potential novel therapeutic modality. To further investigate their therapeutic efficacy, the oligonucleotides were taken forward into cellular studies.

**Chapter 5: The *in vitro* determination of the potential  
of radiolabelled oligonucleotide inhibitors of  
telomerase as novel radiopharmaceutical agents**

## **Chapter 5: The *in vitro* determination of the potential of radiolabelled oligonucleotide inhibitors of telomerase as novel radiopharmaceutical agents**

### **5.1. Introduction**

This chapter describes the investigation of the therapeutic potential of radiolabelled oligonucleotide inhibitors of telomerase *in vitro*. The potential therapeutic efficacy of experimental radiopharmaceuticals is best examined using the clonogenic assay to establish the ability of treated cells to form colonies (Franken *et al.*, 2006). Additional mechanistic studies provide key evidence of the specificity of action of radiotherapeutics. Such investigations constitute an essential part of the pre-clinical validation of novel therapeutic entities, often identifying candidate molecules suitable for *in vivo* study.

As described in Chapter 4, oligonucleotides complementary to the RNA component of telomerase, hTR, act as potent catalytic inhibitors in cell-free systems. As a result of this, such molecules have progressed into cell studies *in vitro* and beyond.

#### **5.1.1. Oligonucleotide telomerase inhibitors reduce cell proliferation**

Oligonucleotides targeted to hTR have been shown to elicit the predicted cellular effects associated with loss of telomerase activity. The phosphorothioate (PS) oligonucleotide-mediated inhibition of telomerase reduced the growth of Burkitt's

lymphoma cells *in vitro* and in a xenograft model (Mata *et al.*, 1997). A peptide nucleic acid (PNA) directed to hTR led to telomere shortening and a reduction in survival in immortalized ataxia telangiectasia cells (Shammas *et al.*, 1999). Similarly, following the lag period required for critical telomere erosion, an inhibitory PNA slowed the proliferation and increased the proportion of apoptotic melanoma cells (Villa *et al.*, 2000). A 2'-O-methyl modified RNA (2'OMeRNA) oligonucleotide induced telomere shortening and reduced the growth of non-small cell lung cancer cells (Beisner *et al.*, 2010)

The best characterized oligonucleotide inhibitor, GRN163L (Imetelstat, Geron), was shown to inhibit telomerase activity, induce telomere shortening and reduce proliferation of malignant cells *in vitro* (Gellert *et al.*, 2006; Goldblatt *et al.*, 2009a; Goldblatt *et al.*, 2009b; Gomez-Millan *et al.*, 2007; Herbert *et al.*, 2005). In MDA-MB-231 cells, treatment with GRN163L at a concentration of 1.25  $\mu\text{M}$ , led to growth inhibition of 80 % (Goldblatt *et al.*, 2009b). A second breast cancer cell line, MDA-MB-435, underwent a senescent response following 6 weeks of treatment with GRN163L at a concentration of 1  $\mu\text{M}$ , whereas the proliferation of normal BJ fibroblasts remained unaffected (Gellert *et al.*, 2006). Treatment of MDA-MB-231 cells with this agent caused significant telomere shortening over 129 days. Similar treatment was sufficient to reduce the ability of breast (Gellert *et al.*, 2006; Gomez-Millan *et al.*, 2007) and lung (Dikmen *et al.*, 2005) cancer cells to form colonies *in vitro*. Furthermore, intraperitoneally administered GRN163L, at doses of 30 mg/kg, significantly reduced the growth of an orthotopically implanted breast cancer xenograft (Goldblatt *et al.*, 2009b). Similar treatment of glioblastoma cells led to inhibited telomerase activity, telomere shortening and reduced proliferation *in vitro* (Marian *et*

*al.*, 2010a). Xenograft tumour growth was significantly reduced by prolonged administration of GRN163L in this study.

An oligonucleotide of identical sequence to that described in Chapter 4, slowed the growth of prostate cancer cells *in vitro* following the anticipated lag period (Herbert *et al.*, 1999). Over the course of a 120 day experiment, treated cells underwent 20 fewer population doublings compared to control. In addition, the telomere restriction fragment, indicating average telomere length, shortened in a progressive manner from 3.6 to 2.0 kbp. The cellular effects of the 2'OMeRNA oligonucleotide were found to be fully reversible upon cessation of treatment.

### **5.1.2. Cellular delivery of inhibitory oligonucleotides enhances their potential for translation into the clinic**

The cellular delivery of the various oligonucleotides described frequently relied upon the use of transfection reagents, or electroporation in the case of PNAs – strategies not readily translated into the clinical context. The *in vivo*-relevant delivery of nucleic acid-based therapeutics across the cell membrane represents perhaps the greatest challenge in the field.

Despite this, delivery strategies designed to enhance the cell-internalization of oligonucleotide telomerase inhibitors have been successfully pursued. Simple lipidation of thio- N3'-P5' phosphoramidates has been shown to improve cell uptake (Herbert *et al.*, 2005). Addition of a 5'-palmitoyl moiety to the inhibitory oligonucleotide facilitated the transfection-free reduction in telomere length and reduction in growth rate in immortalized mammary epithelial cells, to a greater extent than the non-modified control. Tumour uptake was observed following intravenous administration of

fluorlabelled palmitoylated-oligonucleotides in a xenograft model of prostate cancer. Such modification facilitated entry of this oligonucleotide into clinical trial (Chanan-Khan *et al.*, 2008; Kozloff *et al.*, 2010). Conjugation of the Antennapedia cell-penetrating peptide to a biotinylated PNA telomerase inhibitor facilitated uptake, as visualized by immunofluorescence against biotin (Villa *et al.*, 2000). Prolonged treatment with this construct slowed the growth and modestly increased the apoptotic proportion of melanoma cells *in vitro*. Chitosan-coated polylactide-coglycolide nanoparticles of 150 – 170 nm were loaded with 2'OMeRNA oligonucleotides primarily through electrostatic attraction (Beisner *et al.*, 2010). Fluorescence-activated cell sorting suggested that > 90 % of non-small cell lung cancer cells internalized fluorlabelled-oligonucleotide nanoparticles, compared to < 5 % of cells treated with the fluorlabelled oligonucleotide alone. Treatment for 102 days elicited telomere shortening from 5.8 to 4 kbp and slowed the growth rate of cells *in vitro*.

As the development of a delivery strategy is an involved process, transfection reagents can be employed to facilitate cell-delivery in the first instance, to evaluate the therapeutic potential of radiolabelled oligonucleotides targeted to telomerase. The clonogenic assay is the gold-standard experiment utilized in radiation biology to judge the ability of radiopharmaceuticals to sterilize cancer cells (Franken *et al.*, 2006), and so to indicate the potential therapeutic efficacy of candidate molecules. As radiation-induced toxicity proceeds via the induction of DNA damage, analysis of the activation of the DNA damage response following treatment provides supporting evidence of the therapeutic activity, and the mechanism of action, of candidate constructs.

### **5.1.3. Aim and outline**

The aim of this work was to evaluate the therapeutic potential of radiolabelled oligonucleotide inhibitors of telomerase, primarily using the clonogenic assay to assess the effect of the molecules on cell survival *in vitro*. Transfection reagents were employed to deliver the oligonucleotides for these initial proof-of-principle studies. The induction of a DNA damage response provides further mechanistic information, and so supplements cell survival data. Analysis of the subcellular localization of the inhibitors may also further explain any therapeutic activity observed.

Thus, this chapter intends to indicate the therapeutic potential of the molecules discussed herein, and so to determine whether radiolabelled oligonucleotides warrant further study *in vitro* and *in vivo*.

## 5.2. Materials and methods

### 5.2.1. Transfection of radiolabelled oligonucleotides

Materials: Transfast transfection reagent (Promega #E2431); 24-well plate (Greiner bio-one #662160)

Cells were seeded in a 24-well plate at a density of  $2 \times 10^4$  cells/well overnight. Oligonucleotides were combined with antibiotic-free medium to give 0.25  $\mu\text{g}/\text{well}$ . Transfection reagent was added, according to the manufacturer's instructions, to a total volume of 250  $\mu\text{L}$ , and samples incubated for 15 minutes at room temperature to allow for formation of transfection complexes. Cell-medium was removed and replaced with transfection complexes and incubated for the desired time.

### 5.2.2. Internalization of radiolabelled oligonucleotides

Materials: 24-well plate (Greiner bio-one #662160); 0.1M Glycine, pH 2.5 (Sigma-Aldrich #G8898); 0.1M Sodium hydroxide (Sigma-Aldrich #72068); Wizard<sup>2</sup> 3'' Automatic gamma-counter (Perkin-Elmer 2480)

Cells were seeded in a 24-well plate at  $2 \times 10^4$  cells/well overnight. Cells were transfected for the indicated time, between 0 and 24 hours. Cell treatment was staggered to allow time-points to be processed together. Following incubation, the medium was aspirated and retained. Cells were then washed twice with 500  $\mu\text{L}$  and 250  $\mu\text{L}$  PBS, and the washes combined with the medium to constitute the free-fraction. Free-fractions were diluted 10-fold to facilitate counting in a  $\gamma$ -counter. Cell membranes were washed using 500  $\mu\text{L}$  glycine (pH 2.5) at 4 °C for 6 minutes and re-washed with 500  $\mu\text{L}$  PBS. 250  $\mu\text{L}$  of sodium hydroxide (0.1 M) was added and plates incubated for 20 minutes at room temperature, to lyse the cells. The internalized fraction was then collected and

combined with two PBS washes of 500  $\mu\text{L}$  and 250  $\mu\text{L}$ . Fractions were counted in a Wizard  $\gamma$ -counter. Cells treated with indium-111 chloride or non-transfected radiolabelled oligonucleotides were included as negative controls.

### 5.2.3. Clonogenic assay

Materials: 24-well plate (Greiner bio-one #662160); 6-well plate (Greiner bio-one #657160); Methylene blue (Alfa Aesar #A18174); Methanol (Fisher Scientific #M/4000/PC17);  $^{137}\text{Cs}$ -Irradiator (IBL-637; Cisbio International; dose rate 1.0 Gy/min)

Cells were seeded in a 24-well plate at  $2 \times 10^4$  cells/well overnight. Cells were transfected with 0 – 0.25  $\mu\text{g}$  of oligonucleotide at 0 – 6 MBq/ $\mu\text{g}$  for 2.5 hours before addition of 250  $\mu\text{L}$  of medium and incubation for a further 24 hours. Non-transfected oligonucleotides acted as a control. Indium-111 chloride with or without transfection reagent was used as a control for non-specific uptake. Cells were then harvested following washing in PBS and addition of 100  $\mu\text{L}$  trypsin. Harvested cells were counted before plating in six-well plates at a density sufficient to give > 100 colonies for counting. Untreated cells were typically seeded at 750 cells/well. Where required, cells were subjected to real or sham  $^{137}\text{Cs}$ -irradiation. Colonies were grown for > 7 days, washed in PBS and stained with 1 % methylene blue (in 50 % methanol). Excess stain was washed-off with water. Colonies containing > 50 cells were counted. The surviving fraction (SF) was calculated using the plating efficiency (PE) of untreated cells. Data was fitted using a one-phase decay curve in GraphPad Prism 5.0. Curves were compared using an exact-sum-of-squares F-test.

### 5.2.4. Confocal microscopy

Materials: 8-well chamber slide (Thermo Scientific #177402);  $^{137}\text{Cs}$ -Irradiator (IBL-637; Cisbio International; dose rate 1.0 Gy/min); Formaldehyde (Sigma-Aldrich #252549); Blocking buffer: 2% BSA (Sigma-Aldrich #A7906), 0.1% Triton X100 (Sigma-Aldrich #T9284) in PBS; Mouse anti- $\gamma\text{H2AX}$  (Ser139) antibody (EMD-Millipore #05-636-KC); Mouse anti-coilin antibody (Abcam #ab87913); Mouse anti-TRF1 antibody (Abcam #ab10579); Rabbit anti-TRF2 antibody (Abcam #ab4182); Rabbit anti-CENP-C antibody (Abcam #ab33034); Goat anti-mouse-AF488 antibody (Invitrogen #A11001); Goat anti-rabbit-AF488 antibody (Invitrogen #A11008); Goat anti-rabbit-AF594 antibody (Invitrogen #A11037); Goat anti-mouse-AF633 F(ab')<sub>2</sub> antibody fragment (Invitrogen #A21053); Vectashield (Vector #H-1200); 24x24 mm coverslips (VWR International #6310127); 6-well plate (Greiner bio-one #657160); Laser-scanning confocal microscope (Zeiss LSM710)

Cells were seeded in 8-well chamber slides at  $2 \times 10^4$  cells/well overnight. Cells were transfected for the indicated time in 100  $\mu\text{L}$ , with an additional 100  $\mu\text{L}$  of medium added after 2.5 hours. A  $^{137}\text{Cs}$ -irradiator was used to deliver 4 Gy of ionizing radiation to induce  $\gamma\text{H2AX}$  foci, as a positive control for staining. Irradiated cells were incubated at 37 °C for 1 hour to allow for foci induction. Cells were washed in PBS before fixation in 200  $\mu\text{L}$  of 4 % formaldehyde for 10 minutes at room temperature. Cells were washed in PBS and permeabilized with 200  $\mu\text{L}$  of 1 % TritonX100 in PBS for 10 minutes at room temperature. Blocking buffer (200  $\mu\text{L}$ ) was added and cells incubated for 2 hours at 37 °C or overnight at 4 °C. Primary antibody was diluted in blocking buffer (1  $\mu\text{g}/\text{mL}$  for anti- $\gamma\text{H2AX}$  and anti-coilin, and 5  $\mu\text{g}/\text{mL}$  for anti-TRF2, anti-TRF1 and anti-CENP-C) and 100  $\mu\text{L}$  added to cells for 1 hour at 37 °C. Cells were washed three times with PBS. Fluorlabelled secondary antibodies were diluted to 8  $\mu\text{g}/\text{mL}$  and 100  $\mu\text{L}$  incubated with cells for 1 hour at 37 °C. Cells were washed three times with PBS, before removal of the medium-chamber and gasket. One drop of

Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) was added to each well before samples were mounted with a coverslip.

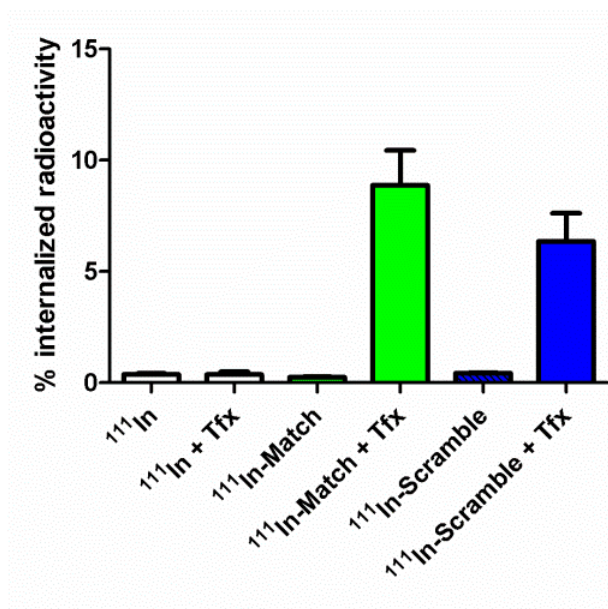
For antibody optimization experiments, cells were seeded overnight on glass coverslips in 6-well plates at a density of  $1.25 \times 10^5$  cells/well. Cells were washed in PBS and fixed in 1 mL of 4 % formaldehyde for 10 minutes at room temperature. Cells were washed in PBS and permeabilized in 1 mL of 1 % TritonX100 in PBS for 10 minutes at room temperature. Non-specific binding was blocked for 2 hours at 37 °C, following addition of 1 mL of blocking buffer. Primary antibodies were added to 100  $\mu$ L at the required concentration, and incubated for 1 hour at 37 °C. Cells were washed three-times in PBS and 100  $\mu$ L of secondary antibodies added at 8  $\mu$ g/mL. Cells were incubated for 1 hour at 37 °C, before washing in PBS. Coverslips were added to glass slides and mounted in Vectashield containing DAPI.

Images were obtained using a laser scanning Zeiss confocal microscope. Confocal planes were imaged using line-scanned consecutive acquisition channels through a 63x/1.40 oil immersion Plan-Apochromat lens (Oil DIC M27), with a pixel dwell time of 0.64  $\mu$ s, and pin-hole of 1 Airy unit, with the following settings: DAPI - illumination 405 nm, 415-493 nm detector; AlexaFluor488 - illumination 488 nm, 494-543 nm detector; AlexaFluor594 - illumination 594 nm, 600-655 nm detector; AlexaFluor633 - illumination 633 nm, 661-755 nm detector; Cy3 - illumination, 543 nm, 563-660 nm detector. For  $\gamma$ H2AX experiments, distinct intranuclear foci were manually counted in > 100 cells per treatment. Data was plotted as mean  $\pm$  standard error about the mean and subjected to a Student's t-test.

## 5.3. Results

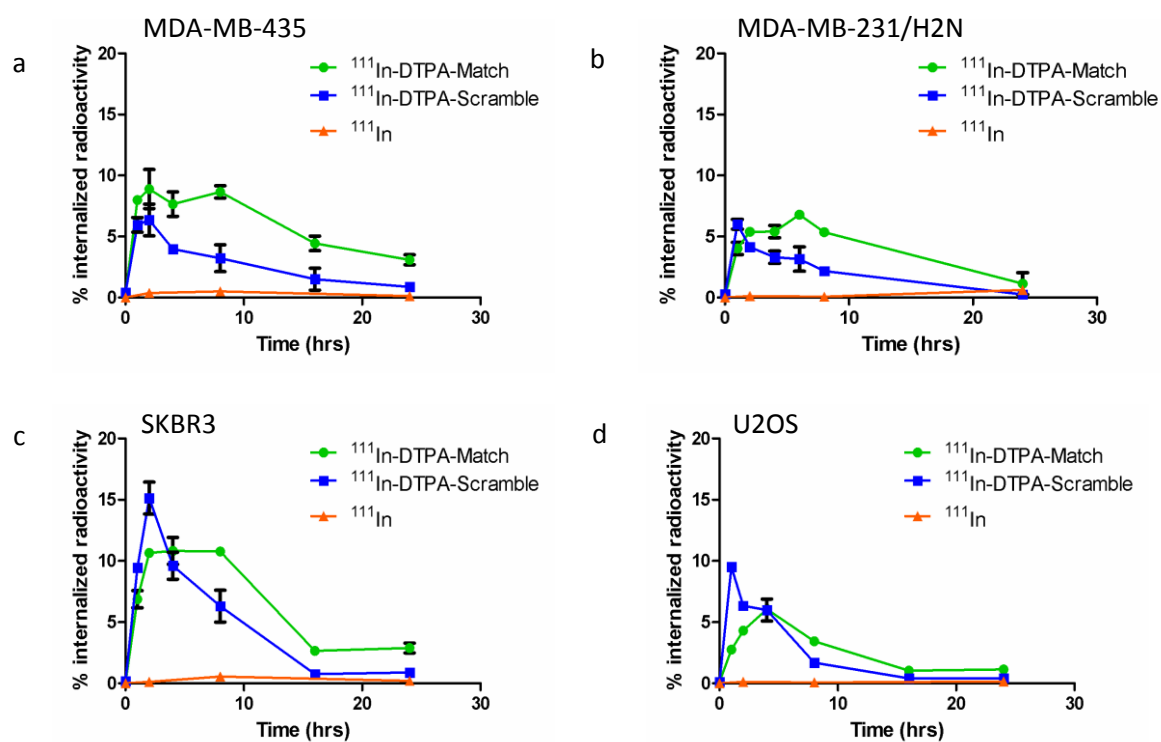
### 5.3.1. Internalization of radiolabelled oligonucleotides

For the initial proof of principle studies, the radiolabelled oligonucleotides were introduced into cancer cells *in vitro* using liposomal transfection reagents. In the absence of transfection, less than 0.5 % of administered radiolabelled oligonucleotides or indium-111 was internalized into cells (*figure 5.1*). Transfected match and scrambled oligonucleotides acted as a positive control and were internalized to > 6 % ( $P > 0.05$ ). For each cell line, the transfection conditions were optimized to approximately equalize the peak delivery of the match and scramble control oligonucleotides. The uptake of radiolabelled oligonucleotides was monitored over 24 hours (*figure 5.2*). The peak uptake of oligonucleotides occurred within four hours in three breast cancer lines (MDA-MB-435, MDA-MB-231/H2N and SKBR3, *figure 5.2 a-c*) and telomerase-negative osteosarcoma cells (U2OS, *figure 5.2 d*). The peak uptake of match oligonucleotide was  $10.8 \pm 1.1$ ,  $8.9 \pm 1.6$ ,  $6.8 \pm 0.3$ ,  $6.1 \pm 0.1$  % of administered radioactivity in SKBR3, MDA-MB-435, MDA-MB-231/H2N and U2OS cells, respectively. After 24 hours,  $2.9 \pm 0.4$ ,  $3.1 \pm 0.4$ ,  $1.2 \pm 0.9$  and  $1.2 \pm 0.3$  % of radioactivity remained internalized in SKBR3, MDA-MB-435, MDA-MB-231/H2N and U2OS cells, respectively.



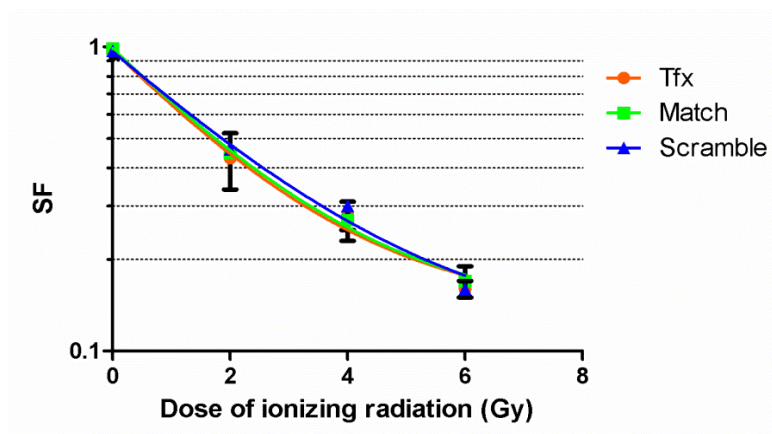
**Figure 5.1: Requirement for delivery of radiolabelled oligonucleotides *in vitro*.**

Oligonucleotides (0.25  $\mu\text{g}$ ) were radiolabelled to 0.8 MBq/ $\mu\text{g}$  and incubated with MDA-MB-435 cells for 2.5 hours. Cells were washed and lysed, and the proportion of internalized radioactivity determined by  $\gamma$ -counting.  $n=2$ , data represents at least three independent repeat experiments.



**Figure 5.2: Internalization of radiolabelled oligonucleotides into cancer cells.**

Oligonucleotides (0.25  $\mu\text{g}$ , 0.8 MBq/ $\mu\text{g}$ ) or indium-111 were combined with transfection reagent and incubated with MDA-MB-435 (a), MDA-MB-231/H2N (b), SKBR3 (c) and U2OS (d) cells. Cells were washed and lysed, and the proportion of internalized radioactivity determined by  $\gamma$ -counting.  $n=6$ , data from three independent repeat experiments.



**Figure 5.3: Effect of oligonucleotides on radiosensitivity.**

Oligonucleotides (0.25  $\mu\text{g}$ ) were transfected into MDA-MB-435 cells for 24 hours before plating for colony formation and exposure to external ionizing radiation ( $^{137}\text{Cs}$ ). Colonies of  $> 50$  cells were counted and the surviving fraction (SF) calculated.  $n=3$ , data represents three independent repeat experiments.

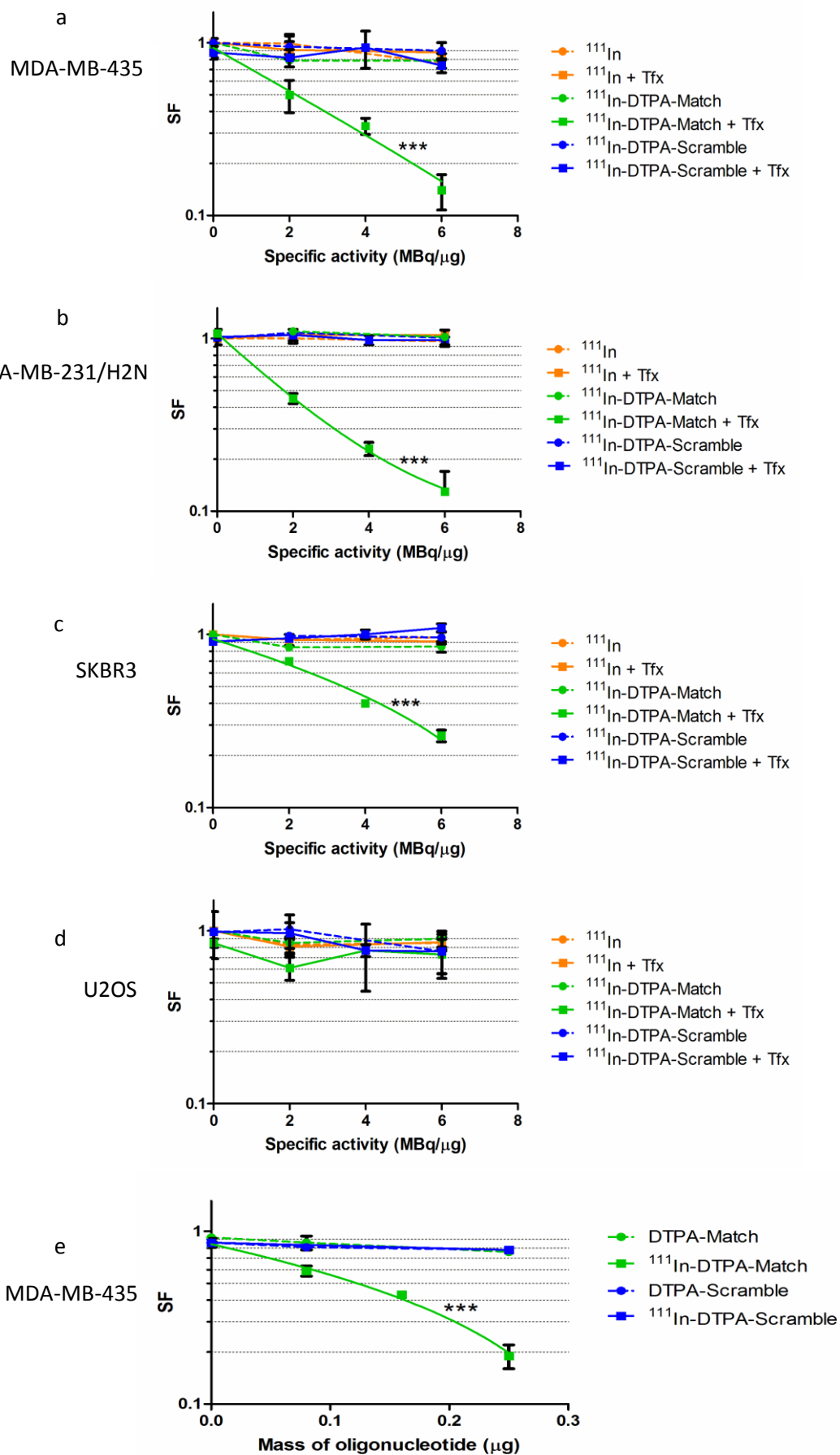
### 5.3.2. Effect of oligonucleotide inhibitors of telomerase on sensitivity to ionizing radiation

To investigate whether oligonucleotide-mediated inhibition of telomerase was sufficient to sensitize cells to ionizing radiation (IR), MDA-MB-435 cells were treated with non-radiolabelled oligonucleotides and subjected to external IR. A clonogenic assay was employed to assess cell survival. Vehicle treated cells irradiated to 2, 4 or 6 Gy exhibited a surviving fraction of  $0.43 \pm 0.09$ ,  $0.28 \pm 0.03$ ,  $0.16 \pm 0.01$  (figure 5.3). Similar results were obtained for cells pre-treated with match or scrambled oligonucleotides ( $P > 0.05$ ).

### 5.3.3. Clonogenic survival of cells treated with radiolabelled oligonucleotide inhibitors of telomerase

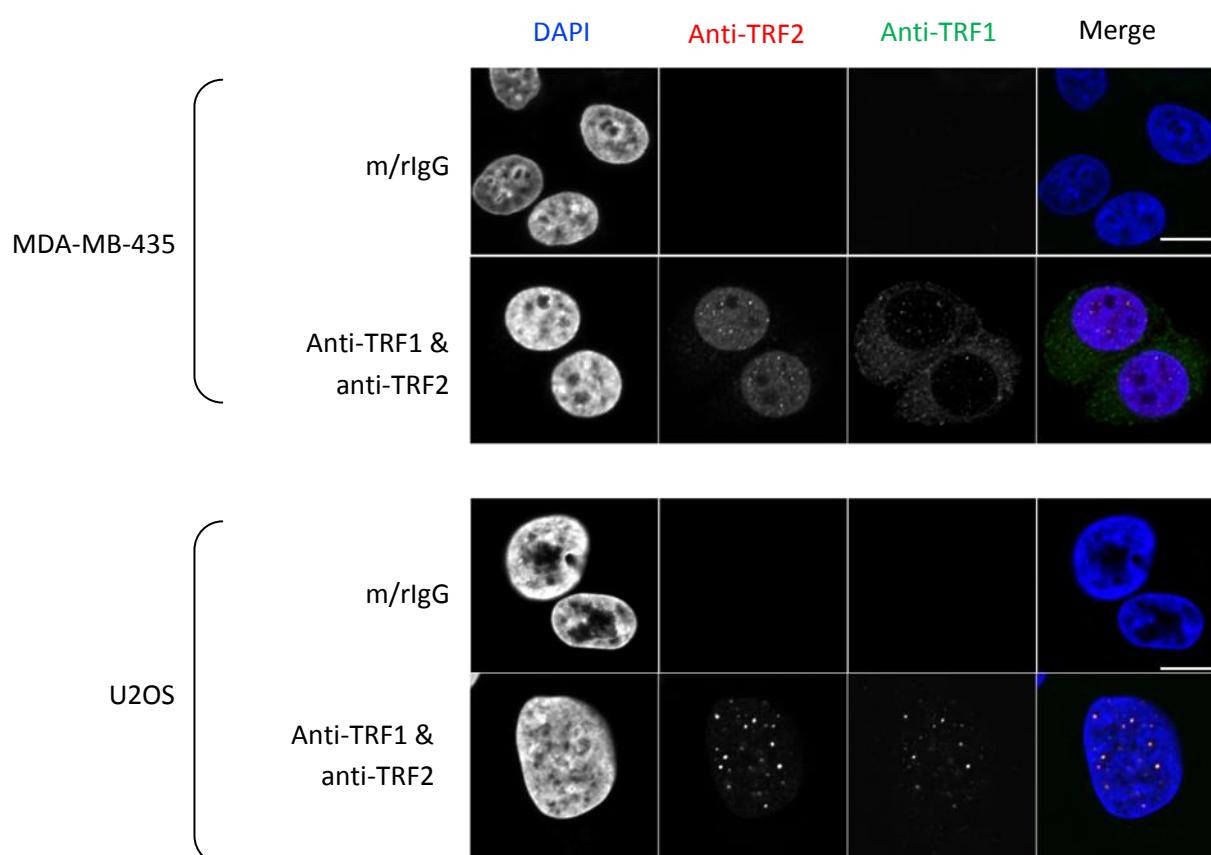
The therapeutic potential of the radiolabelled oligonucleotides was assessed by analysis of the oligonucleotide-mediated reduction in clonogenic cell survival *in vitro*. Following treatment with 0.25  $\mu\text{g}$  of oligonucleotide for 24 hours, viable cells were allowed to form colonies of  $> 50$  cells before subsequent staining and counting. For each cell line tested (MDA-MB-435, MDA-MB-231/H2N, SKBR3, U2OS), the radiolabel alone or in combination with transfection reagent had no significant effect on cell survival (*figure 5.4 a-d*). Likewise, non-transfected oligonucleotides did not affect survival as specific activity was increased. Transfected scrambled oligonucleotides did not significantly reduce survival, up to high specific activity (6 MBq/ $\mu\text{g}$ ). In contrast to this, the transfected match oligonucleotide elicited a significant specific activity-dependent reduction in clonogenic cell survival in the breast cancer cell lines (*figure 5.4 a-c*). The surviving fraction of cells following treatment with transfected match oligonucleotides radiolabelled to 6 MBq/ $\mu\text{g}$  was  $0.14 \pm 0.03$ ,  $0.13 \pm 0.04$ ,  $0.26 \pm 0.02$  for MDA-MB-435, MDA-MB-231/H2N and SKBR3, respectively. The transfected match oligonucleotide was unable to significantly reduce the survival of telomerase-negative U2OS cells compared to control, giving a surviving fraction of  $0.73 \pm 0.16$  at 6 MBq/ $\mu\text{g}$  (*figure 5.4 d*).

The specificity of the effect of the match oligonucleotide, radiolabelled to 6 MBq/ $\mu\text{g}$ , was investigated by varying the mass of oligonucleotide delivered to MDA-MB-435 cells, before plating for colony formation (*figure 5.4 e*). In this assay, non-radiolabelled oligonucleotides had no effect on cell survival. The radiolabelled scrambled oligonucleotide was also unable to reduce clonogenic survival. A dose-dependent effect was observed with the match oligonucleotide, with the surviving fraction being reduced



**Figure 5.4: Clonogenic survival of cancer cells treated with radiolabelled oligonucleotides.**

Varying specific activities (a-d) (at 0.25  $\mu\text{g}$ ) or masses (e) (at 6 MBq/ $\mu\text{g}$ ) of oligonucleotides were transfected into MDA-MB-435 (a & e), MDA-MB-231/H2N (b), SKBR3 (c) and U2OS (d) cells for 24 hours before plating for colony formation in the clonogenic assay. Colonies of > 50 cells were counted and the surviving fraction (SF) calculated. \*\*\*  $P < 0.001$ ,  $n=6$ , data from at least two independent repeat experiments.



**Figure 5.5: Immunofluorescent staining of TRF1 and TRF2 in cancer cells.**

MDA-MB-435 and U2OS cells were stained for the shelterin proteins TRF1 and TRF2. Primary antibodies, or mouse/rabbit (m/rIgG) isotype control antibodies, were detected using secondary antibodies conjugated to AlexaFluor488 and AlexaFluor594 for anti-TRF1 and -TRF2, respectively. Nuclei were stained using DAPI. Images represent several fields of view acquired using a 63x/1.4 objective lens, with 4x digital zoom. Scale bar 40  $\mu\text{m}$ .

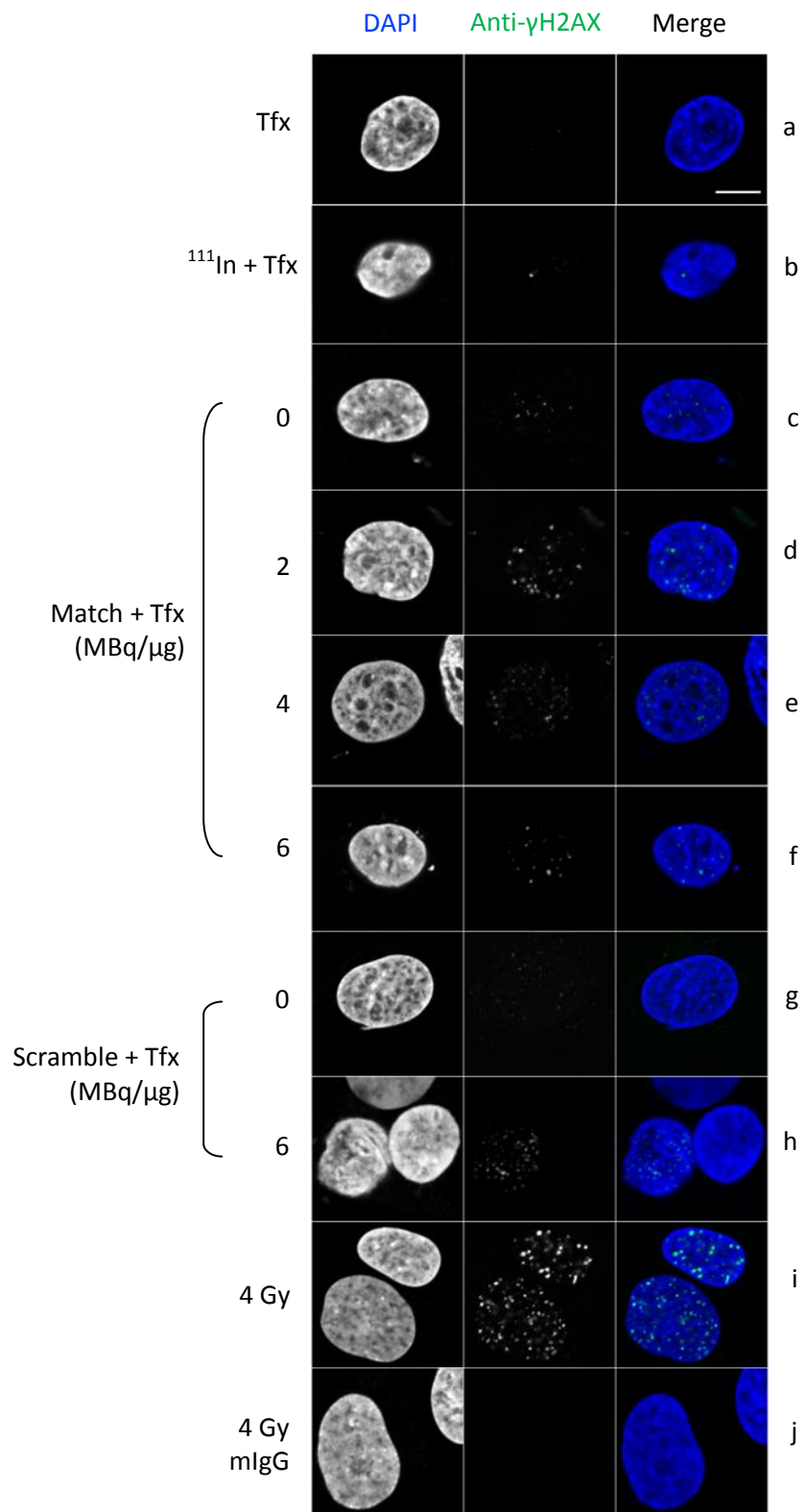
to  $0.19 \pm 0.03$  following treatment with  $0.25 \mu\text{g}$  of oligonucleotide.

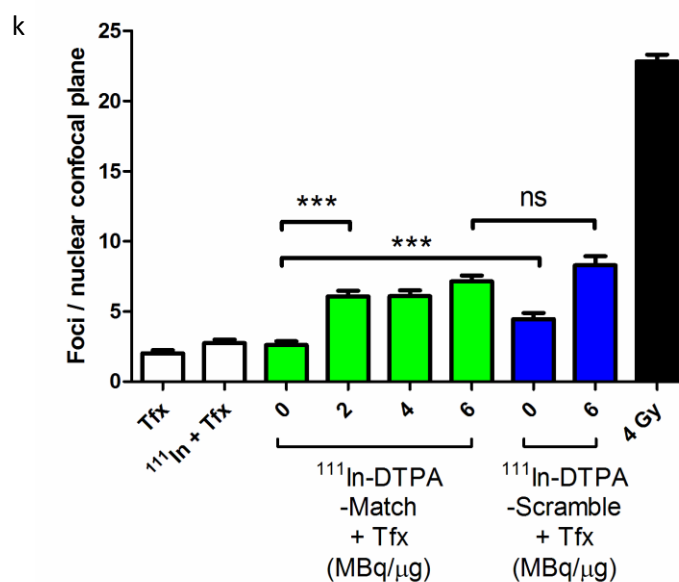
#### **5.3.4. Immunofluorescent staining of shelterin proteins to identify sites of telomeric DNA**

To confirm the specificity of immunofluorescence for shelterin, untreated MDA-MB-435 and U2OS cells were stained for the shelterin proteins TRF1 and TRF2 (Broccoli *et al.*, 1997). Antibody cross-reactivity was not observed. Focal staining was present in the nuclei of cells stained for TRF2 in both cell lines (*figure 5.5*). Similarly, TRF1 staining was discrete and nuclear in U2OS cells. In MDA-MB-435 cells, punctate TRF1 staining was observed in the nucleus, along with cytoplasmic and membrane staining in some cells. Colocalization of the nuclear TRF1 and TRF2 signal was recorded in the cell lines. Little signal was recorded when the primary antibodies were exchanged for non-specific isotype controls.

#### **5.3.5. Induction of a DNA damage response following treatment with radiolabelled oligonucleotides**

To elucidate a potential mechanism of the observed reduction in survival, cells were treated with radiolabelled oligonucleotides and stained with antibodies to the DNA damage response protein,  $\gamma\text{H2AX}$  (Rogakou *et al.*, 1998). Following 4 Gy of external IR, U2OS cells exhibited robust expression of  $\gamma\text{H2AX}$  (*figure 5.6 i*). The mean number of  $\gamma\text{H2AX}$  foci present in the nuclei in a single confocal plane of  $> 100$  cells was found to be  $22.8 \pm 0.5$  (*figure 5.6 k*). No staining was observed in the absence of the relevant primary antibody (*figure 5.6 j*).



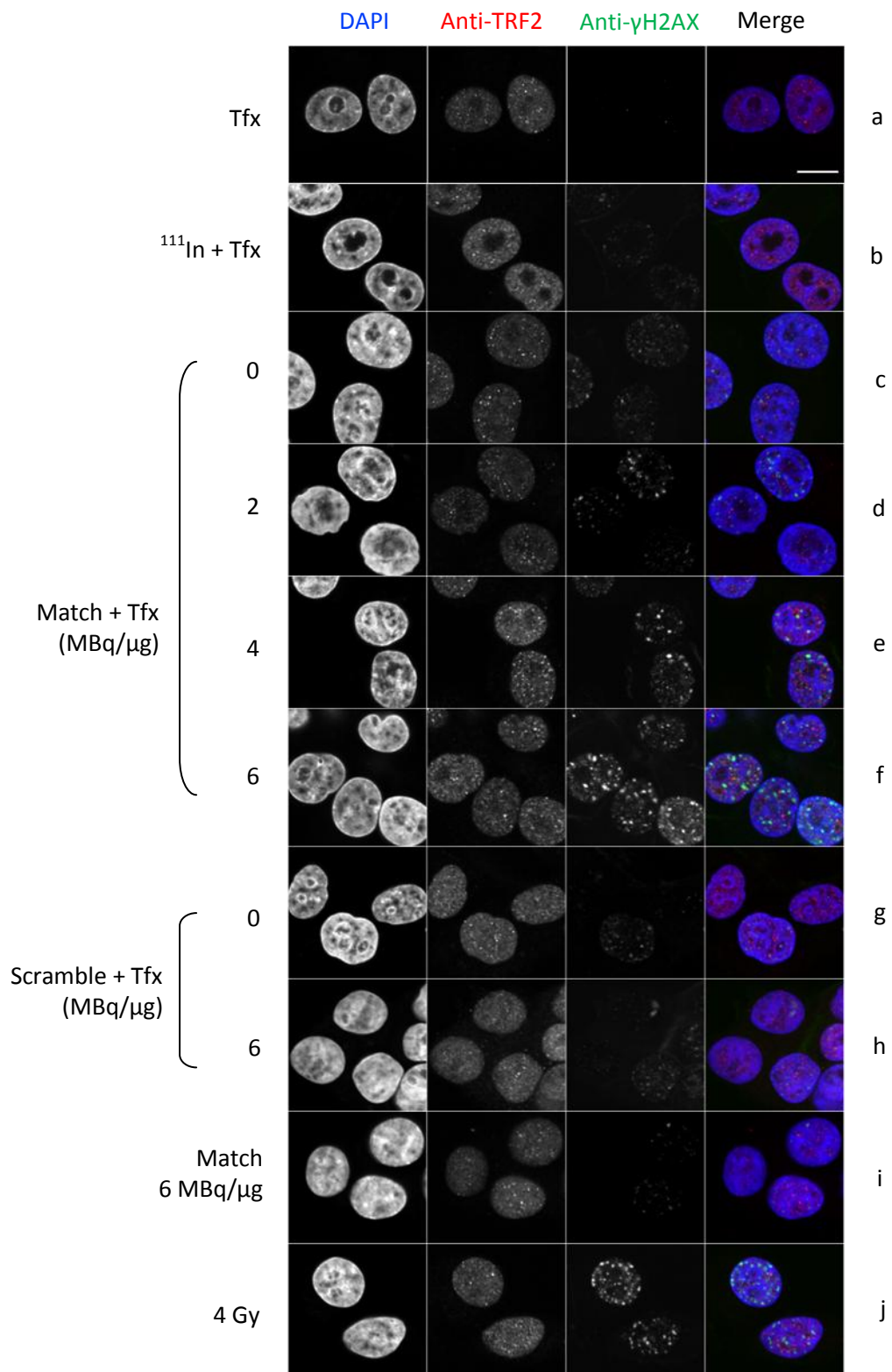


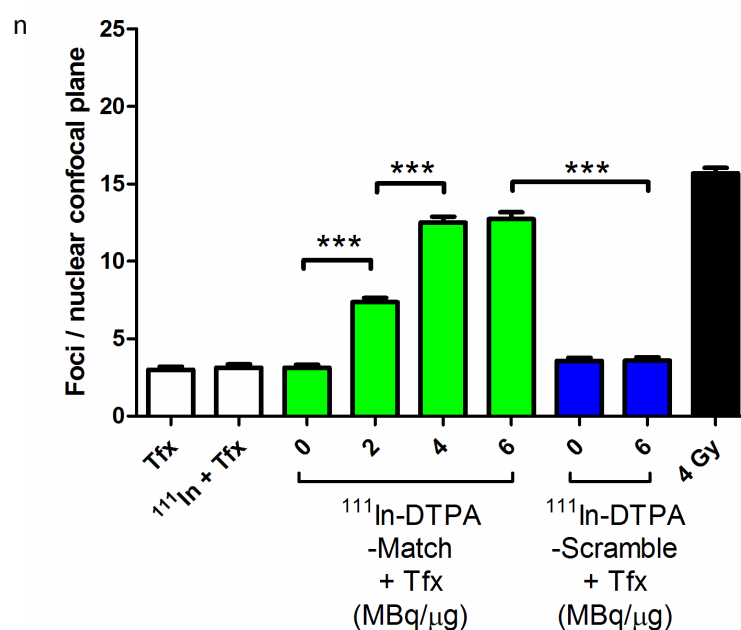
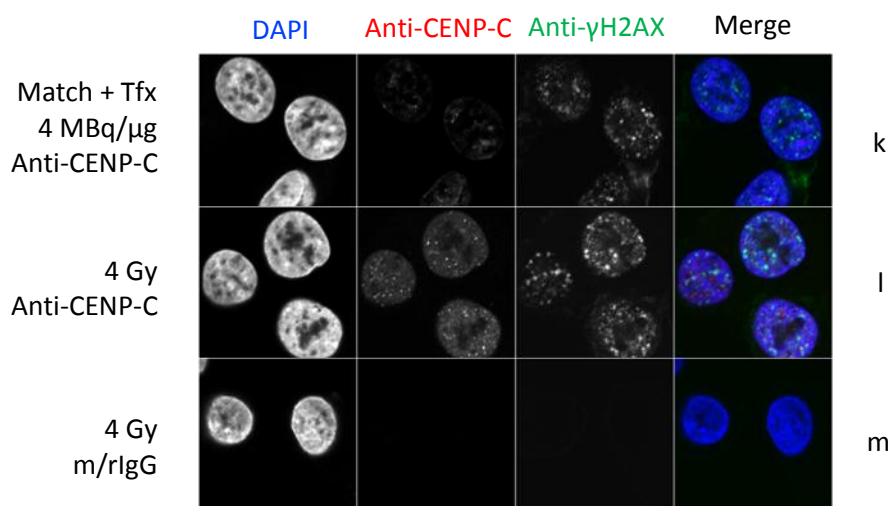
**Figure 5.6: Immunofluorescent staining of  $\gamma$ H2AX in oligonucleotide-treated U2OS cells.**

Cells were transfected with oligonucleotide (0.25  $\mu$ g) or control for 6 hours before fixation and staining for  $\gamma$ H2AX. Images represent several fields of view acquired using a 63x/1.4 objective lens, with 4x digital zoom. Primary antibodies, or mouse (mIgG) isotype control antibodies, were detected using secondary antibodies conjugated to AlexaFluor488. Nuclei were stained using DAPI. For quantification (k), the foci contained within a single confocal plane of > 100 nuclei were counted. \*\*\*  $P < 0.001$ ,  $n > 300$ , data from three independent repeat experiments. Scale bar 40  $\mu$ m. Bars represent mean  $\pm$  standard error.

Treatment with transfection reagent alone or in combination with indium-111 led to  $2.0 \pm 0.2$  and  $2.7 \pm 0.3$   $\gamma$ H2AX foci/nuclear confocal plane, respectively (*figure 5.6 a, b & k*). The non-radiolabelled match and scrambled oligonucleotides induced  $2.6 \pm 0.3$  and  $4.5 \pm 0.4$  foci/nuclear confocal plane, respectively (*figure 5.6 c, g & k*). Match oligonucleotides radiolabelled to 2, 4 and 6 MBq/ $\mu$ g induced  $6.1 \pm 0.4$ ,  $6.1 \pm 0.4$ ,  $7.2 \pm 0.4$  foci/nuclear confocal plane, respectively (*figure 5.6 d-f & k*). Scrambled oligonucleotides radiolabelled to 6 MBq/ $\mu$ g induced  $8.3 \pm 0.7$  foci/nuclear confocal plane (*figure 5.6 h & k*).

Robust  $\gamma$ H2AX staining was recorded following 4 Gy of external IR in MDA-MB-435 cells, with  $15.7 \pm 0.4$  foci/nuclear confocal plane being recorded (*figure 5.7 j, l & n*). No foci were detected following staining with an irrelevant primary antibody (*figure 5.7 m*). Few foci were observed after treatment with transfection reagent or indium-111, with  $3.0 \pm 0.2$  and  $3.1 \pm 0.2$  foci/nuclear confocal plane, respectively (*figure 5.7 a, b & n*). Non-transfected oligonucleotides failed to upregulate  $\gamma$ H2AX foci number (*figure 5.7 i*). Radiolabelled and non-labelled scrambled oligonucleotides induced  $3.6 \pm 0.2$  and  $3.6 \pm 0.2$  foci/nuclear confocal plane, respectively (*figure 5.7 g, h & n*). A specific activity-dependent increase in foci number was observed following treatment with match oligonucleotides (*figure 5.7 c-f*). Treatment with match oligonucleotide led to upregulation of  $\gamma$ H2AX to  $7.4 \pm 0.3$ ,  $12.5 \pm 0.4$ , and  $12.7 \pm 0.4$  foci/nuclear confocal plane following labelling to 2, 4 and 6 MBq/ $\mu$ g, respectively (*figure 5.7 n*). Treatment with non-radiolabelled match oligonucleotides led to  $3.1 \pm 0.2$  foci/nuclear confocal plane (*figure 5.7 c & n*).





**Figure 5.7: Immunofluorescent staining of  $\gamma$ H2AX in oligonucleotide-treated MDA-MB-435 cells.**

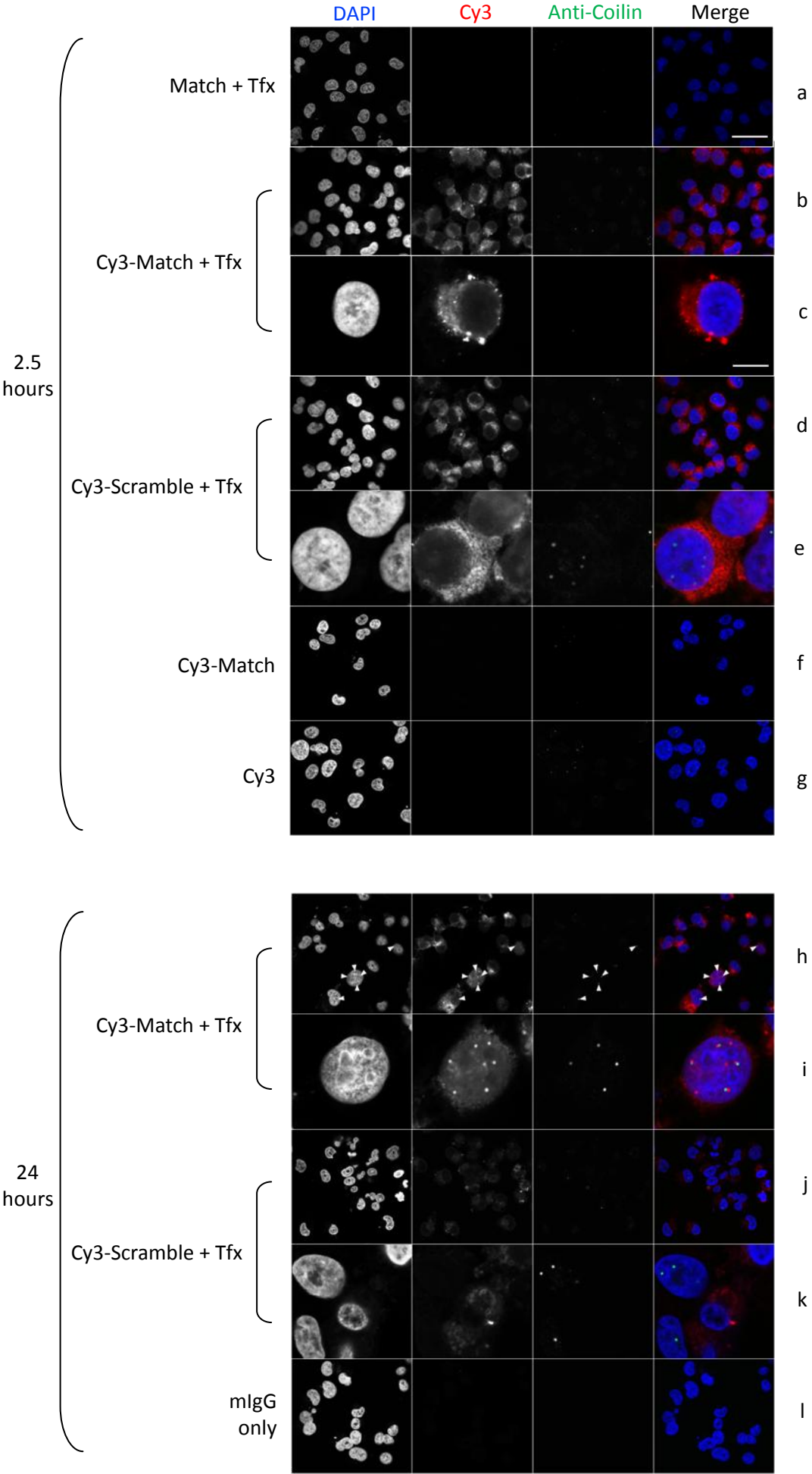
Cells were transfected with oligonucleotide (0.25  $\mu$ g) or control for 6 hours before fixation and staining for  $\gamma$ H2AX. Images represent several fields of view acquired using a 63x/1.4 objective lens, with 4x digital zoom. Primary antibodies, or mouse/rabbit (m/rIgG) isotype control antibodies, were detected using secondary antibodies conjugated to AlexaFluor488 and AlexaFluor594 for anti- $\gamma$ H2AX and anti-TRF2/CENP-C, respectively. Nuclei were stained using DAPI. For quantification (n), the foci contained within a single focal plane of > 100 nuclei were counted. \*\*\*  $P < 0.001$ ,  $n > 300$ , data from three independent repeat experiments. Scale bar 40  $\mu$ m. Bars represent mean  $\pm$  standard error.

In addition to  $\gamma$ H2AX, the breast cancer cell line MDA-MD-435 was also stained for the telomere binding protein TRF2 (Broccoli *et al.*, 1997) to elucidate any spatial distribution of the foci; or CENP-C, a centromere binding protein (Earnshaw and Rothfield, 1985), as an irrelevant control. No clear association of  $\gamma$ H2AX foci with CENP-C (*figure 5.7 k*) or TRF2 (*figure 5.7 d-f*) staining was observed.

### **5.3.6. Subcellular distribution of fluorlabelled oligonucleotides**

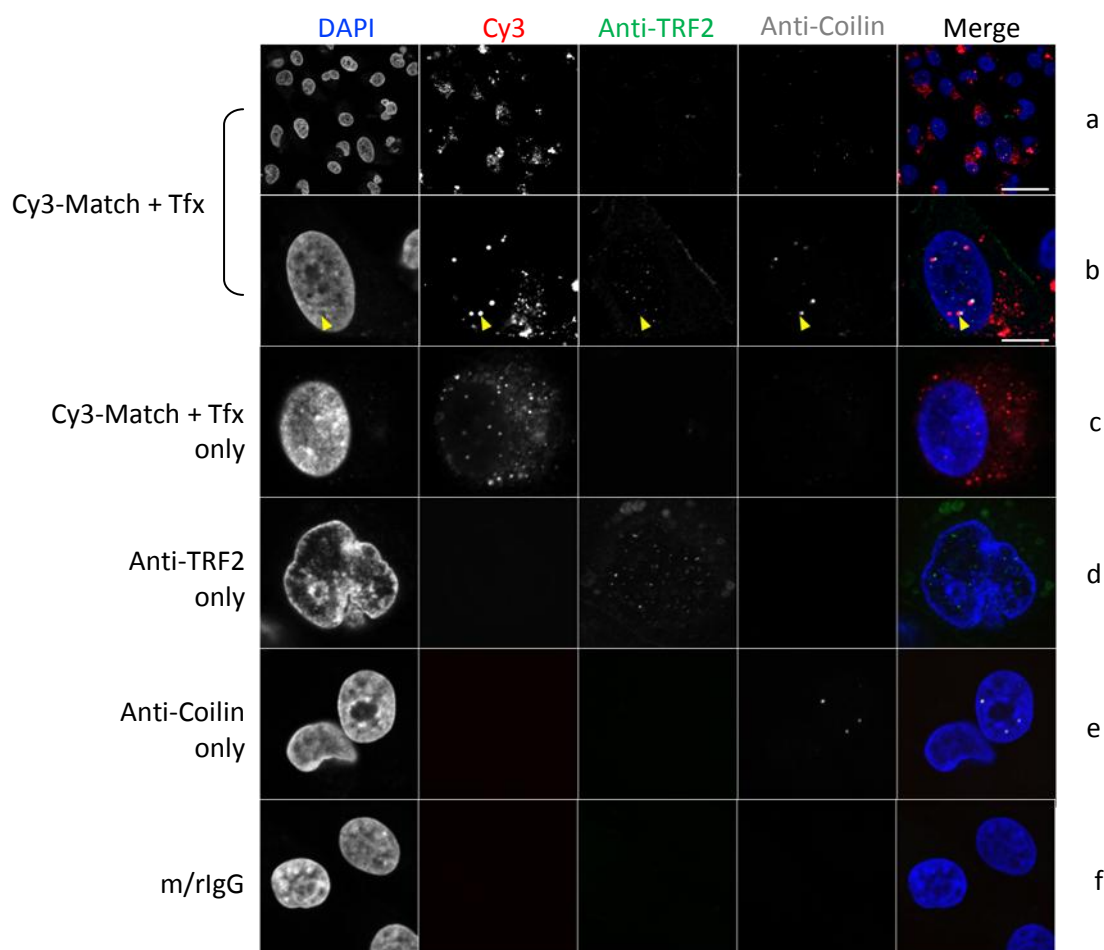
The subcellular distribution of constructs in MDA-MB-435 cells was explored using fluorlabelled oligonucleotides, as fractionation of cells treated with radiolabelled oligonucleotides was not possible, owing to the requirement for transfection. Following transfection for 2.5 hours, a robust Cy3 signal was observed in cells treated with Cy3-Match or Cy3-Scramble oligonucleotides (*figure 5.8 b-e*). In both these cases, the Cy3 signal was predominantly excluded from the cell nucleus (*figure 5.8 c & e*). A Cy3 signal was absent from cells treated with non-fluorlabelled oligonucleotides (*figure 5.8 a*). In addition, little Cy3 signal was recorded in cells treated with Cy3 alone or non-transfected Cy3-oligonucleotides (*figure 5.8 f & g*).

After 24 hours of incubation, Cy3-oligonucleotide-transfected cells showed a decrease in Cy3 signal intensity and in the case of Cy3-Scramble, this remained predominantly cytoplasmic (*figure 5.8 h-k*). In contrast, Cy3-Match oligonucleotides exhibited a more focal distribution after 24 hours, with some discrete signal being recorded in the nucleus in approximately 30 % of cells (*figure 5.8 h & i, arrows*). A counter stain for coilin, a protein marker of Cajal bodies (Raska *et al.*, 1990), was employed to explicate the observed pattern of localization of the Cy3-labelled oligonucleotides. An association between a proportion of the punctate Cy3 signal and coilin staining was observed,



**Figure 5.8: Subcellular distribution of fluorlabelled oligonucleotides in MDA-MB-435 cells.**

Cells were transfected with Cy3-oligonucleotide (0.25  $\mu\text{g}$ ) or control before fixation and staining for coilin. Images represent several fields of view acquired using a 63x/1.4 objective lens, with additional 4x digital zoom where required. Primary antibodies, or mouse (mIgG) isotype control antibodies, were detected using secondary antibodies conjugated to AlexaFluor488. Nuclei were stained using DAPI. Scale bar 10  $\mu\text{m}$ , a,b,d,f,g,h,j,l; and 40  $\mu\text{m}$ , c,e,i,k.



**Figure 5.9: Colocalization of fluorlabelled oligonucleotides with coilin and TRF2 in MDA-MB-435 cells.**

Cells were transfected with Cy3-oligonucleotide (0.25  $\mu\text{g}$ ) for 24 hours before fixation and staining for coilin and TRF2. Images represent several fields of view acquired using a 63x/1.4 objective lens, with additional 4x digital zoom where required. Primary antibodies, or mouse/rabbit (m/rIgG) isotype control antibodies, were detected using secondary antibodies conjugated to AlexaFluor488 and AlexaFluor633 for anti-TRF2 and anti-coilin, respectively. Nuclei were stained using DAPI. Scale bar 10  $\mu\text{m}$ , a; and 40  $\mu\text{m}$ , b-f.

although precise colocalization was not demonstrated (*figure 5.8 i*). Instead, a peripheral association was consistently detected. Additional staining for the shelterin protein TRF2, suggested this protein may also colocalize with the punctuate Cy3 and coilin signals after 24 hours in some cells (*figure 5.9 a & b, arrow*). The signals associated with TRF2 and coilin staining were only recorded following staining with primary antibodies directed to the relevant protein (*figure 5.9 d-f*). Likewise, Cy3 signal was dependent on treatment with Cy3-oligonucleotide (*figure 5.9 c*). In addition, cross-reactivity between primary and irrelevant secondary antibodies was not observed.

## 5.4. Discussion

Radiolabelled oligonucleotide inhibitors of telomerase have been validated in cell-free systems. To evaluate the potential therapeutic benefits of such molecules, critical *in vitro* cell-based investigations were conducted.

### 5.4.1. Radiolabelled oligonucleotides were delivered to cancer cells *in vitro*

Studies designed to monitor the internalization of radiolabelled oligonucleotides highlighted the requirement for a delivery vehicle for efficient uptake of nucleic acids, as shown previously (Beisner *et al.*, 2010). Non-transfected radiolabelled oligonucleotides were not significantly internalized into breast cancer cells *in vitro* above indium-111 control (*figure 5.1*). To assay the effect of radiolabelled

oligonucleotides on whole cells, transfection reagents were employed for these proof-of-principle studies. Uptake of indium-111 was not enhanced following incubation with transfection reagent (*figure 5.1*).

The internalization of radiolabelled oligonucleotides was monitored over time and peak uptake found to occur within 4 hours and to be > 6 % in breast carcinoma and osteosarcoma cell lines (*figure 5.2*). Previous reports suggested that *in vitro* internalization of radiopharmaceuticals to a similar extent was sufficient for molecular imaging (Wang *et al.*, 2009a) and therapy (Cornelissen *et al.*, 2012b; Costantini *et al.*, 2007) *in vivo*. Area under the curve analysis of these data allows for the calculation of the cumulative activity of exposed cells to be calculated. Assuming a cell radius of approximately 10  $\mu\text{m}$  and uniform radionuclide distribution, the medical internal radiation dosimetry (MIRD) scheme S-value ( $S_{C\leftarrow C} 2.29 \times 10^{-4} \text{ Gy/Bq s}$ ) was used to calculate the approximate dose per cell (Goddu *et al.*, 1994). Doses of 1.64, 1.14, 1.46, 0.65 Gy/cell and 0.69, 0.57, 1.11, 0.50 Gy/cell were recorded for match and scrambled oligonucleotides radiolabelled to 0.8 MBq/ $\mu\text{g}$  in MDA-MB-435, MDA-MB-231/H2N, SKBR3 and U2OS cells, respectively.

Match and scrambled oligonucleotides exhibited divergent internalization profiles, despite optimization of transfection designed to equalize the peak uptake, likely due to differences in transfection efficiency. Thus, the exposure of cells to the radionuclide was not precisely equal between the match oligonucleotide and scramble control. A component of the difference in uptake over time may have been due to enhanced retention of the match oligonucleotide through binding to target. The interpretation of future cell studies must therefore take this difference in internalization into consideration.

#### **5.4.2. Oligonucleotide telomerase inhibitors do not radiosensitize cells to ionizing radiation over 24 hours**

The delivery of telomerase-inhibitory oligonucleotides did not significantly alter the clonogenic survival of MDA-MB-435 cells treated with external IR compared to control ( $P > 0.05$ ) (*figure 5.3*). These data suggest that the oligonucleotides were not sufficient to sensitize breast cancer cells to IR *in vitro*. This finding is in contrast to previous work where radiosensitization following elimination of telomerase activity was reported. However, studies demonstrating such radiosensitization depended on long-term elimination of telomerase activity via knockout of *mTERC* (Goytisolo *et al.*, 2000; Wong *et al.*, 2000) or knockdown of hTERT (Masutomi *et al.*, 2005; Nakamura *et al.*, 2005; Papanikolaou *et al.*, 2009). In addition, radiosensitization potentiated by oligonucleotide-mediated telomerase inhibition *in vitro* and *in vivo* required 42 days of treatment, for critical telomere shortening to occur (Gomez-Millan *et al.*, 2007). Thus, prolonged treatment with inhibitor and so telomere shortening may be necessary for radiosensitization. In support of this requirement, delivery of a shortened telomere-mimetic oligonucleotide capable of the initiation of a DNA damage response, sensitized tumour cells to IR *in vitro* and *in vivo* (Weng *et al.*, 2010). Such a long term effect was not considered in the investigations described here, which assessed cell survival following treatment for 24 hours, but may still be of relevance for radiolabelled telomerase inhibitors in the clinical context.

#### **5.4.3. Radiolabelled oligonucleotides reduce the clonogenic survival of telomerase-positive cells *in vitro***

The clonogenic assay is the gold standard platform for determining the response of cells to ionizing radiation (Franken *et al.*, 2006). This assay was employed to assess the

effect of radiolabelled inhibitors of telomerase on the ability of treated cells to proliferate over five to six rounds of replication and so to succumb or survive following exposure.

In breast carcinoma and osteosarcoma cells treatment with indium-111, even in the presence of transfection reagent, had no significant effect on clonogenic survival up to an activity equivalent to 6 MBq/ $\mu$ g of oligonucleotide (*figure 5.4*). Likewise, in the absence of transfection, oligonucleotides labelled to high specific activity failed to reduce cell survival in all cell lines tested. Such findings highlight the greatly enhanced therapeutic efficacy of Auger electron-emitting radionuclides following internalization into cells (Goddu *et al.*, 1994; Hofer *et al.*, 1975). Radiolabelled oligonucleotides of scrambled sequence, following artificial delivery to cells, also failed to significantly affect clonogenic survival.

In contrast, a specific activity-dependent reduction in clonogenic survival of telomerase-positive breast cancer cells was recorded, following treatment with hTR-targeted radiolabelled oligonucleotides (*figure 5.4 a-c*). Non-radiolabelled telomerase inhibitory-oligonucleotides failed to affect cell survival in this short-term assay, highlighting the requirement for prolonged treatment with existing inhibitors, as shown previously (Gellert *et al.*, 2006; Herbert *et al.*, 1999). Survival curves generated following administration of high linear energy transfer (LET) radiation-emitting radionuclides are expected to show a linear relationship with increasing activity, as the contribution of sublethal damage is negated. Such an effect was observed following treatment of MDA-MB-435 and MDA-MB-231/H2N cells with transfected match oligonucleotides of increasing specific activity. In contrast, a shoulder was observed at low specific activity with SKBR3 cells, perhaps representing an indirect induction of

DNA damage for example as a result of a predominantly cytoplasmic localization. Alternatively, such a finding may reflect the presence of a mixed population of differentially treated cells. Despite demonstrating the second greatest integrated uptake of the radiolabelled match oligonucleotide, the reduction in survival of SKBR3 cells was more modest than that of MDA-MB-435 and MDA-MB-231/H2N cells. Interestingly, SKBR3 cells exhibited lower telomerase activity than the other cell lines (*figure 4.3 d*), raising the possibility that the effect on survival may be proportional to telomerase expression or activity, rather than extent of uptake alone.

As the uptake of the scrambled control oligonucleotide was not precisely equal to that of the match, irrefutable evidence of the specificity of the effect has not been obtained. However, at high specific activity, match oligonucleotides reduced survival by > 70 % whereas controls had no significant effect. This was the case when the uptake of match and scrambled control was similar, for example in SKBR3 cells (*figure 5.2 c*). These findings provide supporting evidence for the sequence-specificity, and so presumably target-specificity, of the effect on survival.

Additionally, the mass of oligonucleotide radiolabelled to 6 MBq/ $\mu$ g and delivered to cells proportionally affected the clonogenic survival of MDA-MB-435 cells (*figure 5.4 e*). Scrambled control and non-radiolabelled oligonucleotides exhibited little influence over survival. These data further confirm the sequence-specificity and dose-dependent effect of the radiolabelled oligonucleotides. A slight shoulder was observed in the survival curve, potentially indicating a lack of sufficient nuclear localization when the match oligonucleotide was administered at low concentration.

Crucially, telomerase-targeted radiolabelled oligonucleotides had no effect on the clonogenic survival of telomerase-negative osteosarcoma cells (Scheel *et al.*, 2001)

(figure 5.4 d), despite being internalized to a similar extent compared to breast cancer cells, for example MDA-MB-231/H2N (figure 5.2 b & d). Such a finding supports the development of radiolabelled oligonucleotides as a potential clinical therapeutic agent, as cytotoxicity is likely to be limited to telomerase-expressing cells.

#### **5.4.4. Radiolabelled oligonucleotides induce a DNA damage response in telomerase-positive cells**

The expected mechanism explaining the radiolabelled oligonucleotide-mediated reduction in clonogenic survival proceeds via the irradiation of DNA and subsequent activation of a DNA damage response. To confirm this effect was occurring, cells were treated with radiopharmaceuticals before being stained for  $\gamma$ H2AX, a marker of the DNA damage response (DDR) (Rogakou *et al.*, 1998).

The survival of osteosarcoma cells was not affected by radiolabelled oligonucleotides and accordingly, minimal upregulation of  $\gamma$ H2AX was observed following treatment (figure 5.6). External irradiation significantly increased the number of  $\gamma$ H2AX foci, validating the assay as a measure of the induction of the DDR. The signal was dependent on presence of the anti- $\gamma$ H2AX antibody, suggesting staining was specific. The number of  $\gamma$ H2AX foci increased modestly upon treatment with radiolabelled match oligonucleotide compared to non-labelled and radiolabel controls, but this was not dependent on specific activity and occurred to a similar extent in cells treated with an oligonucleotide of scrambled sequence. Thus, the increase in foci number reflects the induction of non-specific DNA damage following internalization of indium-111, but was not sufficient to affect clonogenic survival. In addition, a significant difference in foci number was observed between the non-radiolabelled match and scramble control

oligonucleotides. The increased number of foci induced by the scrambled construct may reflect the requirement for a greater concentration of transfection reagent for oligonucleotide delivery, in this cell line.

In contrast, radiolabelled oligonucleotides induced a specific activity-dependent increase in the number of  $\gamma$ H2AX foci in telomerase-positive MDA-MB-435 cells (*figure 5.7*). The oligonucleotide-induced increase in foci number seemed to saturate between 4 and 6 MBq/ $\mu$ g, although the intensity and size of foci appeared greater at the higher activity. Treatment with match radiolabelled oligonucleotides led to robust upregulation of  $\gamma$ H2AX. Non-transfected oligonucleotides, on the other hand, failed to significantly upregulate  $\gamma$ H2AX foci number, confirming the requirement for internalization for therapeutic effect. No induction of  $\gamma$ H2AX above background was observed for cells treated with the scramble control oligonucleotide, even when radiolabelled to 6 MBq/ $\mu$ g. These findings are in clear agreement with the cell survival data, where complementarity to target was necessary for reduction in clonogenic survival. External irradiation led to the robust upregulation of  $\gamma$ H2AX, albeit to a lesser extent than observed with U2OS. The increased number of foci observed in U2OS may reflect the aneuploid status, and so increased genomic content, of this cell line (Mukherji *et al.*, 2006).

A triplex forming oligonucleotide labelled with an Auger electron-emitting radionuclide was shown to induce DNA damage within close vicinity of the targeted sequence *in vitro*, owing to the short range of the emitted electrons (Sedelnikova *et al.*, 2002; Sedelnikova *et al.*, 2000). This induction of damage focused around the targeted site

coupled to the possibility that catalytically inhibited telomerase may still be recruited to telomeres, for example through allosteric interaction with the telomeric substrate itself (Matthes and Lehmann, 1999) or via recruitment by shelterin proteins such as TPP1 (Zhong *et al.*, 2012), prompted the hypothesis that damage may exhibit a geographical preponderance for telomeric DNA. Zhao *et al.* (2011) first raised the possibility that oligonucleotide-bound telomerase may be pre-positioned on telomeric DNA in a similar fashion to uninhibited molecules. Treated cells were co-stained for  $\gamma$ H2AX and TRF2, a telomere binding protein (Broccoli *et al.*, 1997); or CENP-C, a centromere binding protein (Earnshaw and Rothfield, 1985), which acted as an irrelevant locus control.

Staining for TRF2 revealed a punctate pattern in MDA-MB-435 and U2OS cells (*figure 5.5*). To confirm the TRF2 stain was a surrogate for telomeric DNA, staining for a second independent telomere-binding protein, TRF1, was employed (Broccoli *et al.*, 1997; Fairall *et al.*, 2001). Unexpected cytoplasmic signal was recorded following TRF1 staining in MDA-MB-435 cells, where some membrane fluorescence was also observed, and is likely a consequence of non-specific antibody binding. Dual staining revealed colocalization of TRF1 and TRF2 in MDA-MB-435 and U2OS cells. Colocalization of these proteins has been reported previously in cancer cells, with or without treatment with IR (Zhu *et al.*, 2000). The observed TRF2 staining closely resembled previously published data, where TRF2 staining was shown to correlate with fluorescence *in situ* hybridization staining of telomeric DNA (Broccoli *et al.*, 1997; Fotiadou *et al.*, 2004; Zhu *et al.*, 2000). Thus, immunofluorescent (IF) staining of TRF2 was validated as a surrogate of telomeric DNA.

In our studies, no clear association between  $\gamma$ H2AX and TRF2 staining was observed (*figure 5.7 d-f*), and so no evidence for the preferential induction of DNA damage at telomeres obtained. However, such a specific effect is difficult to discern using IF

staining, owing to the high number of foci of both  $\gamma$ H2AX and TRF2 – due to the need to disambiguate random coincidence of staining. Furthermore, damage-induced  $\gamma$ H2AX foci have been estimated to span megabases of DNA (Rogakou *et al.*, 1999; Rogakou *et al.*, 1998), potentially concealing association with the relatively short human telomeres (< 20000 nucleotides). Recent work has suggested that DNA damage inflicted at the telomeres may be particularly persistent; owing to the necessary inhibition of the DNA damage response at the chromosomes ends (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). Thus, analysis of the proportion of  $\gamma$ H2AX foci associated with telomeres over time, following treatment with radiolabelled telomerase inhibitors, may facilitate the statistical detection of any spatial predilection. In addition, the precipitation of chromatin using antibodies directed to DDR factors coupled with sequencing (ChIPSeq) may be useful in addressing this question.

#### **5.4.5. Fluorlabelled oligonucleotide inhibitors associate with Cajal bodies in cancer cells**

The subcellular localization of the inhibitors following delivery was elucidated using fluorlabelled oligonucleotides. Cells treated with non-fluorlabelled or non-transfected fluorlabelled oligonucleotides exhibited little Cy3 staining, suggesting the signal was specific to internalized fluorlabelled oligonucleotides. These studies supported the assertion that transfection was required for oligonucleotide internalization. In further agreement with the internalization studies performed using radiolabelled oligonucleotides, a robust signal, approximately equal between match and scramble controls, was observed after 2.5 hours (*figure 5.8 b-e*). At this early time-point, oligonucleotides were predominantly excluded from the cell nucleus, where telomerase was shown to reside in cancer cells *in vitro* (Lee *et al.*, 2010).

After 24 hours of treatment, the oligonucleotide-associated fluorescence signal had decreased in both cases, again supporting the obtained internalization data (*figure 5.8 h-k*). The non-targeting oligonucleotides remained mainly cytoplasmic, whereas oligonucleotides complementary to hTR were found within the cell nucleus (*figure 5.8 h & i*). Furthermore, a punctate nuclear distribution was observed in a proportion of cells, which appeared to be peripherally associated with a coilin counter-stain. Cajal bodies (CBs), characterized by the presence of punctate coilin, are nucleolus-associated nuclear suborganelles, involved in ribonucleoprotein assembly, frequently found in proliferative cells (Raska *et al.*, 1990). Fluorlabelled oligonucleotides were therefore shown to associate with CBs in MDA-MB-435 cells *in vitro*. In agreement with these findings, the nuclear localization of fluorlabelled 2'OMeRNA (Beisner *et al.*, 2010) and PNA oligonucleotide telomerase inhibitors (Shammas *et al.*, 1999; Villa *et al.*, 2000), has been reported previously in cancer and immortalized cells. Furthermore, unassigned focal accumulation was recorded in the nuclei of a subset of cells (Beisner *et al.*, 2010; Shammas *et al.*, 1999). Thus, these studies showed for the first time that fluorlabelled oligonucleotide telomerase inhibitors associate with nuclear CBs in telomerase-positive cancer cells *in vitro*.

Fluorescence *in situ* hybridization staining for the RNA component of telomerase, performed post-fixation, revealed similar hTR and CB colocalization (Zhu *et al.*, 2004), particularly with a peripheral association (Jady *et al.*, 2006; Tomlinson *et al.*, 2006). Thus, the CB-associated inhibitory oligonucleotides are likely bound to their target, hTR, in these subnuclear domains. Furthermore, during G1-phase of the cell cycle, no nuclear hTR foci were observed (Jady *et al.*, 2006; Tomlinson *et al.*, 2006), explaining

the appearance of oligonucleotide foci only in a proportion (~ 30 %) of cells in these studies.

As reports had suggested CB-associated hTR foci occasionally colocalize with telomeric DNA (Cristofari *et al.*, 2007; Jady *et al.*, 2006; Tomlinson *et al.*, 2006) fluorlabelled oligonucleotide-treated cells were stained for both coilin and TRF2. Single-stained control cells were used to ensure the fluorescence signals were not detected in the other channels, as the use of four fluorophores increases the possibility of cross-talk (*figure 5.9 c-e*). Using this technique, the occasional colocalization of fluorlabelled oligonucleotide with both coilin and TRF2 was observed (*figure 5.9 b, arrow*). The low frequency of occurrence of this staining may be explained by work that suggested hTR was found at telomeres only during S-phase of the cell cycle (Jady *et al.*, 2006; Tomlinson *et al.*, 2006). In addition, the presence of CBs at the telomere was also shown to be dependent on cell cycle position (Cristofari *et al.*, 2007).

Thus, the accumulation of fluorlabelled oligonucleotides in discrete intranuclear foci may be dependent on the presence of hTR in CBs during the cell cycle. The oligonucleotides may associate with hTR either in the telomerase complex or prior to holoenzyme assembly, although the accumulation of hTR in CBs was shown to be dependent on hTERT (Tomlinson *et al.*, 2008; Zhu *et al.*, 2004). The formation of hTR foci was absent in telomerase-negative cells (Zhu *et al.*, 2004). These studies suggested that fluorlabelled oligonucleotides accumulate in the nuclei of telomerase-positive cells, and may bind to hTR located in CBs.

Nuclear accumulation of Auger electron-emitting radionuclides enhances their therapeutic efficacy (Goddu *et al.*, 1994; Hofer *et al.*, 1975), and likely explains the

highly significant effect of the radiolabelled oligonucleotides on cell survival. The range in water of the Auger electrons emitted during indium-111 decay was estimated to be 0.25 nm – 13.6  $\mu$ m, with most in the nanometre range (Cornelissen and Vallis, 2010). Thus, radiolabelled oligonucleotides located in CBs are likely to preferentially irradiate the CBs themselves (diameter < 1  $\mu$ m) and surrounding genomic DNA. Furthermore, the lack of nuclear accumulation of hTR in telomerase-negative cells (Zhu *et al.*, 2004) may contribute to the specificity of the effect. However, the intracellular behaviour of the fluor- and radiolabelled oligonucleotides may not be identical due to differences in size, charge and hydrophobicity. Once the requirement for transfection is overcome, studies employing cellular fractionation following administration of radiolabelled oligonucleotides would provide an accurate, if lower resolution, sub-cellular distribution profile. Despite this, studies performed with fluorlabelled oligonucleotides provide an indication of the possible localization of the radiolabelled versions. The use of micrometre-resolution photoresist autoradiography in combination with IF, a technology under development; may be useful in confirming the distribution of the radiolabelled oligonucleotides (Falzone *et al.*, 2012).

#### **5.4.6. Conclusions and summary**

The aim of this chapter was to assess the potential of radiolabelled oligonucleotides as novel anti-cancer therapeutics. To do this, the *in vitro* delivery of radiolabelled oligonucleotides into cancer cells was optimized, for initial proof-of-principle studies. Telomerase inhibitory oligonucleotides did not significantly sensitize cells to external  $\gamma$ -irradiation over 24 hours. Treatment of telomerase-positive cells with radiolabelled oligonucleotide inhibitors was sufficient to induce a highly significant decrease in

clonogenic survival. Investigation of the subcellular effect of the treatment revealed the induction of DNA damage. Fluorlabelled oligonucleotides were found to associate with Cajal bodies in cancer cells, providing an indication of the distribution of the radiolabelled molecules.

Thus, radiolabelled oligonucleotide inhibitors of telomerase have been demonstrated to be promising candidate radiopharmaceuticals, with good therapeutic efficacy and specificity. As such, further pre-clinical characterization of the molecules is recommended – with particular focus on improvement of the *in vivo* stability and delivery of the radiolabelled oligonucleotides, which are currently the major limitations to such a strategy.

## **Chapter 6: Discussion**

## Chapter 6: Discussion

### 6.1. Novel radiolabelled inhibitors of telomerase

The development of molecularly-targeted radiotherapy (mRT) agents directed to telomerase-overexpressing cells represents a novel therapeutic modality, potentially applicable to the treatment of the majority of metastatic cancers. The work discussed herein describes the synthesis and *in vitro* validation of radiolabelled variants of small molecule (Chapter 3) and oligonucleotide (Chapters 4 and 5) inhibitors of telomerase.

#### 6.1.1. Radiolabelled small molecule inhibitors

Small molecules of different classes have been developed as telomerase inhibitors with limited success. The lack of a high-resolution crystal structure of human telomerase has meant most inhibitors have been identified following screening of natural products (Menichincheri *et al.*, 2004; Seimiya *et al.*, 2002). However, recently a low-resolution structure of human telomerase was reported (Sauerwald *et al.*, 2013), representing some progress in this area. An alternative screening strategy for the identification of small molecule telomerase inhibitors was also recently proposed (Cohn *et al.*, 2012), highlighting the current limitations in the identification of such molecules. These advances are already yielding potent, novel small molecule telomerase inhibitors, based around pyrazole-derived compounds (Liu *et al.*, 2013; Luo *et al.*, 2013). Halogenated variants have also been synthesised, suggesting radioiodine-labelling may be possible, although stringent validation will be required.

Thus, the small molecule telomerase inhibitors used as seed compounds in our radiolabelling strategy were likely suboptimal, in terms of stability, potency, specificity of action and bioavailability. As such, candidate radiolabelled small molecules were not identified. Novel compounds improving upon these characteristics may provide a more suitable basis for future radiolabelling studies.

In a similar strategy, the therapeutic potential of addition of an Auger electron-emitting radionuclide to a small molecule has been previously demonstrated following the radioiodination (iodine-125) of quercetin, a DNA-binding agent (Hosseinimehr *et al.*, 2011). In this study, radiolabelled quercetin accumulated in the nuclei and induced apoptosis in prostate cancer cells *in vitro*. Thus, the possibility of developing an efficacious telomerase-targeted Auger electron-emitting small molecule radiopharmaceutical remains, although alternative parent molecules should be employed.

### **6.1.2. Radiolabelled oligonucleotide inhibitors**

Oligonucleotides targeting the template region of the RNA component of telomerase (hTR) have been extensively shown to act as telomerase inhibitors *in vitro* and *in vivo*. The best characterized oligonucleotide inhibitor, GRN163L, is currently undergoing clinical trial (Chanan-Khan *et al.*, 2008; Kozloff *et al.*, 2010). Oligonucleotide-mediated inhibition of telomerase activity in breast cancer cells, including MDA-MB-435 cells, has been shown previously (Gellert *et al.*, 2006; Herbert *et al.*, 2005) and is in good agreement with the findings reported here.

The radiolabelling of an antisense oligonucleotide targeting the mRNA of hTERT was pursued to potentiate the molecular imaging of telomerase positive cancer *in vivo* (Liu *et al.*, 2007). These radiolabelled oligonucleotides retained their capacity to inhibit hTERT expression, indicative of preservation of interaction with target. In addition, following administration *in vivo*, a tumour to muscle ratio of ~ 9 was achieved six hours after administration, providing sufficient contrast for imaging purposes. Thus, the *in vivo* accumulation of radiolabelled oligonucleotides in telomerase-positive tumour tissue was demonstrated. This work also suggested significant accumulation of radiolabelled oligonucleotides in the kidneys and liver, prohibiting the imaging of tumours therein and potentially leading to significant off-target irradiation of normal tissues, in a therapeutic context. Thus, therapeutically radiolabelled oligonucleotides must be validated extensively in the pre-clinical context *in vivo*. Further examples of therapeutically radiolabelled oligonucleotides have been reported (Cheng and Huang, 2009; Sedelnikova *et al.*, 2002; Sedelnikova *et al.*, 2000; Zhang *et al.*, 2008), including indium-111 labelled molecules (He *et al.*, 2004b; Liu *et al.*, 2009; Watanabe *et al.*, 2006). Thus, the therapeutic potential of Auger electron-emitting oligonucleotides has been shown previously, supporting our finding of the significant and telomerase-dependent reduction in clonogenic survival of treated cancer cells *in vitro*. Previously reported oligonucleotides have targeted genomic DNA or mRNA in antigene and antisense strategies, respectively. In contrast, the therapeutic oligonucleotides described here target non-translated RNA and function as catalytic inhibitors, and therefore represent a novel approach.

The subcellular distribution of the oligonucleotides was explored using fluorolabelled molecules. A consistent association between inhibitory oligonucleotides and Cajal

bodies (CBs) was observed in a subpopulation of cells. The RNA component of telomerase contains a CB-localization sequence (CAB box) (Jady *et al.*, 2004) and accordingly accumulates in CBs (Broome and Hebert, 2013; Cristofari *et al.*, 2007; Raska *et al.*, 1990). Furthermore, a functional role for CBs in telomerase biogenesis was recently demonstrated (Broome and Hebert, 2013). Incubation of total HeLa cellular RNA with coilin *in vitro*, led to a marked reduction in the immature form of hTR. The accumulation of hTR in CBs was found to be dependent on the expression of hTERT, and so was only observed in telomerase-positive cancer cells (Tomlinson *et al.*, 2008; Zhu *et al.*, 2004). Loss of CB-localization, following depletion of the hTR binding protein TCAB1 (Venteicher *et al.*, 2009) or mutation of the hTR CAB box (Cristofari *et al.*, 2007), led to manufacture of functionally active telomerase that failed to be recruited to telomeres, and so was incapable of their maintenance. Thus, inhibitory oligonucleotides hybridized to hTR may perturb telomerase assembly and trafficking, further contributing to inhibition in the cellular context. Interestingly, the putative accumulation of radiolabelled oligonucleotides in nuclear CBs, which is dependent on hTERT expression, may promote the irradiation of other CB-associated factors – potentially inflicting further injury upon the cell in a telomerase expression-dependent manner.

## 6.2. Further refinement of radiolabelled oligonucleotide inhibitors of telomerase

### 6.2.1. Oligonucleotide sequence

The selection of the sequence targeted by an inhibitory oligonucleotide is clearly of critical importance. As hTR is the site of recruitment of several protein components of the telomerase complex (Egan and Collins, 2012; Ly *et al.*, 2003; Mitchell and Collins, 2000; Venteicher *et al.*, 2009), it is likely that several sequence motifs are concealed and so unavailable for oligonucleotide targeting. Indeed, comparison of complementary oligonucleotides demonstrated a limit to affinity with increasing length, suggesting some sequence is unavailable for hybridization (Norton *et al.*, 1996). In direct contrast, the substrate-binding template sequence of hTR is necessarily available for interaction with telomeric DNA and therefore exogenous nucleic acids. Oligonucleotides associated with this sequence were not readily competed-off by excess complementary DNA, indicating a stable association (Pitts and Corey, 1998). Oligonucleotides targeting the template sequence exhibited more potent telomerase inhibition compared to those directed to other regions within hTR (Glukhov *et al.*, 1998; Herbert *et al.*, 1999; Norton *et al.*, 1996). In addition, targeting the sequence that prescribes the reverse transcribed telomeric repeat limits the potential for acquisition of resistance by mutation of the target. Mutation of the template region of hTR would lead to an altered telomeric repeat sequence and so loss of shelterin binding, telomere uncapping and activation of a DNA damage response (DDR) (Pitts and Corey, 1998) – as demonstrated by the low-level exogenous expression of a mutant hTR (Kim *et al.*, 2001, Marusic *et al.*, 1997).

### 6.2.2. Oligonucleotide chemistry

As discussed in Chapter 4, the choice of oligonucleotide chemistry is of critical importance to maximise target binding and bioavailability. This study has further confirmed the ability of phosphorothioate (PS) oligonucleotides to inhibit telomerase activity in a sequence-independent fashion (Matthes and Lehmann, 1999; Norton *et al.*, 1996; Pitts and Corey, 1998). Oligonucleotides modified at the 2'-position were shown to exhibit improved characteristics compared to DNA and peptide nucleic acid (PNA) molecules (Pitts and Corey, 1998). Modified 2'-O-methyl RNA (2'OMeRNA) oligonucleotides are incapable of activating RNase H and so antisense cleavage of the targeted RNA (Monia *et al.*, 1993). However, regions of partial complementarity between oligonucleotides and cellular RNAs were shown to be sufficient for activation of RNA cleavage by RNase H, potentially leading to off-target effects (Giles and Tidd, 1992). Thus, the specificity of action of 2'OMeRNA oligonucleotides may be less contentious. This, coupled to the advantageous potency of 2'OMeRNA oligonucleotides (Glukhov *et al.*, 1998; Herbert *et al.*, 1999; Norton *et al.*, 1996), makes them a useful therapeutic tool. Often, the comparison of several oligonucleotides of identical sequence but alternative chemistry provides useful corroboration of a sequence-specific effect.

### 6.2.3. Choice of therapeutic radionuclide

The selection of the radiolabel employed for therapy may be used to manipulate the applications of an mRT agent. Within the context of Auger electron-emitting radionuclides, the decay profile of the isotope employed determines its efficacy and depends on the physical and biological half-life; as well as the yield and energy of the

emitted electrons (reviewed in Cornelissen and Vallis, 2010). Exchange of the radionuclide may require the use of an alternative method of conjugation, as was the case with small molecule inhibitors labelled with iodine-123 and oligonucleotides labelled with indium-111. Emission of imageable  $\gamma$ -photons may also be advantageous in the clinic, both for tumour imaging and therapeutic dose determination. Thus, the use of other radionuclides, to enhance particular characteristics, is likely to affect the function and biodistribution of the therapeutic molecules – and therefore require independent validation. For example, simple exchange of chelating agents was shown to affect the *in vivo* distribution of lutetium-177-labelled antibodies targeted to a mutant epidermal growth factor receptor (Hens *et al.*, 2010). Despite having the greatest yield of Auger electrons per decay, iodine-125 is not suitable for clinical use, due to its long half-life (*table 1.1.*). Thus, radionuclides such as iodine-123 and particularly indium-111 provide a reasonable deposition of energy, whilst offering other suitable characteristics, including the emission of keV photons and clinically-relevant physical half-lives.

#### **6.2.4. Delivery of oligonucleotides *in vivo***

The *in vivo* delivery of oligonucleotides perhaps presents the greatest challenge to their therapeutic use, as naked oligonucleotides are not readily internalized into cells. Several non-targeting strategies have been employed to deliver telomerase-inhibitory oligonucleotides. Addition of a cell-penetrating peptide to an oligonucleotide was suggested to enhance uptake (Villa *et al.*, 2000). Inhibitory oligonucleotides were also delivered to cells using a nanoparticle strategy (Beisner *et al.*, 2010). Other antisense

oligonucleotides have been delivered to cells using cationic porphyrins (Benimetskaya *et al.*, 1998).

Targeted delivery strategies have also been investigated. The addition of a tumour-targeting internalizing antibody potentiates delivery of oligonucleotides dependent upon expression of a cell-surface tumour antigen. One such approach employed trastuzumab, a humanized antibody specific to the HER2/Neu receptor frequently upregulated in breast cancer, amongst others (Cho *et al.*, 2003; van't Veer *et al.*, 2002). The antibody was conjugated to a radiolabelled oligonucleotide via a streptavidin-biotin linkage (Wang *et al.*, 2009a). Using this construct, radiolabelled oligonucleotides were specifically delivered to HER2-overexpressing cells *in vitro* and significant accumulation in tumour xenografts observed. Interestingly, a therapeutic interaction between trastuzumab and a telomerase-inhibitory oligonucleotide was reported *in vitro*, in breast cancer cells (Goldblatt *et al.*, 2009a). Indeed, telomerase inhibition resensitized cells that had acquired resistance to trastuzumab. A correlation between HER2/Neu expression and telomerase activity has been previously identified (Papanikolaou *et al.*, 2009) and, moreover, a regulatory link established (Goueli and Janknecht, 2004). Thus, an oligonucleotide delivery construct based around trastuzumab may offer increased selectivity of delivery, in addition to a potential therapeutic advantage. Such macromolecular delivery constructs are likely to increase the biological half-life and tumour accumulation of these agents (Muggia, 1999). The use of streptavidin also readily facilitates the inclusion of additional functionality, such as fluorophores for optical imaging (Liang *et al.*, 2010) and nuclear localization sequence (NLS) -containing peptides for enhanced nuclear targeting (Liu *et al.*, 2009). In the case of Auger electron-emitting triplex forming oligonucleotides, addition of an NLS enhanced the cleavage of target sequence, which otherwise required an excess of

ballast oligonucleotide, presumably to saturate cytoplasmic oligonucleotide-binding factors (Panyutin *et al.*, 2003).

### **6.3. Telomerase inhibition in combination with ionizing radiation**

In addition to exploiting the cellular distribution of telomerase for delivery of Auger electron-mediated irradiation, the development of radiolabelled telomerase inhibitors for the mRT of metastatic cancer aimed to improve upon the current limitations of telomerase-inhibitory therapy.

#### **6.3.1. Radiolabelling of telomerase inhibitors to enhance their function**

Non-radiolabelled inhibitors of telomerase had no effect on cell survival in a 24 hour clonogenic assay in these studies, highlighting the reliance of current inhibition strategies on the erosion of telomeres below a critical threshold for the initiation of growth arrest. Thus, such strategies are expected to exhibit a lag-time before effect, raising difficulties associated with the long-term administration of inhibitors in the clinical setting. In addition, functional p53 within tumour cells may be required for the adoption of senescence (Brassat *et al.*, 2011), although continued proliferation would eventually lead to death following crisis. Telomerase inhibitors may therefore be of limited use in the context of tumours with long telomeres and mutant p53. Addition of a

therapeutic radionuclide circumvents these limitations by eliciting cell death in a telomere-length- and p53-independent manner. Likewise, treatment with radiolabelled inhibitors reduces the opportunity for acquisition of resistance by switching from telomerase-dependent telomere maintenance to alternative lengthening of telomeres (ALT), which has been reported following the loss of telomerase function (Hu *et al.*, 2012). In addition, some ALT cells were shown to upregulate hTR expression (Cairney *et al.*, 2008), raising the possibility of targeting cancer cells with radiolabelled oligonucleotides to hTR, even in the absence of telomerase activity.

The low abundance of the enzyme in cancer cells represents a challenge for the development of radiopharmaceuticals targeting telomerase (Cohen *et al.*, 2007). Thus, targeting hTR in preference to hTERT for mRT may offer a further advantage, as hTR is present in elevated abundance in cancer cells (Cao *et al.*, 2008; Yi *et al.*, 2001). However, targeting hTR may lead to a modest loss of selectivity, as this molecule exhibits a wider expression profile than hTERT (Yashima *et al.*, 1998). Despite this, hTR is consistently expressed at greater levels in cancer cells than normal tissue (Nakamura *et al.*, 1997; Yashima *et al.*, 1998; Yi *et al.*, 2001; Yi *et al.*, 1999), and is further stabilized when co-expressed with hTERT (Yi *et al.*, 1999). The cell survival data obtained in these studies indicates that the accumulation of radioactivity in telomerase-positive cancer cells *in vitro*, albeit following artificial delivery, was indeed sufficient to specifically reduce survival – so mitigating the potential limitation regarding the low cellular abundance of telomerase. In this context, the selection of therapeutic radionuclides such as indium-111, with a reasonable yield of Auger electrons per decay (~7), may partially compensate for the low abundance of target. Calculation of the cellular dose suggested that, even when labelled to low specific activity (0.8 MBq/ $\mu$ g), match oligonucleotides delivered in excess of 1 Gy/cell over 24

hours in telomerase-positive populations. This dose corresponded to approximately 100 mBq/cell, and accordingly these oligonucleotides reduced clonogenic cell survival *in vitro*.

Telomerase inhibitory oligonucleotides were shown to induce haematological dose limiting toxicities in patients, specifically concerning thrombocytopenia and reduced coagulation (Chanan-Khan *et al.*, 2008; Ratain *et al.*, 2010). Radiolabelling of such inhibitors may reduce the necessary treatment time as discussed, but may indeed exacerbate off-target toxicity, for example following irradiation and sterilization of blood progenitor cells. Low level telomerase activity was detected in blood cell progenitors, as well as stimulated T and B cells (Hiyama *et al.*, 1995; Morrison *et al.*, 1996). In addition, telomerase activity was readily detected in germ cells, in rats (Ravindranath *et al.*, 1997). Telomerase expression in normal stem-cells was reported to be modest and transient (Feng *et al.*, 1995; Kim *et al.*, 1994), perhaps presenting a therapeutic window. The telomerase activity expressed by patient-derived mesenchymal stem cells, for example, was significantly lower than that of cancer cells (Jeon *et al.*, 2011). Masutomi and colleagues (2003) reported transient upregulation of hTERT during the cell cycle in immortalized fibroblasts, although telomerase activity was not detected, even in synchronized populations. Differentiated normal cells express no telomerase activity and are unaffected by telomerase inhibition *in vitro* (Damm *et al.*, 2001; Gellert *et al.*, 2006; Goldblatt *et al.*, 2009a; Goldblatt *et al.*, 2009b).

As few *in vitro* models representing the stem cell niche exist, especially without immortalization leading to the upregulation of telomerase activity, whole organism toxicity is difficult to predict, necessitating *in vivo* experimentation. Biodistribution data

obtained *in vivo* for existing oligonucleotide telomerase inhibitors may assist in the prediction of possible off-target effects (Dikmen *et al.*, 2005; Liu *et al.*, 2007). Likewise, late generation telomerase-null murine models and analysis of telomerase-mutated disease phenotypes may provide an indication of the likely toxicities associated with the depletion of telomerase-positive stem-cell populations (Hiyama and Hiyama, 2007; Wong *et al.*, 2000). The lack of effect on the survival of telomerase-negative cells in this work, suggests that toxicity would be largely limited to cells expressing robust telomerase activity, although this must be extensively verified in pre-clinical models of cancer.

### **6.3.2. Loss of telomerase activity may radiosensitize cancer cells**

As discussed in Chapter 5.4.2., evidence exists to suggest that loss of telomerase activity may promote sensitivity to ionizing radiation (IR). Knockdown of hTERT expression increased the radiosensitivity of breast (Papanikolaou *et al.*, 2009) and cervical (Nakamura *et al.*, 2005) cancer cells *in vitro*. Similar results suggested that loss of telomerase activity led to altered chromatin organization and inhibition of the DDR (Masutomi *et al.*, 2005). A putative correlation between telomerase activity and the response to IR has been established. Irradiation of cancer cells led to increased telomerase activity following post-translational modification of hTERT, via the PI3K/Akt pathway (Ram *et al.*, 2009). Telomerase activity was also upregulated in response to irradiation in telomerase-positive leukaemia cells (Finnon *et al.*, 2000). More recently, 2 Gy of IR was found to upregulate the telomerase activity of squamous cell carcinoma cells, likely via the NF $\kappa$ B-mediated activation of hTERT transcription (Aravindan *et al.*, 2013). Further to this, exogenous expression of hTERT increased the rate of repair of plasmid DNA in fibroblasts exposed to ultra-violet radiation (Shin *et*

*al.*, 2004). Forced hTERT expression in foreskin fibroblasts affected chromatin organization, gene expression and increased the repair of IR- and cisplatin-induced DNA damage (Sharma *et al.*, 2003). The link between telomerase activity and DNA damage repair may partially explain the relative radiosensitivity of cells where telomerase expression has been artificially silenced. However, similar short-term radiosensitization was not observed following catalytic inhibition of telomerase in our assay, perhaps indicating telomere-elongation-independent functions of telomerase in the DDR.

Late generation telomerase-deficient mice, which have shortened telomeres, were demonstrated to have increased sensitivity to whole body irradiation (Goytisolo *et al.*, 2000; Wong *et al.*, 2000). Prolonged catalytic inhibition, sufficient to lead to critical telomere erosion, has also been shown to sensitize cells to DNA damage, probably due to the excess of telomere-associated damage foci. Long-term telomerase inhibition was required to reduce the rate of repair of cisplatin- or doxorubicin-induced DNA damage, visualized using the comet assay, in sarcoma cells (Uziel *et al.*, 2010). Prolonged treatment of putative glioblastoma tumour-initiating cells with GRN163L elicited telomere shortening and enhanced radiosensitivity in combination with temozolomide *in vitro* (Marian *et al.*, 2010a). Likewise, long-term administration of GRN163L enhanced the radiosensitivity of breast cancer cells *in vitro* and moreover, pre-treatment combined with IR significantly slowed xenograft tumour growth compared to single treatment *in vivo* (Gomez-Millan *et al.*, 2007). Thus, loss of expression of hTERT or telomere shortening appear to be the critical factors necessary for radiosensitization.

In addition to the effect of radiolabelled oligonucleotides on clonogenic survival demonstrated in this study, further mechanistic investigations were performed. The subcellular distribution of the induction of the DDR protein  $\gamma$ H2AX was analysed. No clear association between damage foci and telomeric DNA was determined, possibly due to limitations in the techniques employed. Radioactive decay focussed at the telomeres may be of particular interest in the context of work that demonstrated the inhibition of repair of telomere-associated DNA damage (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012; Rochette and Brash, 2010). In addition to potential colocalization of radionuclide-bound telomerase with telomeric DNA, telomerase has also been suggested to interact with other genomic loci, including the promoters of Wnt-response genes (Park *et al.*, 2009). Thus, the distribution of DNA damage following application of a radiolabelled inhibitor of telomerase is likely to depend upon multiple factors.

#### **6.4. Telomerase as a therapeutic target**

In addition to the high contrast generated by the restricted expression profile of telomerase in normal tissue and upregulation in cancer, other therapeutic advantages of telomerase inhibition have been described.

### 6.4.1. Reduction in metastatic potential

The repression of telomerase activity during differentiation is thought to act as an important tumour suppressive mechanism. A link between the Wnt-signalling pathway, involved in stem-cell signalling, and telomerase activity has been established (Park *et al.*, 2009). Furthermore, abrogation of telomerase activity was found to promote the differentiation of glioma cells *in vitro*, assessed by upregulation of the gliomal differentiation markers GFAP and cyclin-dependent kinase inhibitor proteins (Kondo *et al.*, 1998b). In support of this, other studies proposed loss of telomerase activity may correlate with reduced invasive potential. Telomerase inhibition reduced the migration of Ewing sarcoma cells *in vitro* and the aggressiveness of xenografted pancreatic tumours, as assessed by presence of necrotic foci and tumour lymphatic emboli, and tumour distance from the epidermis (Uziel *et al.*, 2010). The treatment of breast cancer cells with the oligonucleotide telomerase inhibitor GRN163L led to dysregulation of actin filaments and sites of focal adhesion (Goldblatt *et al.*, 2009b). This translated into a reduction in invasive potential *in vitro*. Similarly, breast cancer cells treated with GRN163L were shown to exhibit reduced invasivity *in vitro*, even when telomeres had not yet been extensively eroded and cell growth was unaffected (Gellert *et al.*, 2006). Thus, inhibition of telomerase activity may reduce the metastatic potential of malignant cells. Indeed, in addition to reducing primary tumour growth, significantly fewer metastatic splenic nodules were observed following subcutaneous infusion of telomerase inhibitors to a mouse bearing a Burkitt's lymphoma xenograft tumour (Mata *et al.*, 1997). In other work, lung cancer cells were pre-treated with GRN163L before intravenous injection into nude mice (Dikmen *et al.*, 2005). Significantly fewer lung nodules were observed in animals receiving treated-cells, as determined by histopathological examination. Moreover, administration of GRN163L to mice

intravenously injected with cells that had not been pre-treated, prevented the formation of detectable lung tumours in this model.

#### **6.4.2. Depletion of cancer-initiating cells**

A subpopulation of cancer cells displaying the characteristics of stem-cells, including the expression of stem-cell-marker molecules and ability to reconstitute a tumour *in vivo* when administered at low concentration, has been described (Bortolomai *et al.*, 2010). Moreover, such stem-cell-like tumour-initiating cells tend to be resistant to both chemotherapeutic agents (Dallas *et al.*, 2009; Shafee *et al.*, 2008) and IR (Bao *et al.*, 2006). Thus, agents exhibiting toxicity towards these cells offer great therapeutic potential.

Aldehyde dehydrogenase-positive (ALDH<sup>+</sup>) non-small cell lung cancer cells were identified as possessing stem-like properties *in vitro* and *in vivo* (Serrano *et al.*, 2011). Such cells were found to be sensitive to telomerase inhibition, mediated by the small molecule MST-312, compared to the bulk cell population. Telomerase inhibition led to a significant reduction in the proportion of ALDH<sup>+</sup> cells *in vitro* and in xenograft tumours. Furthermore, MST-312 sensitized ALDH<sup>+</sup> tumour-initiating cells to IR *in vitro*. Likewise, the long-term treatment of prostate cancer cells with GRN163L significantly reduced the number of CD44<sup>+</sup> or CD133<sup>+</sup> tumour-initiating cells and, accordingly, inhibited the ability of the cells to form holoclones and spheroids *in vitro* (Marian *et al.*, 2010b). This oligonucleotide telomerase inhibitor also depleted the proportion of CD44<sup>+</sup> and ALDH<sup>+</sup> tumour-initiating cells from breast and pancreatic cell populations (Joseph *et al.*, 2010). Telomerase inhibition reduced the self-renewal and anchorage-independent spheroid formation of these cells *in vitro* and significantly

reduced their *in vivo* tumorigenicity. Interestingly, in all cases, no apparent difference in telomerase activity between tumour-initiating and bulk cells was observed.

### 6.4.3. Combination therapy

As discussed in the context of IR in Chapter 6.3.2., telomerase inhibition in combination with additional therapeutic intervention may be beneficial. Evidence of a therapeutic interaction between loss of telomerase activity and chemotherapeutic agents was established in *mTERC*<sup>-/-</sup> mouse embryonic fibroblasts, which exhibited increased sensitivity to doxorubicin, daunorubicin and actinomycin D (Lee *et al.*, 2001). Cervical cancer cells were sensitized to the DNA-damaging agents doxorubicin, bleomycin and etoposide, by the short-hairpin RNA (shRNA) mediated knockdown of hTERT (Nakamura *et al.*, 2005).

As a result, the potential of the combination of telomerase inhibition and chemotherapy has been explored. The small molecule telomerase inhibitor BIBR-1532 further reduced the colony forming ability of both wild-type and resistant cells, following melphalan treatment (Ward and Autexier, 2005). Pre-treatment with the telomerase inhibitor GRN163L significantly sensitized breast and colorectal cancer cells to etoposide and irinotecan (Tamakawa *et al.*, 2010). Catalytic inhibition of telomerase also increased the sensitivity of hepatoma cells to doxorubicin *in vitro* (Djojsubroto *et al.*, 2005). Breast cancer cells treated with GRN163L and paclitaxel showed reduced cell growth and plating-efficiency *in vitro*, seemingly with synergistic interaction (Goldblatt *et al.*, 2009b). This combination treatment significantly slowed the growth of tumour xenografts above that mediated by the single agents. Oligonucleotide-mediated

telomerase inhibition has also been reported to increase the sensitivity of sarcoma cells to cisplatin *in vitro* (Uziel *et al.*, 2010). Pancreatic tumour xenograft growth was further inhibited by this combination treatment.

Similar advantageous combination effects have been demonstrated with molecularly targeted agents. GRN163L exhibited synergistic inhibition of the growth of HER2/Neu overexpressing breast cancer cells in combination with trastuzumab *in vitro* (Goldblatt *et al.*, 2009a). In this setting, telomerase inhibition was sufficient to re-sensitize trastuzumab-resistant cells.

Interestingly, the possibility that telomerase inhibition may yield beneficial modulation of tumour microenvironment has also been proposed. Overexpression of hTERT correlated with an increase in VEGF expression in cancer cells *in vitro* (Zhou *et al.*, 2009). The co-implantation of glioblastoma and endothelial cells xenografted into immunocompromised mice led to the development of vessel-like structures (Falchetti *et al.*, 2008). Inhibition of telomerase activity in the angiogenic endothelial cells, by knockdown or expression of dominant-negative hTERT, abolished tubule formation and reduced xenograft growth. In addition, knockdown of hTR expression suppressed genes implicated in angiogenesis and metastasis in cancer cells *in vitro* (Li *et al.*, 2005). Thus, inhibition of telomerase may have some anti-angiogenic activity. Furthermore, the oligonucleotide-mediated inhibition of telomerase was found to reverse hTERT-associated increases in hyperphosphorylation of the retinoblastoma protein (pRB), E2F activity and activation of proliferation in lens epithelial cells (Xiang *et al.*, 2002), suggesting inhibition may limit telomerase-associated proliferative signalling in this context.

Thus, targeting telomerase for mRT may facilitate a reduction in tumour proliferation and aggressiveness, along with a depletion of therapy-resistant malignant cell populations. In some cases, such additional effects were shown to require prolonged treatment to allow for telomere shortening. However, short-term effects, mediated over a few days, including reduction in invasive potential, depletion of cancer stem-cells and sensitization to other therapeutic modalities have also been demonstrated *in vitro* and *in vivo*, and so may be clinically relevant in the context of radiolabelled telomerase inhibitors. As cancer cells typically have shorter telomeres than normal cells (Marian *et al.*, 2010a; Meeker *et al.*, 2002), and telomere shortening may be an additional factor for combination effects, sensitization to chemo- and especially radiotherapy may preferentially occur in malignant tissue – further widening the therapeutic window. The interaction of telomerase inhibition with mRT and other therapeutic strategies may therefore enhance treatment outcome.

### **6.5. Radiolabelled oligonucleotides for the molecular radiotherapy of disseminated cancer cells**

The addition of radiolabelled oligonucleotides with therapeutic potential described here, expands the range of radiopharmaceuticals under experimental development. This work supplements the emerging potential of Auger electron-emitting oligonucleotides, capable of reducing the clonogenic survival of cancer cells *in vitro*, as molecular radiotherapeutic agents (He *et al.*, 2004b; Liu *et al.*, 2009; Sedelnikova *et al.*, 2002; Sedelnikova *et al.*, 2000). The intrinsic characteristics of this class of agent offer

therapeutic advantages over existing mRT approaches. The short range of emitted Auger electrons means nuclear targeting is a therapeutic requirement but negates the cross-fire irradiation of adjacent normal tissue. In addition, the high linear energy transfer (LET) associated with Auger electrons leads to an enhanced biological effectiveness, due to the increased complexity of inflicted DNA damage. The molecular weight of the radiolabelled oligonucleotides is likely to be lower than that of peptide and antibody-based radiopharmaceuticals, hastening the clearance of radioactivity from normal tissues. Oligonucleotides also provide a high degree of selectivity and favourable binding affinity. In addition, oligonucleotides labelled with indium-111, which emits penetrating gamma photons, facilitate molecular imaging *in vivo* (Amirkhanov *et al.*, 2010; Dewanjee *et al.*, 1994; Liu *et al.*, 2009; Wang *et al.*, 2003). Thus, indium-111-labelled oligonucleotides targeting telomerase may function as so-called theranostic agents, simultaneously delivering high LET therapeutic irradiation whilst acting as a single photon-emission computed tomography (SPECT) tumour-imaging agent. Strategies to circumvent limitations associated with the stability and delivery of oligonucleotides *in vivo* are being considered, and include the use of alternative oligonucleotide chemistry, and nanoparticle peptide- and antibody-targeted delivery constructs.

## 6.6. Conclusion

This work has sought to develop and validate novel radiopharmaceuticals targeting telomerase. The labelling of a panel of existing small molecule inhibitors of telomerase with Auger electron-emitting radionuclides, did not yield a suitable candidate radiopharmaceutical. Such a negative finding mirrors the current difficulties associated with development of small molecule inhibitors of telomerase.

In contrast to the situation with the small molecules, the development of oligonucleotide-based strategies is more promising. Accordingly, radiolabelled oligonucleotide inhibitors of telomerase exhibited therapeutic potential, at least in the *in vitro* setting discussed herein. This novel class of molecule significantly and specifically inhibited the clonogenic survival of telomerase-positive cancer cells. Further characterization of these molecules suggested that the inhibitory oligonucleotides may occupy discrete intranuclear domains upon administration, in agreement with previous work. Limitations of such a therapeutic strategy include the requirement to improve upon oligonucleotide stability and targeted delivery; as well as the potential induction of radiotoxicity, associated with off-target and non-specific irradiation of normal tissues *in vivo*.

This project potentially improved upon the characteristics of existing telomerase inhibition strategies, which currently require long-term administration for effect, whilst aiming to develop a novel mRT agent capable of targeting disseminated cancer cells. The pharmacological targeting of telomerase may offer benefits, in addition to providing a means for selecting malignant in preference to normal tissue, applicable to a

broad range of clinical cancer presentations. Telomerase inhibition has been suggested to reduce tumour aggressiveness, crucially including the reduction of metastasis. In addition, telomerase inhibition may preferentially deplete therapy resistant cancer cell populations, for example by targeting putative tumour-initiating cells. Auger electron mRT, here potentiated by the nuclear localization of telomerase, generally aims to sterilize disseminated tumour cells in the micrometastatic niche, usually in the adjuvant setting. Thus, this strategy would necessarily be combined with other clinical therapeutic modalities, including molecular-targeted agents and chemotherapy, and IR. Telomerase inhibition has been demonstrated to exert a therapeutic interaction with all these modalities, potentially mutually optimizing treatment outcome. Indeed, radiosensitization mediated by an oligonucleotide simultaneously delivering high linear energy transfer (LET) radiation is an exciting possibility, although more work is required to ascertain this connection.

Thus, further development, and critically *in vivo* characterization, of Auger electron-emitting radiolabelled oligonucleotides targeted to telomerase is recommended.

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