HARNESSING A SECONDARY DNA STRUCTURE AT THE TELOMERES USING A SMALL MOLECULE, TMPyP4: IMPLICATION IN CANCER THERAPY

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information, which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Shriram Venkatesan

29 September, 2013

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"Nothing in biology makes sense, except in the light of evolution."

Theodosius Dobzhansky

CONTENTS

SYNOPSIS

Telomeres – arrays of G-rich DNA and the terminal part of chromosomes – interact dynamically with specialised proteins, shelterins, to protect chromosomal and hence, genetic integrity. Shelterins ensure telomere maintenance by allowing or denying access to various telomere-interacting factors, depending on the cellular context, in a highly evolved manner. Despite the barrage of mutations that accumulate in a cell that is straying away from normalcy towards becoming cancerous over time, functional telomeres prevents mitotic catastrophe and in about 90% of the cases, it happens by activation of an otherwise mostly repressed enzyme – telomerase, which interacts with the telomere dynamically and adds on telomeric repeats. Some key DNA repair orchestrators such as DNA-PK and ATM kinase form the second line of defence, by a prolonged cell arrest upon telomere dysfunction, in order to repair the damage and prevent genomic instability. Any perturbation in the interaction between the shelterin complex at the telomeres, and telomerase is likely to challenge the immortality of cancer cells. This study involves harnessing the tendency of the G-rich single-stranded overhang at the telomere to form secondary structures. G-quadruplexes, which are stacks of tetrads of guanine molecules, is the major type of secondary structure that can form preferentially at the telomeres and thus, small molecules that bind to and stabilises these structures have achieved the destruction of normal telomere homeostasis. One such small molecule, meso-5,10,15,20-Tetrakis-(N-methyl-4-pyridyl)porphine, Tetratosylate, also known as TMPyP4 – one of the first ligands to be explored for their quadruplex-binding – has been shown hitherto by other

groups, to preferentially localise to the nucleus, diminish telomerase activity, enforce cell arrest and apoptosis in a range of cancer cell types. High-grade human brain tumours –glioblastoma multiforme and medulloblastoma, owing to their poor survival rate and prognosis, and lack of efficient treatment strategies apart from surgery, radiotherapy and DNA damage inducer, temozolamide. Thus, they require immediate attention for drug development. This study delved into the mechanistic insights behind the above-mentioned observations upon TMPyP4 treatment; validated its combination with DNA repair inhibition; and also explored the potential of TMPyP4 to sensitise brain tumour cells to radiation-induced cell death.

Short-term, proof-of-principle assessment of TMPyP4 exhibited a dose-dependent effect on the viability, and also arrested the proliferation of the brain tumour cells at IC_{50} dose, while having a negligible effect on the control cell type – normal human lung fibroblasts. The observation in this study that the levels of TRF2 (a key shelterin protein) steeply reduced as early as 2 hours after treatment seems to corroborate with previous studies by other groups on the selective binding of TMPyP4 to telomeric sequences *in vitro*. Our study also found that this was accompanied by a relatively more gradual reduction of hTERT (the catalytic subunit of human telomerase) levels and telomerase activity, along with a great increase in the DNA damage levels, assessed after 48 hours of treatment. TMPyP4 thus seemed to specifically bind to the telomeres, interfere with telomere architecture, and inhibit telomerase activity indirectly.

Over short-term treatment, TMPyP4 enhanced the DNA damage, potentiated the cell arrest and hampered to a greater extent, the viability of the brain tumour cells upon irradiation, thus demonstrating a potential and much-needed radiosensitising property for a cancer drug. There are reports of some drugs antagonising the inhibition of DNA repair, but this study on TMPyP4 showed that, the inhibition of either of the two DNA repair lynchpins – ATM kinase and DNA-PK, led to the potentiation and increased the irreversibility of TMPyP4-induced DNA damage, cell arrest and viability.

Assessment treatment of the effects of TMPyP4 over therapeutically relevant long-term treatment on glioblastoma cells revealed concordance with the predictions of the short-term assessment on glioblastoma and medulloblastoma cells, showing that TMPyP4 exhibited a dose- and time- dependent effect on the viability, and DNA damage. More relevantly, TMPyP4 induced telomere aberrations, caused telomere shortening over time, and led to potential telomere-recombination in the glioblastoma cells, culminating in cell death *en masse*.

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LIST OF ABBREVIATIONS

ALT Alternative Lengthening of Telomeres

APBs ALT-associated promyelocytic leukaemia bodies

AT Ataxia telangiectasia

ATM Ataxia telangiectasia- mutated kinase

BBB Blood-brain-barrier

BFB Breakage-Fusion-Bridge

DDR DNA Damage Response

DNA Deoxyribose nucleic acid

DNA-PK DNA-dependent Protein Kinase

DSBs Double strand breaks

dsDNA Double-stranded DNA

GBM Glioblastoma multiforme

H2AX Histone 2AX

HAATI Heterochromatin Amplification-mediated and Telomerase-independent Survivors

HR Homologous recombination

hTERT Human telomerase reverse transcriptase

hTR/ hTERC Human Telomerase RNA

NHEJ Non-homologous end joining

PARP 1 Poly [ADP-ribose] polymerase 1

PML Promyelocytic Leukaemia

POT1 Protection of Telomeres 1

RAP1

Rb Retinoblastoma

RHPS4 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate

RNA Ribosenucleic acid

RTEL	Regulator of Telomere Elongation
SSBs	Single strand breaks
ssDNA	Single-stranded DNA
TERRA	Telomere Repeat containing RNA
TIN2	
TMPyP4	Porphyrin
TPP1	Adrenocortical dysplasia protein homologue (TIN2-interacting protein 1)
TRF1	Telomere Repeat-binding Factor
TRF	Telomere Restriction Fragment length analysis
CIN	Chromosome instability
FISH	Fluorescence in-situ Hybridization
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride

RESEARCH PUBLICATIONS

- **Venkatesan S**, Teo ZX, Sethu S, Jayapal M, Hande MP; The effect of the G-quadruplex ligand TMPyP4 on telomere-telomerase equilibrium in human brain cancer cells; Manuscript in preparation.
- Pavagadhi S, Betha R, **Venkatesan S**, Balasubramanian R, Hande MP; Physicochemical and toxicological characteristics of urban aerosols during a recent Indonesian biomass burning episode, *Environ Sci Pollut Res Int.* 2012 Sep 13
- Halaby MJ, Hakem A, Li L, El Ghamrasni S, **Venkatesan S**, Hande PM, Sanchez O, Hakem R*;* Synergistic interaction of Rnf8 and p53 in the protection against genomic instability and tumorigenesis; *PLoS Genet*. 2013;9(1):e1003259. doi: 10.1371/journal.pgen.1003259. Epub 2013 Jan 31.
- El Ghamrasni S, Pamidi A, Halaby MJ, Bohgaki M, Cardoso R, Li L, **Venkatesan S**, Sethu S, Hirao A, Mak TW, Hande MP, Hakem A, Hakem R*;* Inactivation of Chk2 and Mus81 leads to impaired lymphocytes development, reduced genomic instability and suppression of cancer; *PLoS Genetics*; 2011 May;7(5)

CONFERENCE PRESENTATIONS

- **Venkatesan S**, Teo ZX, Sethu S, Jayapal M, Hande MP. Targeting telomere-telomerase equilibrium in human brain cancer cells by a G-quadruplex stabilising agent. *Cold Spring Harbour meeting on Telomeres and Telomerase*. New York, USA. April 30 - May 4, 2013.
- **Venkatesan S**, Nandakumar S, Chinmayee BN, Sethu S, Jayapal M, Hande MP. Targeting telomere-telomerase equilibrium in brain cancer cells by a G-quadruplex interacting agent. *The 70th Annual Meeting of the Japanese Cancer Association*. Nagoya, Japan. October 3-5, 2011
- **Venkatesan S**, Sethu S, Jayapal M, Hande MP. Harnessing a Secondary DNA Structure at the Telomere for Cancer Therapy. *XXXVI Annual Conference of Environmental Mutagen Society of India (EMSI) and International Symposium on Environmental Exposures to Mutagens and Carcinogens on Human Health*. Vellore, India. February 4-7, 2011.

Chapter 1

Introduction

1.1 TELOMERE BIOLOGY AND CANCER THERAPEUTICS – A HISTORICAL PERSPECTIVE

1.1.1 Cancer, genetic material, and DNA – uncovering the link

In 1914, at a time when we did not even know that the genetic material was composed of nucleic acids, key research on sea urchin eggs revealed an association between inappropriate chromosome segregation during cell division with changes in cellular growth. This led to the hypothesis that abnormal chromosomes cause cancer (Boveri, 1914).

As Gregor Mendel's work was being rediscovered and its significance becoming more apparent by the day, and about a decade after the word "somatic mutation" had been coined, genetic mutations were being attributed as the origin of cancer (Bauer, 1928; Tyzzer, 1916).

It was long known that chronic occupational exposure to some chemicals could cause cancer. By 1915, there was even an established mouse model to recapitulate carcinogenesis by coal tar (Yamagiwa, 1915). However, the pathogenesis of such environmental exposures wasn't known and people never fancied that the genetic material would be mutated directly. The perception was however set to change radically in a few years. By 1928, X-rays were shown to be mutagenic in nature, in *Drosophila melanogaster* (Muller, 1928).

The first speculation at the existence of a special structure at the ends of chromosomes was in a lecture given by Hermann Muller in 1938 (Muller, 1938). Experimental observation of the same was seen by Barbara McClintock with the

observation of fusion of broken ends of chromatids in meiotic anaphase in *Zea mays* (McClintock, 1941). Only four decades later would they be named 'telomeres' and a completely new perspective of its effects on cell physiology would be unravelled.

After DNA and not protein was proven to be the genetic material, and possible mechanisms of DNA replication postulated, the important concept of DNA repair was demonstrated in Chinese hamster ovary cells. The surviving population of cells following irradiation, lacked heritable change in their DNA and seemed to repair it before they divided. This clearly established the presence of well-regulated DNA repair mechanisms in cells and possibly their modulation in cancer (Elkind and Sutton, 1959).

1.1.2 Telomeres – implications in ageing and cancer

Although way back in 1881 itself, it was proposed that tissues get worn out due to limitation in the number of cell divisions and that this affects the organ's performance, it was not until Leonard Hayflick in 1961 showed the first experimental evidence of limited lifespan of somatic cells (Hayflick and Moorhead, 1961). Hayflick co-cultured 'old' normal human male fibroblasts with 'young' normal female human fibroblasts, and had unmixed controls as well. He found that after a few doublings in culture, when the control male cells stopped dividing, there were only female fibroblasts in the mix. This was proof enough that not all cells in culture are immortal: fibroblasts had limited lifespan in culture. This showed that although there are 'young' cells in the vicinity, cells had an inherent control mechanism to know that they were 'old' (Shay and Wright,

2000). Despite this elegant demonstration, scepticism was still rife. According to Nobel Laureate Peyton Rous who reviewed and rejected Hayflick's first attempt at publishing it: "the largest fact to have come from tissue culture in the last fifty years is that cells inherently capable of multiplying will do so indefinitely if supplied with the right milieu *in vitro*" (Shay and Wright, 2000). Later work in Hayflick's lab by doctoral student Woodring Wright showed that the cell's "replicometers" lay in the nucleus (Wright and Hayflick, 1975).

James Watson, in 1972, proposed that the 3' end of linear DNA cannot be fully copied during replication, owing to the inability of DNA polymerase to add nucleotides without a primer. This was called the 'end replication problem' (Figure 1.1) (Watson, 1972).

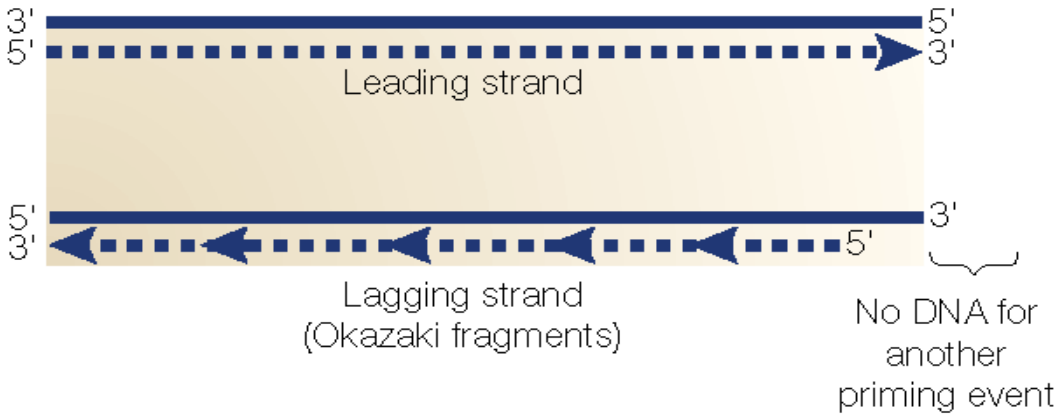


Figure 1.1 **DNA end-replication problem:** DNA replication at the leading strand poses no problem to DNA polymerase (Polα) as it can extend the leading strand in a continuous manner. Replication at the other strand (the lagging strand), however, takes place by using primers at various points and synthesis in the form of short Okazaki fragments as Polα cannot add nucleotides in 3'-5' direction. The lagging strand also cannot replicate completely, as a primer cannot attach beyond the end of the template DNA. This leaves a 3' overhang. The leading strand is also processed to leave a 3' overhang. Image source: (Shay and Wright, 2000).

Olovnikov later recalled his 'Eureka' moment while returning home after attending a lecture where Hayflick's work was discussed:

> "The Theory of Marginotomy came to me in that Moscow subway station. I heard the deep roar of an approaching train coming out from the tunnel into the station itself. I imagined the DNA polymerase to be the train moving along the tunnel that I imagined to be the DNA molecule. I thought that this polymerase cannot begin to copy from the very beginning because there is a dead zone between the front end of the polymerase molecule and its catalytic centre. This is analogous to the dead zone between the front end of a subway car standing at the beginning of the subway platform and the nearest entrance door to the first car." (Olovnikov, 1996)

Thus, Olovnikov went a step further from Watson's finding of the end-replication problem and proposed that the ends of chromosomes of linear DNA (telomeres) shorten with time (i.e. with every cell division cycle) and this could be the mechanism behind Hayflick's observation on cellular ageing. Experimental proof backing the theory and bringing it back to focus would only happen two decades later though.

Meanwhile, study of telomeres gathers pace. In 1978, Elizabeth Blackburn, doing her post-doctoral fellowship at Yale University, was mapping DNA sequences of the unicellular eukaryotic ciliate, *Tetrahymena*. They can have up to hundreds of chromosomes upon differentiation. Blackburn observed one particular repeat sequence $(TTGGGG)_n$, at the ends of chromosomes (Blackburn and Gall, 1978).

Jack Szostak, a postdoctoral fellow at Harvard Medical School, had just observed that minichromosomal linear DNA when introduced into yeast, underwent rapid degradation (Szostak and Wu, 1979). McClintock's and Muller's proposition was proven right when Blackburn and Szostak introduced the Tetrahymena end sequence into the yeast minichromosomes. Astonishingly, the repeat element protected them from the anticipated rapid degradation and was named 'telomeres'. ('Telo' means 'end' and 'meros' means part in Greek.) The protection of yeast chromosomes by DNA from the completely unrelated *Tetrahymena* species also pointed out to the existence of telomeres as a fundamental mechanism across many organisms (Szostak and Blackburn, 1982). This would soon lead to the unravelling of a distinct pattern conserved across all eukaryotes – a guanosine-rich hexameric DNA repeats at the chromosome ends. The sequence of human telomeres was later revealed to be composed of hexameric 5'-TTAGGG-3' repeats (Moyzis et al., 1988).

Carol Greider, graduate student in Elizabeth Blackburn's lab in 1985 observed that the *Tetrahymena* telomeres were getting longer with every generation during its logarithmic phase of growth. Greider realised that due to the DNA end-replication problem, the telomeres should not lengthen, if not shorten with every generation. Greider and Blackburn then looked for an unknown enzymatic activity that could lead to the addition of repeats in 3'-5' direction. This led to the discovery of a terminal transferase activity, which they found was an enzyme that they named 'telomerase (Figure 1.2) (Greider and Blackburn, 1985).

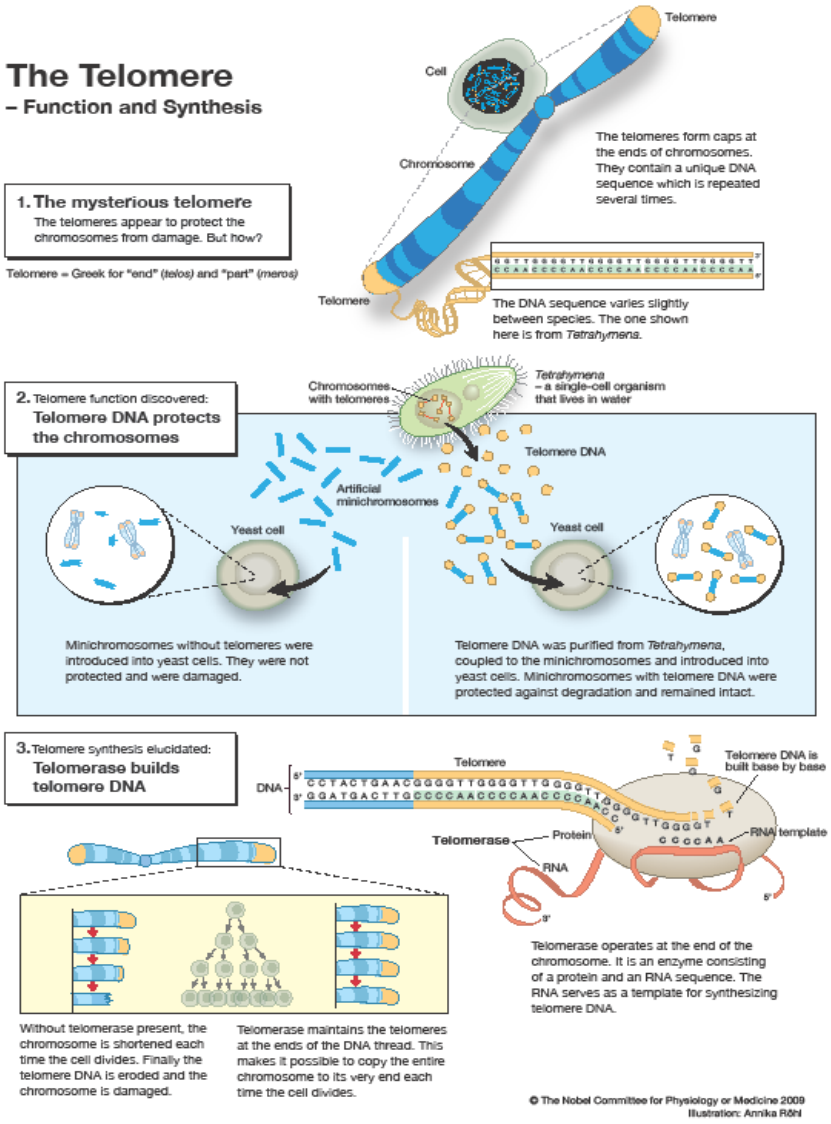


Figure 1.2 **The discovery of telomere and telomerase** Image source(Rohl, 2013)

Telomere shortening was already being speculated to happen with cellular ageing. The first experimental hints were obtained from the finding that telomere lengths varied considerably among different tissues (Cooke and Smith, 1986). Direct evidence of telomere shortening with increasing passage number followed soon

after by studies on cultured human fibroblasts *in vitro* and primary human skin cells (Harley et al., 1990; Lindsey et al., 1991).

This was followed by investigations on identifying telomerase activity in various human tissues. Studies revealed telomerase activity in, germline cells and cancer cells (Kim et al., 1994; Morin, 1989). Telomere shortening, thus, underlies an elegant method adopted by human system for maintaining cellular homeostasis and cell turnover.

While the core components of human telomerase holoenzyme – human telomerase RNA (hTR) and catalytic subunit, human telomerase reverse transcriptase (hTERT) were being identified, signs of existence of an alternative telomere lengthening mechanism in humans, leading to cell immortalisation was being observed as well (Blasco et al., 1997; Bryan et al., 1995; Feng et al., 1995; Nakamura et al., 1997). Another landmark study was the finding that the introduction of hTERT into normal human cells led to the extension of their lifespan by at least 20 population doublings (Bodnar et al., 1998). This was the first study to demonstrate the tantalising link between telomeres and senescence. This led to a host of studies on the nature of the telomerase components, and eventually led to explosion of studies on a new field by itself – telomere biology.

1.2 HALLMARKS OF CANCER

Since the advent of molecular biology, there has been immense improvement in our understanding of the molecular events underlying carcinogenesis. Major hallmarks of cancer (Figure 1.3) have been unravelled from a body of work and have been reviewed extensively. Cancer cells invariably possess or eventually

gain the abilities to sustain incessant growth signalling; inactivate or repress cell death signalling; maintain factors needed for perpetual cell division; reprogramme their metabolism to survive challenging micro-environments; evade destruction by the immune system; induce formation of angiogenic lymph and blood vessels; and invade and colonise in tissues of distant organs (Hanahan and Weinberg, 2000, 2011).Understanding the precursor of all the hallmarks is key to designing therapeutic strategies for cancer.

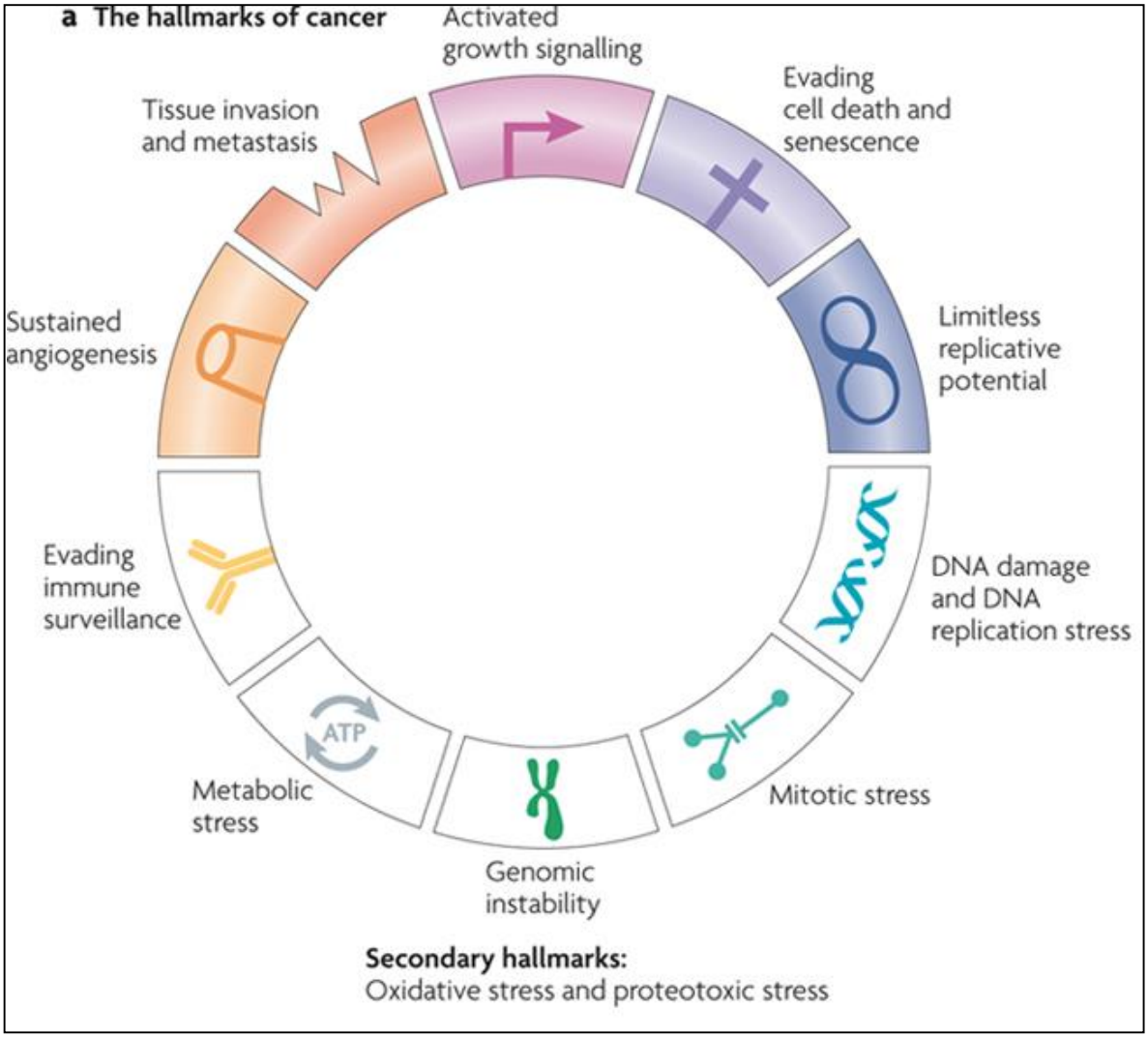


Figure 1.3 **Hallmarks of cancer** Image source: (Negrini et al., 2010)

1.2.1 Genomic instability

It has been well-established that telomeres guard chromosome integrity and thus genome stability by preventing attack by exonucleases; protection from illegitimate recombination; proper positioning of chromosomes in the nucleus; and facilitating alignment of chromosomes to allow for meiotic recombination, among others (Blackburn, 1991; Greider, 1991, 1996; Hande, 2004; Tomita and Cooper, 2007; Zakian, 1995). Genome stability is also threatened by spontaneous mutations that occur owing to rare errors inevitable during DNA replication and

proof-reading and to a greater extent by damage due to by-products of cellular metabolism, chronic exposure to environmental agents such as X-rays, UV-radiation, and various chemicals lead to damaging of cellular structures, mainly the DNA (Hoeijmakers, 2009; Hughes and Reynolds, 2005; Johnson et al., 2000; Wilson et al., 2008). Mammalian cells deploy hundreds of DNA repair proteins belonging to specific pathways, whose activation depends on the type of DNA damage and the phase of cell cycle (Figure 1.4) (Hoeijmakers, 2001; Matsuoka et al., 2007).

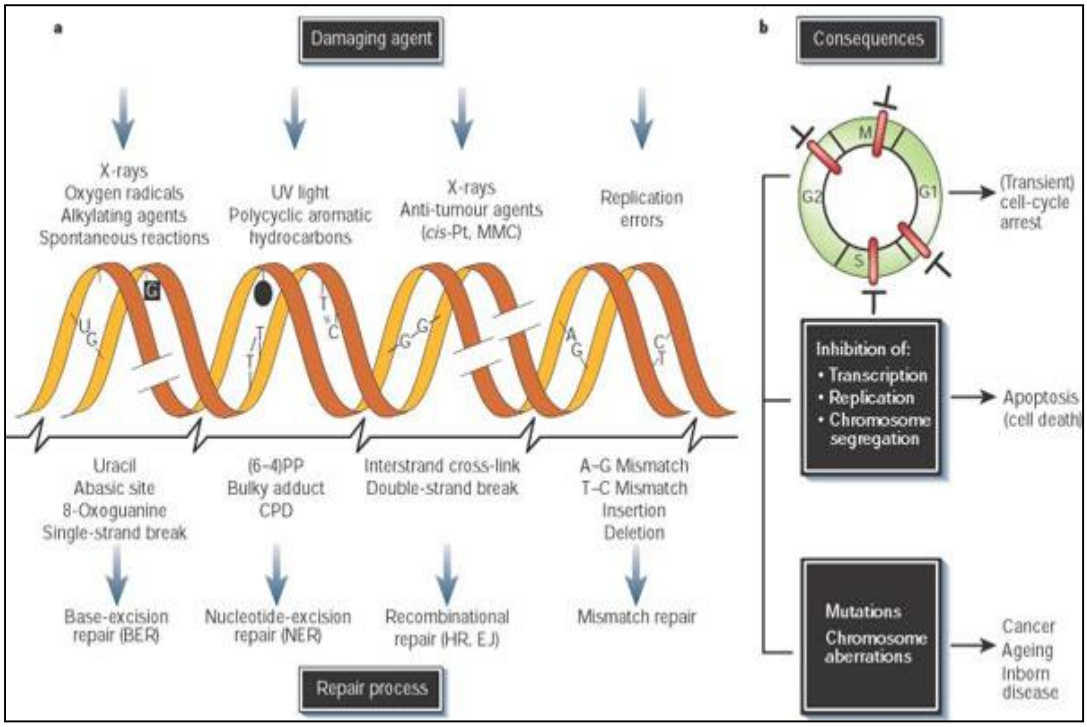


Figure 1.2 **DNA repair pathways in mammalian cells** Cells employ specialised pathways for repair od DNA damage depending on the type and intensity of damage and the phase of cell cycle. Image source: (Hoeijmakers, 2001).

DSBs are the major threat to a cell's survival and are often catastrophic to the stability of the genome. Unrepaired SSBs can get converted to DSBs as a result of replication (Khanna and Jackson, 2001). Classical DSB repair is executed by two

major pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) (Khanna and Jackson, 2001). HR mediates accurate repair of DSBs by using available homologous DNA as template, while NHEJ operates by involving ligases to join the broken segments in an error-prone, homology-independent manner (Hoeijmakers, 2001).

Cells encountering irreparable DNA damage or possessing critically short telomeres undergo senescence or apoptosis. This is ensured by cellular checkpoint machinery like p53 and Rb (Evan and Vousden, 2001). Cells that acquire the ability to bypass these barriers in this turmoil do so by virtue of mutations in checkpoint proteins or by telomere elongation (Evan and Vousden, 2001). This underscores the importance of genome maintenance mechanisms in cells.

Not in all cases are cancer genomes unstable (Bodmer et al., 2008; Muleris et al., 1995; Sieber et al., 2003). Nevertheless, genomic instability, if not the primer for carcinogenesis, accelerates the process in an overwhelming majority of cases (Negrini et al., 2010; Sieber et al., 2003). Although somatic mutations are rampant in cancers, not all mutations cause genomic instability. Mutations in DNA repair factors could lead to lack of repair or erroneous repair, both of which would give rise to a mutator phenotype and hence, genomic instability, have been shown to be the cause of a majority of hereditary cancers (Hoeijmakers, 2001; Loeb, 2001).

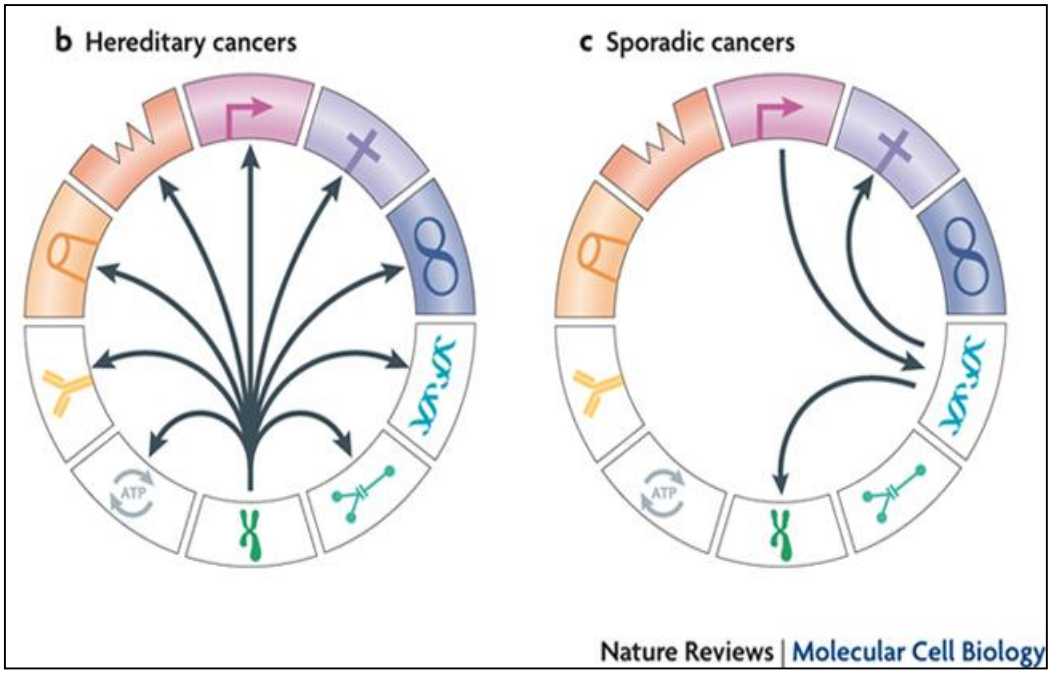


Figure 1.5 **Temporal order of acquisition of the hallmarks in hereditary and sporadic cancers** Image source: (Negrini et al., 2010).

Oncogene activation in sporadic cancers has been shown to cause genomic instability via DNA replication stress. The emergence of genomic instability, when coupled with the frequently observed p53-incativating mutations would fuel the acquisition of other hallmarks and hence facilitate carcinogenesis (Figure 1.5) (Halazonetis et al., 2008; Negrini et al., 2010).

Most full-blown cancers are genetically unstable and have been shown to be sensitive to therapeutic interventions by way of DNA repair inhibition, hitherto and thus has been an active area of drug research and development (Ashworth, 2008; Fong et al., 2009; Jacinto and Esteller, 2007; Shay and Wright, 2002).

Mounting evidence indicates that genomic instability by way of telomere dysfunction results in more aggressive tumours than those arising without telomere dysfunction (Artandi and DePinho, 2010). In any case, telomeres would be maintained stably during malignant progression, and hence would be over-

dependent on telomere stability to survive the challenges thrown by the microenvironment along with build-up of inherent and potentially catastrophic genomic instability.

1.3 TELOMERES IN HUMANS

The biology of telomeres has been an enigmatic one, involving various molecules interacting dynamically in an evolutionarily well-trimmed fashion. Telomeres consist of tandem hexameric DNA repeats, with specialised protein complexes that envelop it also regulating access to the ends to legitimate enzymes involved in telomere metabolism. Telomeres also transcribe into repetitive RNA which also seems to be playing significant roles in telomere maintenance. Telomeres thus form the intersection of DNA, protein, and RNA molecules acting in concert to maintain chromosome and genomic integrity. Telomeres are cancer's Achilles heel and manipulating the same in view of therapy is a promising strategy.

1.3.1 Telomere sequence

Telomeres are composed of hexameric tandem repeats with each repeat containing three or more guanosine residues. Vertebrates, regardless of chromosome number or chromosome length, have highly-conserved 5'-TTAGGG-3' terminal repeats with a long double-stranded region and a short single-stranded overhang or the G-tail (Blackburn, 2000; Collins, 2000; Moyzis et al., 1988). The telomeric repeats protects chromosome integrity and also serves as buffer for the loss of terminal DNA due to the inherent 'end-replication' problem. A few telomeric repeats are also found in interstitial chromosome regions (ITS or interstitial telomeric sequences) (Azzalin et al., 1997). It has been postulated that

ITS not only signifies past evolutionary telomere recombination-based events of genetic instability, but also reveal potential sites of future evolutionary rearrangement events (Zhdanova et al., 2007).

Telomeres are separated from the main chromosomal body of coding regions by sub-telomeric DNA. Those regions usually consist of clusters of duplicated sequences interspersed with canonical telomeric repeats (Lin and Yan, 2008). Sub-telomeric regions are characterised by their dynamic nature, being regarded as hypervariable, and also highly liable to recombination and amplification (Mefford and Trask, 2002). Sub-telomeric regions code for a diverse range of proteins, including transcription factors, in addition to harbouring genes that code for proteins of unknown functions (Linardopoulou et al., 2005). Since these regions are prone to stochastic recombination, it is possible that telomeric integrity might also get affected during such events. Interestingly, sub-telomeric rearrangements have also been implicated in some mental retardation cases (Flint et al., 1995).

1.3.1.1 G-quadruplex structures

G-rich stretches of DNA or RNA are known to form stable secondary structures called G-quadruplexes *in vitro*. Intramolecular G-quadruplex just requires a single molecule of nucleic acid, while intermolecular G-quadruplexes are formed with at least two strands of nucleic acid (Figure 1.6) (Bochman et al., 2012; Burge et al., 2006). Although they have been shown to exist *in vivo* in *Stylonichia* and yeast, their existence in human cells has largely been a colloquium with compelling,

multiple lines of indirect evidence by means of studies mimicking physiological conditions (Paeschke et al., 2008; Paeschke et al., 2005; Schaffitzel et al., 2001). However, their existence *in vivo* in human cells has been only recently demonstrated; and the study shows about 25% of the G-quadruplex forming regions are at the telomeres (Biffi et al., 2013). Due to the repetitive occurrence of guanosine residues, telomeres are prone to forming secondary structures such as G-quadruplexes at the ssDNA overhang or the G-tail or during replication when they are unwound.

Emerging evidence that numerous helicases like Dog1 and RTEL assist with the removal of secondary structures such as G quartets reveals a variety of ironing-out processes that the cell has evolved to counteract the innate problem posed by telomeric composition and elsewhere in other G-rich regions too (Cheung et al., 2002; Ding et al., 2004; Wu et al., 2009). However, recent findings of such structures serving to maintain telomeres by occurring transiently in lower organisms like yeast and *Stylonichia* implies an either possibly well-regulated roles or evolutionarily suppressed roles for G-quadruplexes (Paeschke et al., 2005; Smith et al., 2011). Emerging evidence points to the former – the presence of potentially G-quadruplex forming regions in more than 40% of gene promoters in humans; and hence, a temporally well-regulated role in gene expression (Huppert and Balasubramanian, 2007). Strikingly, potential G-quadruplexes seem to be concentrated in promoters of proto-oncogenes and seem rare in those of tumour suppressor genes (Eddy and Maizels, 2006). G-quadruplex formation at telomeric repeat-containing RNA has also been found to be involved in telomere

heterochromatinisation (Biffi et al., 2012). It is becoming evident that G-quadruplex forming regions in the genome are not merely present as coincidence.

Overall, although G-quadruplexes can form at many places in the human genome, the greatest concentration of such regions is at the telomeres, and they have a greater propensity to form G-quadruplexes due to favourable energy of stabilisation, abundance of long tracts of G-rich repeats, and the more dynamic nature of telomere configurations as compared to the rest of the genome (Huppert, 2010; Lipps and Rhodes, 2009).

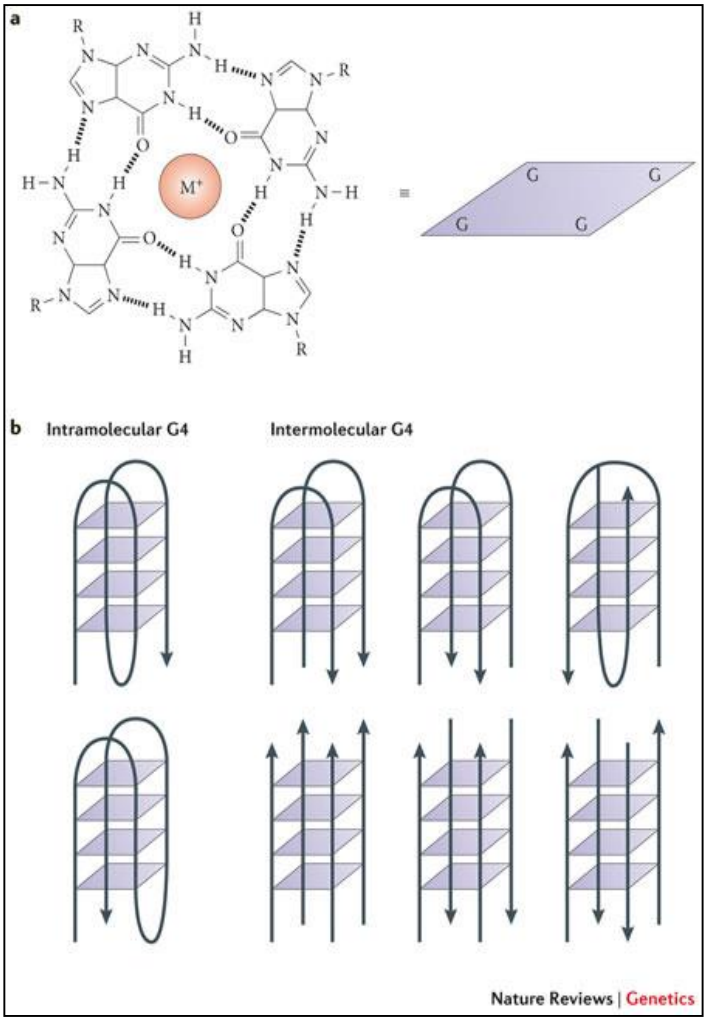


Figure 1.6 **Graphical representation of the possible G-quadruplex structures** Four guanine nucleotides in proximity, associate with each other through Hoogsteen bonding to form a G-quartet. Three or more G-quartets from the same strand or two strands of DNA when stacked upon one another gives rise to intra- or inter-molecular G-quadruplexes respectively Image cource: (Bochman et al., 2012).

The study assesses the usefulness of harnessing this tendency of telomeres to be folded into stable quadruplexes, in the purview of cancer therapy.

1.3.2 Telomere maintenance mechanisms

Telomere length and the integrity of the telomere complex are both important in constituting a functional telomere (Blackburn, 2000). Dysfunctional telomeres may cause the cell to undergo replicative senescence or apoptosis. If the cell is not arrested in a chance event, sister chromatids of the concerned chromosome would fuse following replication, owing to lack of functional telomeres (Figure 1.7).The cell could possibly continue cycling in a phenomenon called breakage – fusion – bridge cycle.

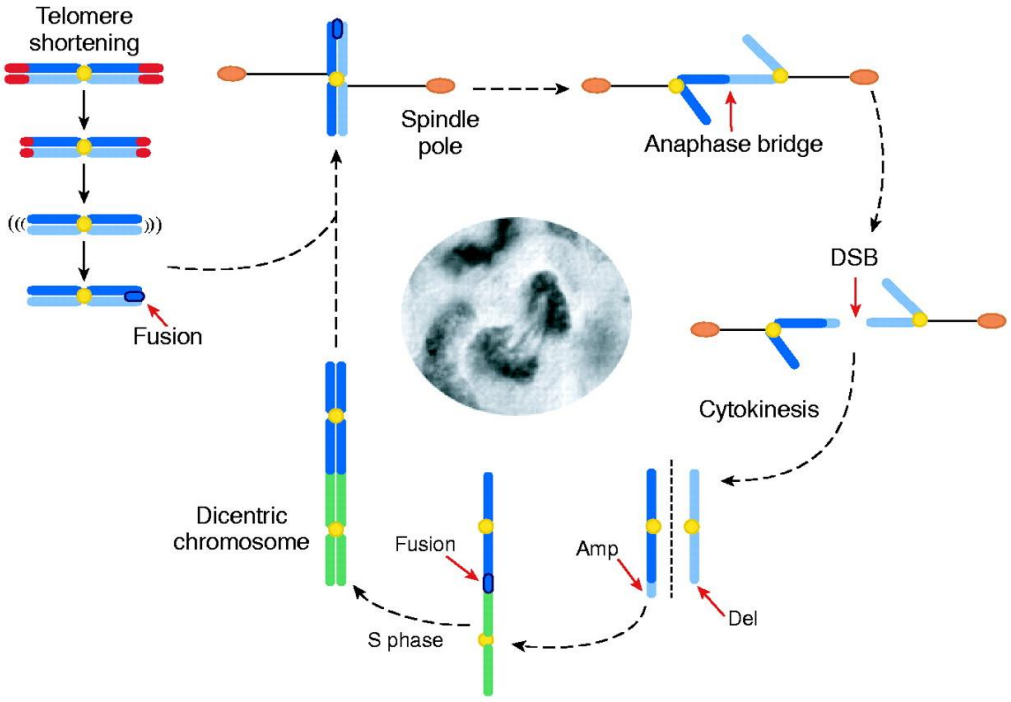


Figure 1.7 **Telomere-mediated chromosome instability.** Fusion of sister chromatids about their dysfunctional telomere would lead to biased distribution of DNA at anaphase. This results in addition of certain regions in the inverted orientation in the chromosome of one daughter cell potentially leading to frame-shift mutations or gene amplifications/ silencing, while the other cell receives a chromosome with potentially large sub-terminal deletions. Image source: (Colnaghi et al., 2011).

In the absence of arrest, this phenomenon continues as a chain and eventually leads to a creation of a mutator phenotype – one that could potentially fuel carcinogenesis (Bailey and Murnane, 2006; Loeb, 2001). It has been shown very recently that cellular immortalisation happens upon ectopic expression of an hTERT mutant incapable of contributing to telomere lengthening or maintenance (Miller et al., 2013). Functional telomeres are nevertheless, essential for genome maintenance and to prevent mitotic catastrophe in the potentially emerging cancerous clones (Hoeijmakers, 2009; Maser and DePinho, 2002; Shay, 1997). Hence, tipping this balance over in cancer cells by way of perturbing telomere

maintenance mechanisms is a guaranteed strategy, universal to managing any rampant cancer.

1.3.2.1 Telomere length maintenance in human cancer

Telomere length in humans typically ranges from 5-20 kb. It is well established that telomeres shorten with every replication cycle in most somatic cells (Allsopp et al., 1995). At some point during carcinogenesis, the cells would experience telomere crisis and those that manage to overcome this crisis are able to successfully gain replicative immortality and survive amidst other control mechanisms in place (Greider, 1998; Tang et al., 1998). In case of normal human fibroblasts at crisis, 1 in 10 million clones manage to escape the checkpoints of cell cycle arrest and execution of apoptosis independent of checkpoint status, mainly governed by telomere length (Figure 1.8) (Shay and Wright, 1989; Wright et al., 1996).

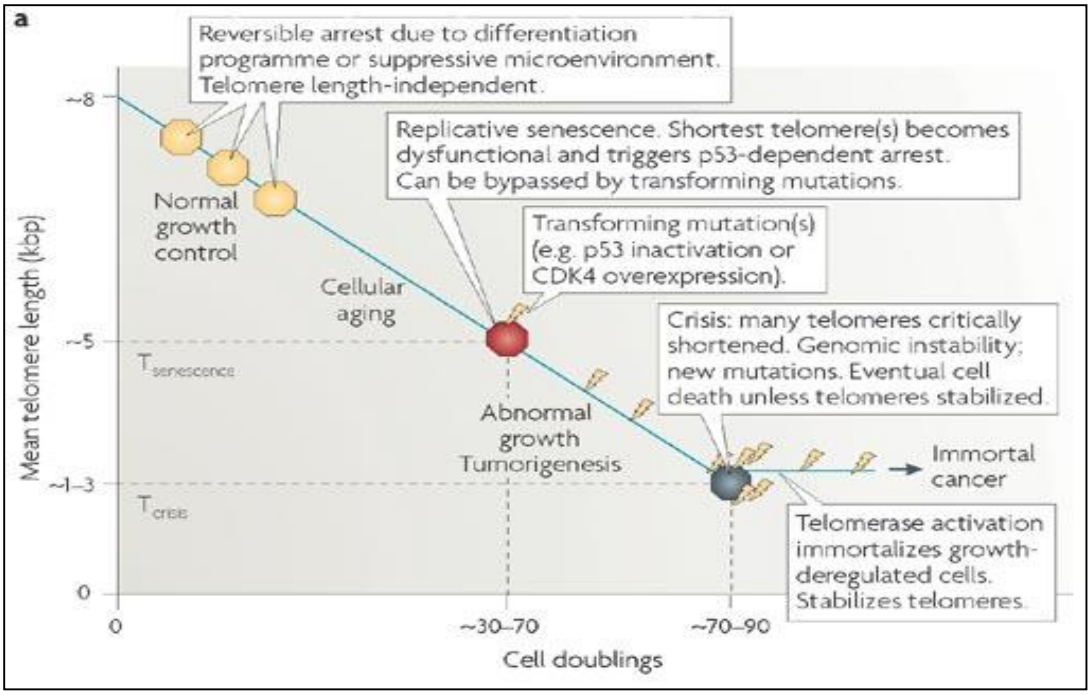


Figure 1.3 **Telomere length dynamics and ageing**As illustrated, germ cells and normal stem cells have long telomeres due to telomerase activity. The observation that telomerase-positive cancer cells typically have shorter telomeres than normal differentiate. Image source: (Harley, 2008).

1.3.2.1.1 Reactivation of telomerase

Access to the telomeres and the extent of elongation by telomerase is tightly regulated by a sophisticated network of telomere-associated proteins. Consequently, approximately 90% of the cases, those clones that reactivate the otherwise repressed telomerase go on to become cancer cells (Kim et al., 1994; Shay, 1997) (Figure 1.9).

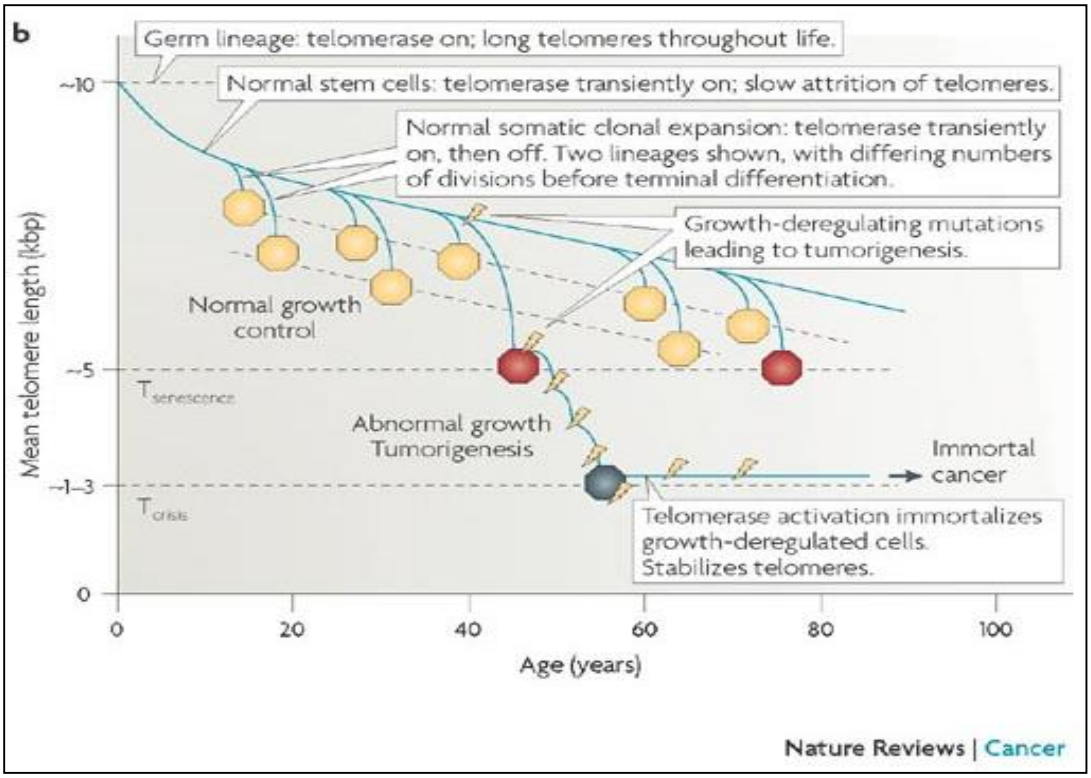


Figure 1.4 **Cell lineages and telomere length dynamics** Stem cells and germline cells have a greater and more stable telomere length over one's lifespan, as compared to normal somatic cells. In most cases, escape from telomere-crisis and progression towards malignancy occurs by telomerase reactivation. Image source: (Harley, 2008).

The temporal frame of telomerase activation in cancers is varied and thus postulated to be highly dependent on the tissue of origin, and the microenvironment (Hahn, 2001). Telomerase reactivation has been shown to happen, in a majority of cases by overexpression due to chromosomal rearrangement to be juxtaposed to a highly active promoter, or alternative splicing of hTERT (Hahn, 2001).Nevertheless, the intricate network of telomerase regulation that would lead to its reactivation is still a vaguely understood niche(Shay and Keith, 2008).

The core of human telomerase holoenzyme consists of a catalytically active protein subunit telomerase reverse transcriptase (hTERT) and a template for

telomere repeat addition – telomerase RNA (hTR) (Collins, 1996; Feng et al., 1995; Lingner et al., 1997; Nakamura et al., 1997). Telomerase also contains a nucleolar protein, dyskerin that has been shown to affect the assembly of hTR, leading to a reduction in telomerase activity (Mochizuki et al., 2004). Dyskerin is part of a small nucleolar ribonucleoprotein complex, H/ACA snoRNP, that is responsible for hTR processing and maturation, which in turn localises hTR to the telomerase holoenzyme complex (Mitchell et al., 1999a; Mitchell et al., 1999b). Overall, active telomerase, when assembled, carries out *de novo* telomeric repeat addition with great processivity (acting on the same telomere again and again) and activity (acting on several molecules of telomeres after processive addition on one molecule).

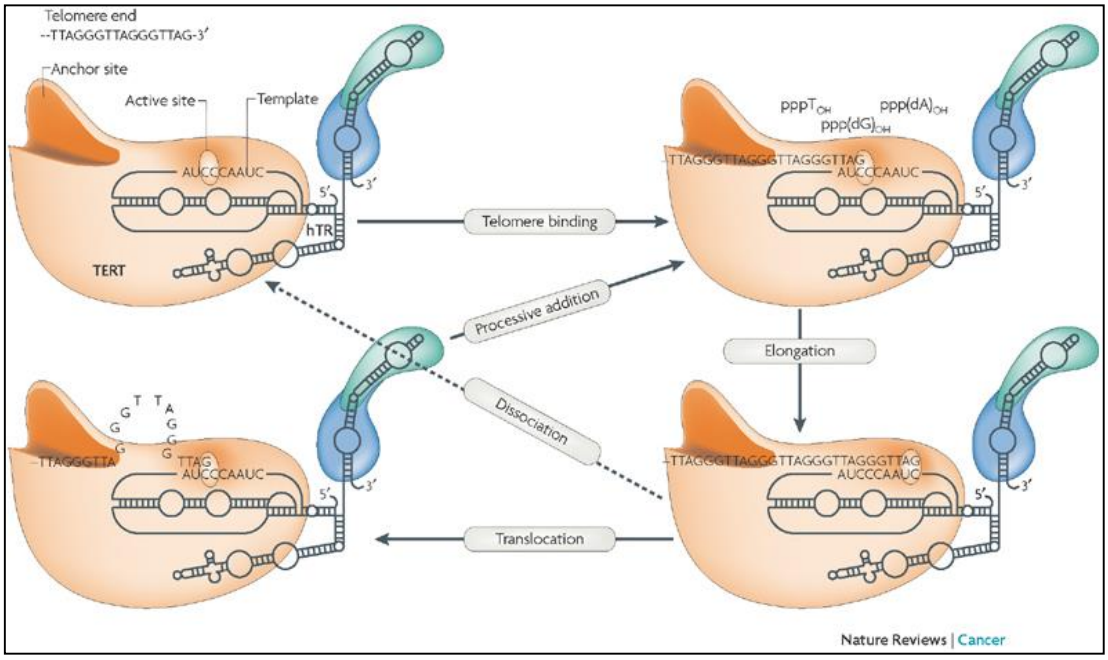


Figure1.5 **Mechanism of lagging-strand telomere elongation by telomerase** hTERT harbours the catalytically active site, while the processed hTR (due to the H/ACA snoRNPs (the blue and green hinges)) serves as a template for primer attachment to the end of the G- tail. Multiple telomerase complexes can act on the same telomere or the same complex can process more cycles of repeat addition following translocation of the newly synthesised telomere end. Image source: (Harley, 2008).

1.3.2.1.2 Alternative lengthening of telomeres (ALT)

The first indications of the presence of telomerase-independent telomere maintenance mechanisms arose from observations of prolonged survival of several mammalian cell type in telomerase-null background, by a few pioneering studies (Hande et al., 1999a; Niida et al., 2000; Rogan et al., 1995). Since then, a body of research has shown that most of the 10-15% of cancers that are telomerase-negative seems to maintain their telomeres by ALT. ALT activity has been observed more frequently in cancers of mesenchymal origin, namely glioblastoma multiforme, and osteosarcoma (Cesare and Reddel, 2010; Lafferty-Whyte et al., 2009). ALT activation is typically characterized by heterogeneity in telomere length, with some unusually long telomeres; the presence of ALT-associated promyelocytic leukaemia (PML) bodies (APBs), which contain extrachromosomal telomeric DNA, TRF1 and TRF2 and proteins implicated in DNA recombination and replication, including the MRN complex — MRE11, RAD51 and NBS — which has been shown to be necessary for APB formation and ALT as well (Henson et al., 2002; Yeager et al., 1999; Zhong et al., 2007). ALT-positive cells also possess extrachromosomal circular telomeres in the form of double-stranded telomeric DNA (t-loops), C-rich strands (C-circles) and G-rich strands (G-circles) whose relative abundance has been speculated with a telomere-trimming mechanism, one that is a result of runaway-telomere elongation by recombination events (Griffith et al., 1999; Henson et al., 2009; Pickett et al., 2009).

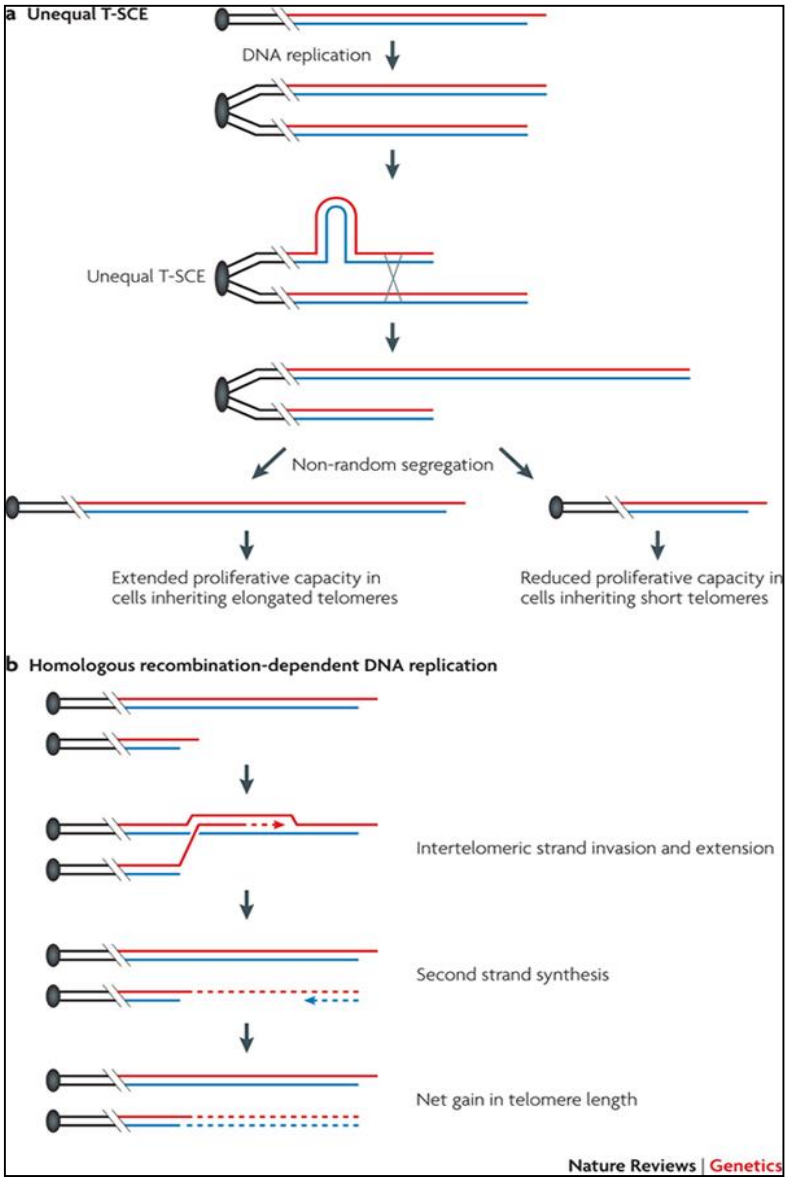


Figure1.11: **Recombination-based models for telomere maintenance by ALT** a. unequal telomere sister-chromatid exchange (T-SCE) and b. homologous recombination-based net telomere lengthening using the sister chromatid as a template. Image source: (Cesare and Reddel, 2010).

The staggering increase in recombination events and also the pattern of telomere length changes observed in ALT cells, points recombination as a compelling mechanism of telomere maintenance by ALT. Although the important proteins

orchestrating the recombination have been revealed, the exact recombinatorial mechanism has not yet been established (Figure 1.11) (Cesare and Reddel, 2010).

The proteins known to facilitate ALT are present in normal cells as well, where they perform normal DNA recombination and repair functions in response to DNA damage (Cesare and Reddel, 2010). The mechanism that helps normal cells prevent those proteins from engaging in ALT-associated telomere recombination is not known. The presence of telomere DNA damage response and the absence of chromosome fusions in ALT cells calls for the abundance of intermediate telomeres – not entirely dysfunctional (Cesare et al., 2009). Some epigenetic modifications are strongly implicated in activation of ALT (Blasco, 2007; Lovejoy et al., 2012).

Telomere elongation by telomerase gives rise to homogenous repeats, unlike that observed in ALT (Conomos et al., 2012; de Lange, 2004). Presumably due to this reason, and also perhaps due to relatively greater ease of telomerase activation over ALT by normal cells due to the selective pressure during telomere crisis, it happens to be the major telomere elongation mechanism.

Although there have been observations of coexistence of ALT and telomerase in normal mouse somatic cells, and in human tumour samples recently, and the interplay between the both and their effect on telomere homeostasis is yet to be brought to light (Neumann et al., 2013; Plantinga et al., 2013; Villa et al., 2008).

Recently, another mechanism reminiscent of telomere maintenance by transposons lacking canonical telomeric sequences in *Drosophila* was observed a in minority of telomerase-negative yeast survivors and named ‘HAATI’ –

heterochromatin amplification-mediated and telomerase-independent survivors (Jain et al., 2010). The fact that evolutionarily distant *Drosophila* and yeast exhibit the same mechanism, hints at the possibility of existence of such a mechanism in humans as well.

1.3.2.2 Telomeres protection by well-coordinated protein complexes and accessory factors

At the core of the telomeres are some protein complexes and other players that expose telomeres to legitimate interacting partners like telomerase, for instance, during telomere replication in S phase; and protect the telomeres from those factors that trigger DNA damage response illegitimate recombination like the MRN complex. Though not all is known of their interactions in various cellular states, the past decade has seen the unravelling of many new and unexpected factors associated with the telomeres.

1.3.2.2.1 The shelterin complex:

The presence of a complex of six proteins that bind to the telomeres specifically has been identified and named ‘shelterin’ (Figures 1.12 and 1.13). The subunits in mammals – TRF1, TRF2, TIN2, Rap1, TPP1, and POT1 were identified by looking for proteins with binding specificity to the telomeric repeats; by using sequence homology with their unicellular counterparts; and by searching for protein-protein interactions at the telomere, within a span of 10 years (Baumann and Cech, 2001; Bilaud et al., 1997; Broccoli et al., 1997; Chong et al., 1995; Houghtaling et al., 2004; Kim et al., 1999; Li et al., 2000; Zhong et al., 1992).

TRF1, TRF2 and POT1 bind to telomeric DNA with exquisite specificity (Bianchi et al., 1999; Broccoli et al., 1997; Court et al., 2005; de Lange, 2005; Palm and de Lange, 2008). POT1 binds to the single-stranded G-overhang and to the single-stranded region of a telomeric secondary structure, elaborated below (Baumann and Cech, 2001; Lei et al., 2004; Loayza and De Lange, 2003; Palm and de Lange, 2008).

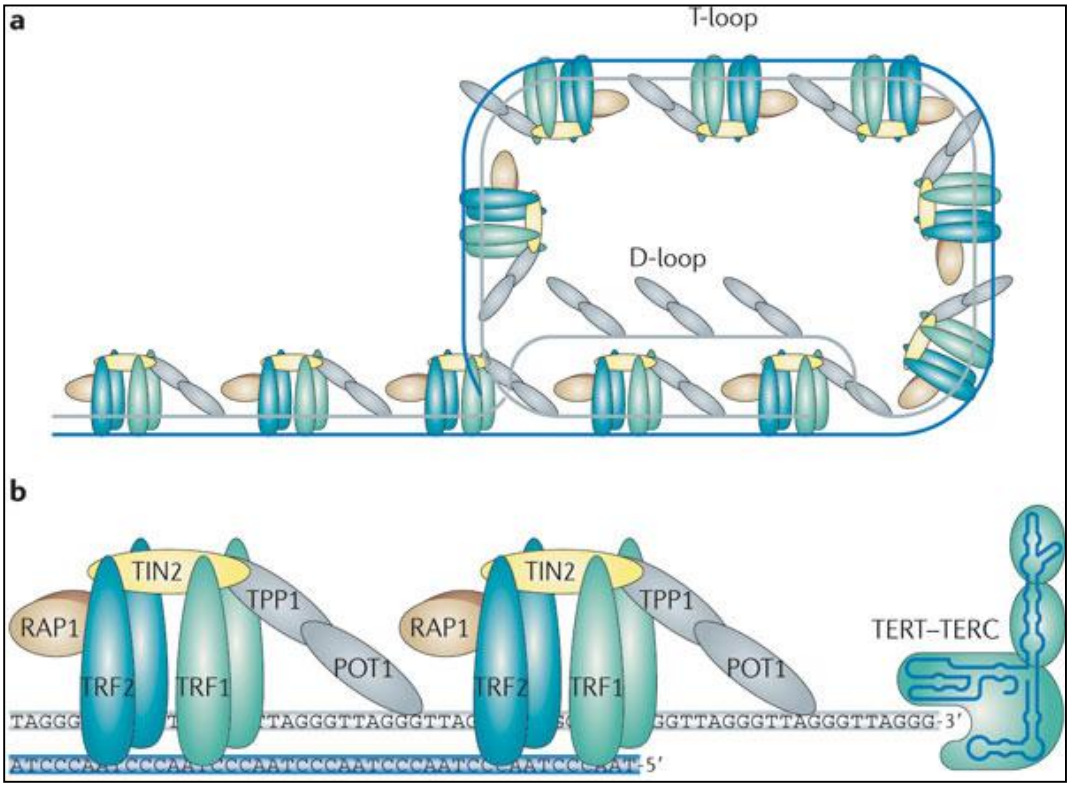


Figure 1.6 **Shelterin – The Border Security Force** a. Shelterin also protects the telomeres by facilitating the formation of a secondary structure – with T-loop (telomere loop) and D-loop (displacement loop). b. TRF1 and TRF2 bind directly to the dsDNA independently, while POT1 binds specifically to the ssDNA overhang or the G-tail. RAP1 associates with TRF2; TIN2 serves as a tether between TRF1 and TRF2; and TPP1 localises POT1 to the G-tail. Image source: (Martinez and Blasco, 2011)

While TRF1 and TRF2 do not interact with each other, and bind to the telomeres independently, TRF1 interacts with and improves the association of POT1 to the

single-stranded region (Loayza and De Lange, 2003). Numerous DDR proteins that also serve to maintain telomeres associate with TRF2 at the telomeres (Blasco, 2005; Smogorzewska and de Lange, 2004). TIN2 acts as the fulcrum between the double-strand binding and the single-strand binding subunits. It binds TRF1 and TRF2 independently and also to the TPP1-POT1 complex (Chen et al., 2008; Kim et al., 2004; Ye et al., 2004). TPP1 while, not binding to telomere directly, recruits POT1 to the telomeric G-overhang by binding to it (Chen et al., 2007; Kibe et al., 2010). In addition, their interaction has also been shown to recruit telomerase to the telomeric single-stranded overhang (Xin et al., 2007). Intact POT1-TPP1 telomere complex has been found to increase telomerase processivity during telomere extension (Wang et al., 2007). The interaction of RAP1 with TRF2 enables its association with telomeres. RAP1 seems to be dispensable for telomere capping but its specific function is largely unknown (Celli and de Lange, 2005; Li and de Lange, 2003; Li et al., 2000). Shelterin proteins, apart from their interactions with each other and also with many other factors ensure dynamic regulation at the telomere. Recent evidence has suggested that multiple POT1-TPP1 complexes along the telomeric single-strand overhang results in a compact and ordered structure. There is emerging evidence that intact shelterin complexes along the length of telomeres are accompanied by significant compaction of the telomeres, and this seems to mute DNA damage response at the telomeres (Baker et al., 2011; Bandaria and Yildiz, 2013; Deng et al., 2009; Martinez et al., 2010; Paeschke et al., 2008; Poulet et al., 2009; Schoeftner and Blasco, 2009).

One problem that the telomere structure poses is the prevention of DDR factors from recognising the G-tail or ssDNA overhang at the telomere as a canonical DNA break. Analysis of human telomeric DNA revealed that the telomere, instead of the expected dsDNA region followed by an ssDNA overhang, exhibited a closed configuration in the form of a loop, called the 't-loop' or the telomeric loop (Griffith et al., 1999). There is compelling evidence of a strand invasion mechanism of the G-tail into the intact dsDNA region, due to the observation of a displaced strand that forms a displacement loop or the 'D loop'.

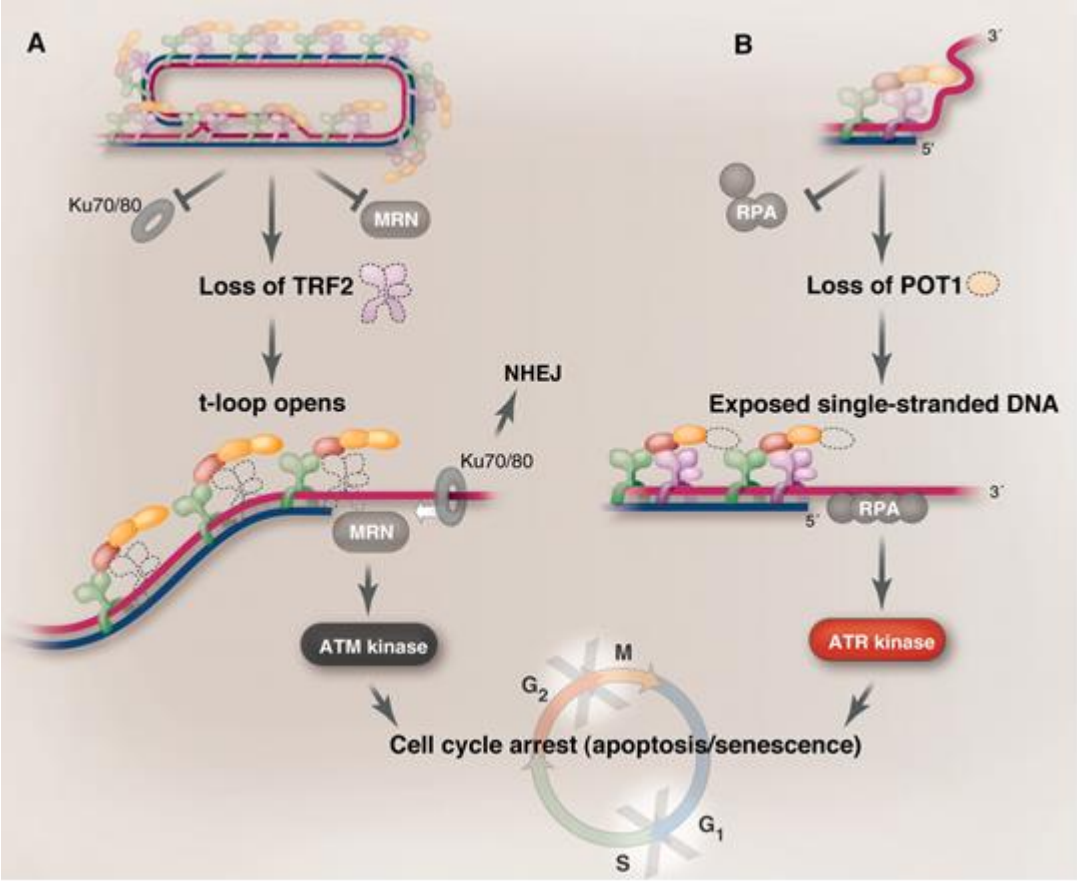


Figure 1.13: **Shelterin – the gatekeeper that prohibits access to DDR orchestrators.** A. TRF2 and B. POT1 are crucial for the integrity of the t-loop structure. TRF2 deficiency activates ATM kinase-mediated DDR, while POT1 deficiency activates ATR kinase-mediated DDR. Image source: (de Lange, 2009).

The t-loop structure seems to be the solution to prevent access of DNA damage sensors with DNA-end binding ability, to the telomeres (Figure 1.13). By preventing the Ku 70/80 complex from loading itself to the terminus, NHEJ activation is thwarted. Also, protection from MRN complex to bind to the terminus means no activation of ATM kinase as well (de Lange, 2009). TRF2 emerges as a linchpin in this context, by virtue of having the innate ability to induce t-loop formation of naked telomeric DNA in vitro and also due to its ability to repress ATM and NHEJ at the telomeres (Griffith et al., 1999; Poulet et al., 2009; Stansel et al., 2001). It is known that while both RPA and POT1 have the ability to bind to the G-tail, RPA is usually detectable at the telomeres only when POT1 is depleted at the telomeres (Barrientos et al., 2008; Denchi and de Lange, 2007). Since RPA is known to activate an ATR-mediated DNA damage response when bound to any ssDNA strand, it has been speculated that the association of POT1 to the telomeric ssDNA protects it from the activation of the ATR pathway (de Lange, 2009). Unlike telomerase-deficient mice (TERC and TERT knock-out mouse models) that survive until they reach adulthood, thorough depletion of at least TRF1, TRF2, POT1a, TPP1 or TIN2 results in early embryonic lethality, while abrogation of RAP1 does not affect mouse viability (Celli and de Lange, 2005; Celli et al., 2006; Chiang et al., 2004; Hockemeyer et al., 2006; Karlseder et al., 2003; Kibe et al., 2010; Sfeir et al., 2010; Wu et al., 2006). Components of the shelterin, when deregulated, result in changes in telomere length, depending on the shelterin protein and the nature of deregulation

(Kim et al., 1999; Loayza and De Lange, 2003; Smogorzewska et al., 2000; van Steensel and de Lange, 1997). Moreover, TRF1, TRF2, POT1 and TIN2 have been documented to be overexpressed in some cancers and TRF2 has been implicated as a major factor in some events of carcinogenesis (Blasco, 2005). This underscores the importance of understanding the biology of the shelterin complex and manipulating it in cancer therapy.

The understanding of more intricate details of the mechanisms by which the shelterin serves to shelter the telomeres, which are still being studied, could lead to building more sophisticated models for the regulation of access to various factors to the telomeres in various cellular contexts.

1.3.2.2.2 CST (Ctc-Stn1-Ten1) complex

One recent scientific discovery based on the approach of looking for human homologues of protein complexes found in yeast was that of the CST complex at the telomere (Miyake et al., 2009; Surovtseva et al., 2009). CST complex consisting of CTC1, STN1 and TEN1, and acting independently of shelterin complex, has been shown to ensure smooth replication at the telomeres and replication restart after stalling of the replication fork (Stewart et al., 2012). STN1 has been shown to be required by DNA-polymerase α for complete extension of the telomere following telomerase action (Wang et al., 2012). CST complex has also been shown to ensure that telomerase acts only once per cell cycle on each telomere (Chen et al., 2012).

1.3.2.2.3 HOT1 (Homeobox telomere-binding protein 1)

hTERT and hTR are brought together and active telomerase assembled in Cajal bodies (Jady et al., 2006). However, the exact mechanism by which telomeres are recruited to the proximity of telomerase in the Cajal bodies is not well understood. HOT1, a newly identified protein that has been shown to directly bind to the telomeres, seems to be aiding in localising telomere sequences to Cajal bodies that contain TERT (Kappei et al., 2013; Tarsounas, 2013).

CST complex, though shown to bind to the telomeres independently of POT1, has been surmised to interact with shelterin to protect the telomeres (Giraud-Panis et al., 2010; Miyake et al., 2009). Thus, the complete understanding of all interactions of the telomeric proteins is being revealed by numerous recent studies and the field is only expanding.

1.3.2.2.4 TERRA (Telomere repeat containing RNA)

Telomeres are transcriptionally active regions, giving rise to long non-coding RNA called TERRA (Azzalin et al., 2007). TERRA levels are tightly regulated through the cell cycle as TERRA has been shown to affect the replication of leading-strand telomeres (Le et al., 2013). TERRA has also been found to orchestrate the binding of POT1 and RPA to the telomere ssDNA and to ensure that POT1displaces RPA promptly after replication is complete as RPA is known to activate DNA damage response (Flynn et al., 2011).Thus, TERRA seems to play an important role too, in regulating telomere capping state, depending on the cellular context. Our understanding about the purpose of TERRA and its crosstalk with the telomere-protecting complexes is only increasing.

1.3.2.2.5 DNA repair factors and telomere maintenance

An early observation that formally linked telomeres with DNA damage response mechanisms included an apparent telomere dysfunction phenotype in cells from Ataxia telangiectasia (AT) patients (Metcalfe et al., 1996). In a study, the first of its kind, cells from mice that lacked ATM exhibited accelerated telomere shortening, accumulation of extrachromosomal telomeres and chromosome alterations (Hande et al., 2001). Eventually, ATM, PARP1, DNA-PKcs, Ku70/80, and XRCC4, among others were all implicated in the maintenance of telomere function, in addition to their role in DNA repair (d'Adda di Fagagna et al., 1999; Gilley et al., 2001; Hande, 2004; Hsu et al., 2000).The MRN complex, that is known to bind to DNA and activate an ATM-mediated DDR, has been shown to associate with TRF2 at the telomeres (Zhu et al., 2000).

One possible reason for this intriguing relationship could be that it may allow for efficient control over cell cycle progression (Gasser, 2000).It also points to the possibility that the DDR proteins, when acting at the telomere at well-regulated cellular contexts such as telomere replication, do not transduce their signal beyond a certain point. Moreover, at least some of the DDR proteins implicated at the telomeres have some novel function, independent of their checkpoint activation and DDR functions (Francia et al., 2007). It is also possible that the stoichiometry of DDR proteins versus the shelterin complex determines the balance between telomere protection and initiation of unrestrained damage response. Also, the observation that there is no striking structural commonality among all the DDR proteins in telomere maintenance points to the possibility that

telomere maintenance is not a discrete function of the cell's machinery, but more integrated with DNA maintenance as such evolutionarily(Slijepcevic, 2006).The exact mechanisms by which many of these factors act to serve in telomere maintenance in various contexts of cell physiology is still an active area of research.

1.3.3. DNA damage response at the telomeres

Studies in senescent cells reveal that telomeres mounting DNA damage-based checkpoint activation seems to be the main trigger behind a senescence programme (d'Adda di Fagagna et al., 2003; Herbig et al., 2004). Even a single DSB at the telomeres of mouse embryonic stem cells seems to have a great effect on chromosomal instability, as opposed to that in interstitial DNA (Zschenker et al., 2009).

The execution of an NHEJ response at the telomeres is controlled greatly by ATM kinase, unlike that in interstitial DNA where ATM regulates NHEJ to a lesser extent; and a failure to activate NHEJ at the telomeres and sub-telomeric regions results in large deletions and gene rearrangements, leading to catastrophic chromosomal instability in human cells (Fumagalli et al., 2012; Miller et al., 2011; Muraki et al., 2012). Damage to interstitial DNA is usually completely reparable; but telomeres exhibit an inability to repair DSBs and also elicit persistent DDR (Fumagalli et al., 2012; Kulkarni et al., 2010; Muraki et al., 2012). Thus, multiple lines of evidence have been accumulating over the recent past about damaged telomeres mounting a unique DDR profile as compared with a damaged interstitial DNA segment.

Nevertheless, targeting these DDR factors in the purview of cancer therapy may serve to dampen canonical DDR as well as weaken telomere protection, even in cancers with stable genomes. Thus, despite the lack of sophisticated understanding behind the intertwined fates of DDR factors and telomeres, targeting telomere maintenance and DNA repair in cancer cells has been a strategy much resorted to in the past decade of research, and is the focus of the study as well.

1.4 TELOMERES AND TELOMERASE IN HEALTH AND DISEASE

Telomere states and telomerase activity have been implicated in wide range of disorders, some of them even rendering one susceptible to cancer. Variant telomere repeats have been shown to be hallmarks of cancers exhibiting ALT (Conomos et al., 2012). Mutations in the shelterin proteins have been implicated in a range of diseases (Armanios et al., 2007; Martinez and Blasco, 2011; Savage et al., 2008). Telomere length has been associated with a staggering variety of disorders and susceptibility to various diseases (Armanios and Blackburn, 2012; Zhu et al., 2011). Mutations in the components of telomerase holoenzyme have also been observed to give rise to cancer susceptibility syndromes (Perona et al., 2009). Mutations in components of the telomeres seem to have a greater penetrance than that of those in components of telomerase holoenzyme, in the aetiology of a wide range of diseases (Figure 1.14).

Telomerase activity has been shown to be diagnostic and prognostic marker for a wide range of cancers. (Reviewed in (Hiyama and Hiyama, 2002))

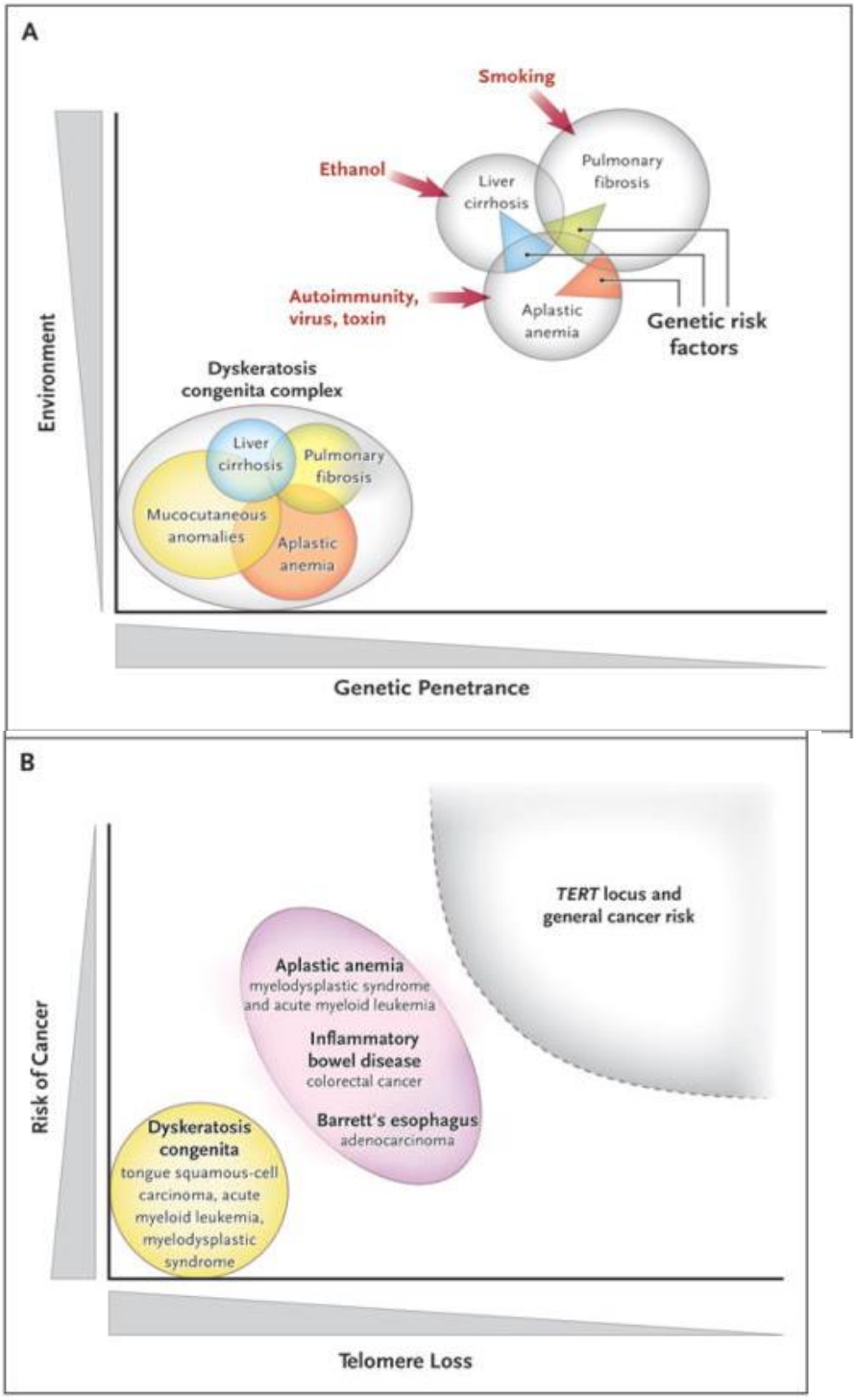


Figure 1.14 **Telomeres and telomerase – aetiology of cancer susceptibility syndromes** A. Penetrance and environmental contribution B. Cancer Risk (as measured by odds ratio) and Telomere erosion. Image source: (Calado and Young, 2009)

Thus, cancer cells maintain their telomeres more vigorously and in a way that is different from normal somatic cells. Targeting telomere homeostasis has been a promising strategy in cancer therapy as normal cells would presumably take more time to get affected as compared to cancer cells.

1.4.1 Targeting Telomere-Telomerase homeostasis in cancer:

Several strategies have been resorted to, that affects telomerase-positive cells.

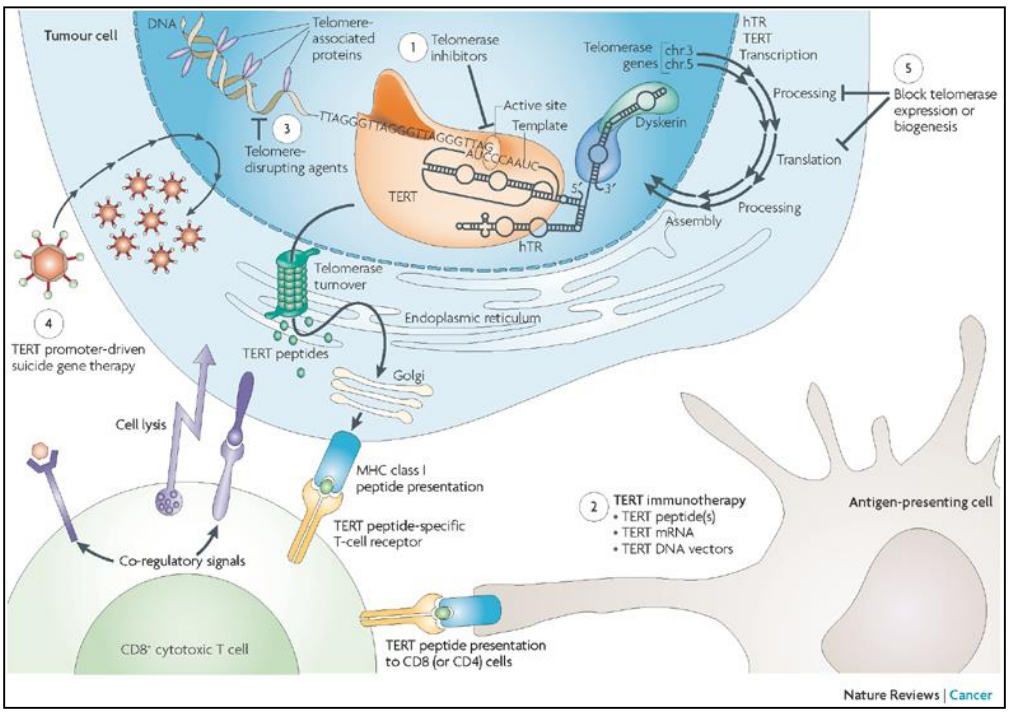


Figure 1.15 **Strategies for targeting telomere maintenance in cancer cells**
The above strategies are expected to work only in telomerase-positive cancers. Although clinical evidence is lacking, studies in mouse models and transformed cell lines have shown that survivors of long-term anti-telomerase treatment in cancer can adapt to ALT to maintain their telomeres and become resistant to telomerase inhibition (Cesare and Reddel, 2010; Chang et al., 2003; Chin et al., 1999; Hu et al., 2012). Image source: Harley, 2008.

Telomere-targeted treatment strategies may serve to affect both, telomerase-positive and telomerase-negative tumours alike. Some of the strategies include the

use of G-quadruplex stabilising ligands, and targeting protein-protein interactions at the telomeres. However, not all is known of such interactions in various cellular contexts yet, and is conceivably, uncharted territory (Bilsland et al., 2011).

1.4.1.1 G-quadruplex stabilising ligands

To reiterate, G-quadruplex structures have been observed to form at many places in the genome, and regulated temporally in contexts of gene transcription, DNA replication, RNA biogenesis, etc. (Maizels and Gray, 2013) However, telomeric G-quadruplexes form with greater ease and telomeres can form more robust ones. Support for this view comes from the fact that many well characterised G-quadruplex ligands like RHPS4, BRACO-19, and Telomestatin, among others exhibit minimal toxicity to normal cells, while largely affecting cancer cells by inducing telomere-dysfunction (Burger et al., 2005; Kim et al., 2002; Leonetti et al., 2004). Moreover, telomere maintenance amidst typically intermittent periods of genomic instability is crucial for the survival of cancer cells (Ding et al., 2012; Shay and Wright, 2002). A wide variety of quadruplex conformations can form naturally in a cell, depending on the cellular milieu (Phan, 2010). Hence, in principle, the addition of drugs that stabilise a specific conformation of quadruplex would ensure specificity and minimise toxicity (Huppert, 2010). Therapeutic intervention by adding ligands that can stabilise G-quadruplexes have shown to result in telomere dysfunction and inaccessibility of telomerase to critically short telomeres and hence a proven therapeutic modality in cancer

treatment, either alone or in combination with conventional therapy (Figure 1.16) (Read et al., 2001; Shay and Wright, 2002).

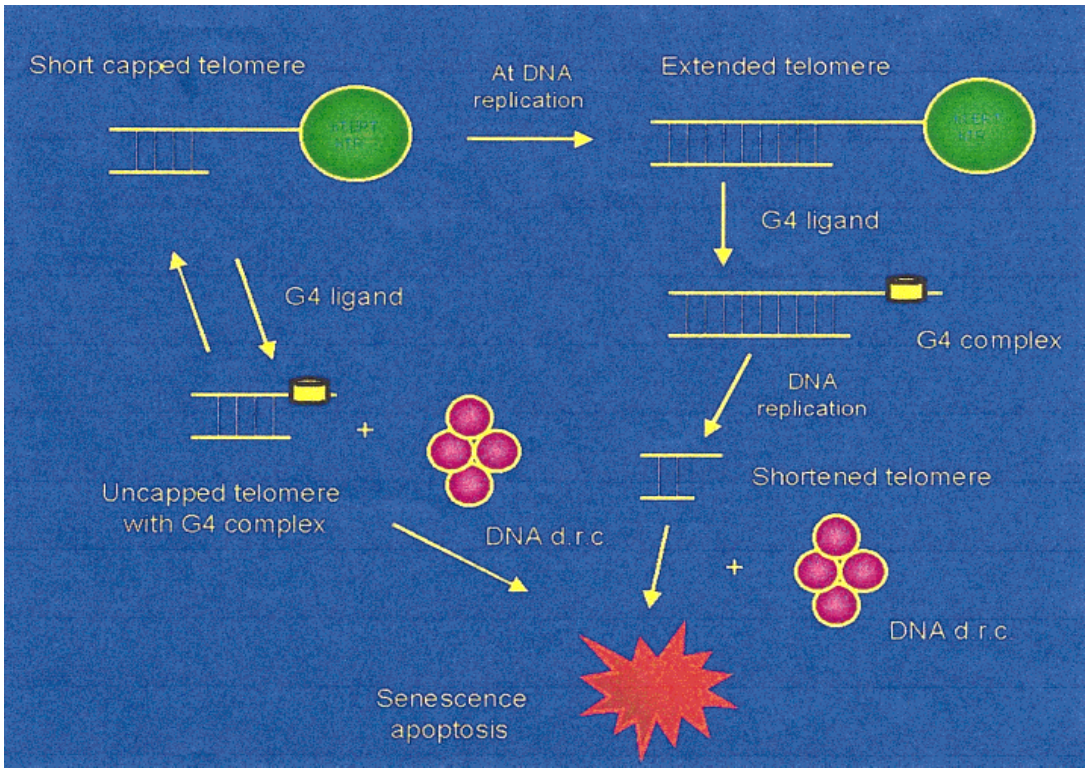


Figure 1.16 **Representative mechanism of action of telomere-G quadruplex-stabilising agents** Typically, G-quadruplex stabilising ligands could affect the access of telomerase to telomere, thereby inhibiting its activity. In addition, by virtue of telomeric G-quadruplex formation, they could even cause telomere length-independent dysfunction, and rapid induction of senescence or apoptosis.
Image source: (Neidle and Read, 2000)

Overall, though the use of G-quadruplex ligands is a great avenue for cancer drug discovery, few drugs have advanced to the clinic. Quarfloxin, a drug that works by selectively inhibiting rRNA synthesis (usually highly upregulated in cancers) is the only one of its kind in clinical trials right now (Drygin et al., 2009). The main problem behind the lack of quadruplex ligand in the clinic has been, in the first place, the unavailability of *in vivo* data for many quadruplex-ligands that showed desirable inhibition *in vitro* (Balasubramanian et al., 2011). This problem has been compounded by the advancement of *in silico* modelling of drug-DNA interactions, as a result of which, many more potential drug candidates are being rolled out.

Among the telomeric quadruplex-stabilising ligands, Telomestatin – a highly specific quadruplex ligand isolated from *Streptomyces anulatus* – has been the most promising drug of its class, but has had problems in terms of bioavailability due to its neutral charge, and mass production efforts (Monchaud et al., 2010; Shin-ya et al., 2001). While xenograft models of RHPS4 and BRACO-19 showed excellent telomeric quadruplex stabilisation with minimal toxicity, their progression to the clinic has been thwarted by the lack of some characteristics that a successful drug must possess, and also due to shifting focus away from telomeric quadruplex ligands due to the emergence of other classes of promising drugs (Balasubramanian et al., 2011).

1.4.1.1.1 TMPyP4 – adopted and then left behind

Porphyrins are a group of naturally occurring compounds with a porphine ring as the common moiety and excellent ability to bind metals. They are present in various forms in cells, and associated with a range of biological functions, e.g., drug efflux pumps on the cell membrane, and constituting haemoglobin, etc. Porphyrins, which can be either anionic or cationic, have been used as photosensitisers photodynamic therapy of some superficial cancers, owing to their specific tumour localisation (Carvalho et al., 2002).

Synthesised cationic porphyrins are the ones of therapeutic relevance for use as nuclear localising, DNA-binding, G-quadruplex stabilising ligands that preferentially affects dividing cells (Han et al., 1999; Shibata et al., 1998). Among the numerous structural isomers analysed in a panel of pioneering efforts to identify promising G-quadruplex ligands, 5,10,15,20 tetrakis-(N-methyl 4-pyridyl)porphine or TMPyP4 emerged the most superior, in terms of protection of telomeric DNA G-quadruplexes from unwinding by helicases, inhibition of telomerase activity, downregulation of hTERT and c-MYC in human cancer cell lines, and extension of survival of tumour-bearing mice (Han et al., 2001; Hurley et al., 2000; Read et al., 2001).

Although TMPyP4 exhibits excellent affinity to quadruplex DNA, *in vitro* studies showed that TMPyP4 has been shown to exhibit only modest specificity for quadruplex DNA over duplex DNA raising concerns over its imminent cytotoxic effects (which is perhaps why no thorough study of its biological effects on

cancer cells has been carried out) (De Cian et al., 2005). The effects of TMPyP4, when carried out in a later study, under molecular crowding conditions that simulates a more realistic intracellular microenvironment shows high degree of specificity to quadruplexes (Martino et al., 2009; Monchaud et al., 2010).

While there have been a few studies on TMPyP4 showing promise hitherto, unfortunately there has not been a thoroughly consistent investigation on its effects on telomere homeostasis, leading to cancer-specific death. While most of them are *prima facie* results on cell fate, in secondary cancer cell lines, only one study using a tumour-bearing xenograft mouse model shows convincing effects on extending its lifespan, albeit lacking mechanistic insights (Table 1).

Study	*Cell type*	*Finding*	**Dose range used (μM)**
Kim et al., (Kim et al., 2003)	SW39, SW26	TMPyP4 acts against telomerase negative cancer cells too	1 for 8 weeks
Parkinson et al., (Parkinson et al., 2007)	SW39, SW26	TMPyP4 forms anaphase bridges between telomeres of different chromosomes	60
Izbicka et al., (Izbicka et al., 1999)	Breast, prostrate, lymphoma, normal	TMPyP4 induces G2M arrest, causes chromosomal aberrations, telomerase inhibition, preferentially localizes in tumor cell nucleus	1, 2.5, 5, 10, 50; for upto15 days
Mikami-Terao et al., (Mikami-Terao et al., 2008)	Leukemic K562, K562/hTERT	TMPyP4 reduced hTERT, c-myc, expression, increased p21, p57, p38 MAPK	100 for 48 hrs
Mikami-Terao et al., (Mikami-Terao et al., 2009)	Retinoblastoma	TMPyP4 causes telomere shortening, increases MAPK, phospho p53, induces apoptosis ; S, G2/M arrest	10, 20, 40, 60 for 48/72 hrs
Shammas et al., (Shammas et al., 2003)	Myeloma	TMPyP4 decreases telomerase activity, causes telomere shortening, growth arrest, death by apoptosis	1, 5, 10, for upto28 days
Aviezer et al., (Aviezer et al., 2000)	CHO cells , *in vivo* xenograft mouse model	EGFR, FGFR gets reduced by TMPyP4 and analogues	25mg/kg for 5 weeks

Table 1 **Summary of key findings on quadruplex-related effects of TMPyP4 in cancer cells**

1.5 MODEL OF STUDY

Malignant gliomas make up approximately 70% of the 22,500 new cases of malignant primary brain tumours diagnosed in adults in the United States per year(Wen and Kesari, 2008). Despite being relatively uncommon, medulloblastoma and malignant gliomas account for disproportionately high morbidity and mortality. Glioblastoma multiforme accounts for approximately 60 to 70% of malignant gliomas, and also the worst prognosis among brain tumours (Wen and Kesari, 2008). The standard of care for GBM is Stupp's regimen, which is maximal surgical resection followed by radiotherapy and concomitant or adjuvant chemotherapy with Temozolamide, a DNA alkylating agent (Stupp et al., 2005). However, while the median survival rate for people with no treatment is around 2 months, the rate went up to only a paltry 7 months in those who received either radiotherapy or chemotherapy alone, and a modest 15 months in those who received both radiotherapy and temozolamide (Yabroff et al., 2012). Although some patients are ineligible for surgical resection, owing to tumour inaccessibility or the criticality of the affected lobe, Stupp's regimen works better in those with surgical resection (Desjardins and Friedman, 2012). Even this adds only a few months' survival, nonetheless.

The past few years has ushered in an era of targeted therapy, where single-molecule treatment has come to the forefront of treating some cancer subtypes with promise. With respect to growth factor inhibition, chances of response is bleak, however, with a response rate of 0-15% and no prolongation of 6-month progression-free survival upon treatment of malignant glioma with single agents

(Sathornsumetee et al., 2007). Another problem with treating brain tumours, in general, has been that of the efficiency of drug delivery across the blood-brain barrier. With more targeted therapeutic agents being developed to respond to many glioma subtypes, and with better drug delivery methods being invented, the search is on to make a giant leap towards significantly extending disease-free survival of glioblastoma, and the hint could lie in attacking cancer's Achilles heel – which is stirring up genetic instability.

1.6 RATIONALE BEHIND THE STUDY

Cancer-related deaths have gone down by 20% in the past two decades, and five-year survival rates increased to 68% from 49% in the 1970s in the U.S. (Siegel et al., 2013). There have been quite a few drugs that remarkably reduced progression of a few cancer sub-types for a while, only for the cancers to evolve into a resistant one eventually. Combination therapeutics has not turned out in the clinic as envisaged either. As such, DNA alkylating chemotherapeutics are still the mainstay of treatment, apart from surgical resection and radiotherapy. Although they work well to debulk tumours, their side effects on normal fast-proliferating cells in many cases, outweigh their benefits. Hence, there is a fervent need for a turnaround in the way we manage cancers. The last two decades has seen the realisation of genomic instability as a main underlying event during tumourigenesis in an overwhelming majority of cases. More importantly, from extensive research on cancer cell lines and animal models, it has emerged that perturbing the same could potentially cause catastrophic genomic rearrangements which would be too much for the cancer cells to adapt. However, the anticipated

effects on normal cells in the tumour is not entirely reassuring, given the past record of cancer treatments that had promise aplenty but proved little. Concurrently though, science has seen the field of telomere biology burgeon into one of the most enigmatic systems in biology. Although not all is known yet, about all the interacting players at the telomeres and in all cellular contexts, it is already evident that this is the direction that cancer therapeutics should explore next. Ironically, as is often the case in biology, the same aspect that drives a strong selection programme for cells on their way to becoming full-blown cancers - telomere dysfunction-induced genomic instability, if induced in cancer cells, would cause mitotic catastrophe that is nearly impossible to cope with. Ways of inducing telomere dysfunction seem, in principle at least, to be highly specific to cancer cells, keeping a therapeutic window in mind. On that note, there have been just a few drugs in clinical trials and the need for more is only getting exigent by the day.

This study investigates the mechanism of one particular small molecule, TMPyP4 that though has shown to be promising in its specificity towards stabilising a secondary DNA structure – the G-quadruplex- at the telomeres and holds promise for inducing telomere dysfunction in cancer cells, has been left rather abandoned. Glioblastoma multiforme and medulloblastoma, the two high-grade malignant brain tumours for which the standard-of-care essentially adds only a few months to two years, is the model of the study.

1.7 HYPOTHESES OF THE STUDY

TMPyP4, by virtue of its ability to stabilise G-quadruplexes (a secondary DNA structure) predominantly at the telomeres, can render telomeric architecture defunct, thereby affecting shelterin binding and telomerase access to the telomeres, eventually signalling DNA damage response, cell cycle arrest, and senescence or apoptosis. Since, it is the cancer cells that maintain their telomeres just above the critical set-point in order to survive, TMPyP4, like other telomere-based agents are expected to act with minimal toxicity.

1.8 OBJECTIVES OF THE STUDY

1. Since TMPyP4 has been shown to be a telomere-perturbing molecule *in vitro*, the study's first objective is to assess the ability ofTMPyP4 in the cancer cells, in affecting telomerase activity, and telomere maintenance over short term, and whether that leads to DNA damage signalling, cell cycle arrest, and cell death, largely in cancer cells.
2. Telomeres and DNA repair orchestrators ATM and DNA-PK are more intertwined functionally, than previously ever thought, with recent research uncovering their importance in regulating signalling events downstream of telomeric DNA damage. The study would ascertain if blocking DNA-PK and ATM kinase pathways, following TMPyP4 treatment would be catastrophic to the cancer cells, thereby highlighting the need for rational combinational strategies to tackle cancer.
3. There has been a tantalising link between telomere destabilisation and radiosensitisation, though it has not been exploited in the clinic, yet. Therefore, the study will assess if TMPyP4 could potentially sensitise the cancer cells to gamma-radiation.
4. Since, the ultimate promise for such a drug lies in exhibiting its effects over a therapeutically relevant treatment regime, the study would investigate the effects of chronic, non-cytotoxic low-dose of TMPyP4 on telomere dysfunction in the cancer cells by affecting at the least, telomere extension by telomerase, and most likely the binding of shelterin proteins to the telomere as well.

Overall, this study aims to ascertain if TMPyP4 continues the promise it showed in *in vitro* studies, *in vivo* as well, in human-derived brain tumour cells. This would then pave way for studies in animal models, and then modifications to its side chains to reduce any unknown toxicity and treatment, and hopefully to clinical trials.

Chapter 2

Materials and Methods

2. MATERIALS AND METHODS

2.1 Experimental design

The study is divided into three phases of investigation into the mechanism of the G-quadruplex ligand TMPyP4 in cancer cells.

The first phase was that of exploring the efficacy of TMPyP4 over a broad range of cell types – human-derived medulloblastoma (ONS76), and glioblastoma (KNS60) cells, with a normal lung fibroblast cell type (IMR90). This involved proof-of-principle studies with doses cytotoxic to the cancer cells but not the normal control, IMR90 over short-term of treatment (48 hours). Since the next phases of study were more focussed on the underlying mechanisms, the model of study was restricted to glioblastoma cells in order to increase the chances of potentially unravelling a cancer-specific mechanism of TMPyP4.

The second phase of study involved the testing of the promise of TMPyP4 in sensitising cancer cells to radiation-induced cell death; and also proof-of-principle studies on the potentiation of TMPyP4 acting in cancer cells with impaired DNA repair response – that mediated by ATM kinase and DNA-PK. The model of study for the radiosensitisation and ATM kinase inhibition parts were KNS60 and A172 cells; and that of DNA-PK inhibition study were M059K and M059J cells which starkly differ in their ability to repair of DNA by DNA-PK-dependent NHEJ.

The third phase of study, also in KNS60 and A172 cells involved studying the mechanism of telomere perturbation in glioblastoma cells at non-cytotoxic concentrations over a few weeks, mimicking a therapeutic window in the clinic.

2.2 Treatment agents

TMPyP4 (Merck, Germany) was obtained as a 25 mg powder and reconstituted to 10 mM with PBS. This was the stock from which the necessary working stock was made for all experiments involving TMPyP4.

KU-60019 (Selleck Chemicals, USA), the ATM kinase inhibitor used was obtained as a 10 mg powder and reconstituted with DMSO to a concentration of 10 mM. Further serial dilutions needed to make the working stock were made using PBS as the diluent.

NU7026 (Merck, Germany), the DNA-PK inhibitor was obtained as a powder and reconstituted to 10 mM using DMSO. Similar to KU-60019, further serial dilutions needed to make the working stock were made using PBS as the diluent.

For the study of γ-radiation, the irradiator used was Gammacell 40 Exactor (Best Theratronics Ltd., Canada) with a Co-60 source and a dose rate of 1.16 Gy/minute.

For chromosome visualisation experiments, GIBCO Karyomax Colcemid (Life Technologies, USA) was used to break the mitotic spindle and arrest cells at metaphase.

2.3 Cell types and cell culture

Human glioblastoma multiforme cell lines A172 (Japanese Collection of Research Bioresources, JCRB0228), KNS60 (Institute for Fermentation, IF050357), and medulloblastoma ONS76 (Institute for Fermentation, IF050355) were obtained from Dr. Masao Suzuki, National Institute for Radiological Sciences, Chiba, Japan. Two more human glioblastoma multiforme cell lines M059K (ATCC CRL-2365) and M059J (ATCC CRL-2366) were obtained from Dr. Susan Loong, National Cancer Centre, Singapore. All the above-mentioned cells were cultured in DMEM (Invitrogen, Life Technologies, USA), supplemented with FBS (Hyclone, Thermo Fisher Scientific, USA) (10% v/v in the final mix) and 100 U/ml Penicillin/ Streptomycin (Pan Biotech, Germany) (1% v/v in the final mix).

Early passage normal lung fibroblasts IMR90 was obtained from Coriell Cell Repositories, USA and cultured in minimum essential medium with Earle's salt, MEM (Invitrogen, Life Technologies, USA), supplemented with MEM essential amino acids (Invitrogen, Life Technologies) (2% v/v in the final mix), 10 mM non-essential amino acids (Invitrogen, Life Technologies, USA) (1% v/v in the final mix), MEM vitamin solution (Invitrogen, Life Technologies, USA) (1 % v/v in the final mix), FBS (15% v/v in the final mix), and 100 U/ml Penicillin/ Streptomycin (1% v/v in the final mix). All cells were maintained in a humidified incubator at 37°C and 5% CO_2, the cell density was kept below 80% generally and culture medium was changed every two days for long-term treatment studies. Subculturing was facilitated by detachment of the cells using a ten-fold dilution of trypsin-EDTA solution (0.05% stock) (GE Healthcare, UK).

2.4 Assays to study cell fate

The study used a wide range of assays to assess cell fate, such as methods to study the metabolic activity, membrane permeability, and caspase activity to find out the effect of a particular treatment on cell viability and the activity of apoptosis of the cells. Flow cytometry was used to assess the effect of treatment on the cell cycle.

2.4.1 MTT assay

This assay works on the principle that MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), a yellow dye that permeates living cells, is reduced to purple formazan by active mitochondrial reductases and the extent of this conversion is proportional to the proportion of viable cells in the mix.

About 2,000 cells were plated in 96 well plates and necessary treatments carried out along with media to a final volume of 200 µl. At the end of the time point, the old media was replaced with a final concentration of 1mg/ml of MTT in 200 µl of fresh DMEM. The plate was incubated at 37C in a humidified chamber for 2.5 hours. Typically, the converted formazan product violet crystals would be observed in the media and need to be solubilised by the addition of a good non-polar solvent, preferably DMSO (50 µl). The absorbance was read at 570 nm using Tecan SpectraFluor Plus multi-well plate reader (Tecan, Switzerland). The viability is then expressed as relative absorbance with respect to the appropriate control.

2.4.2 Cell Titer Glo assay kit

Another method used in the study for measuring cell viability was the Cell Titer Glo assay kit (Promega, USA) that uses a reagent that penetrates the cells and binds to ATP, giving out a luminescent signal. Since ATP in dying cells is rapidly degraded by ATPase, the amount of ATP detected is taken to be proportional to the number of viable cells present in a population of cells. About 2,000 cells were plated in white-walled 96 well plates preferably, and necessary treatments carried out along with media to a final volume of 100 µl. After the time-point, 100 µl of the supplied reagent was added to each well, then shaken for about 5 minutes and let to settle for another 5 minutes on the bench. Once the luminescence stabilises, the reading was taken using Tecan Infinite 200 Pro multi-well plate reader. The luminescence was read using Tecan SpectraFluor Plus multi-well plate reader (Tecan, Switzerland). The viability is then expressed as relative luminescence with respect to the appropriate control.

2.4.3 Apotox Glo Triplex assay kit

Apotox Glo Triplex assay (Promega, USA) assesses cell viability, cytotoxicity and caspase activity simultaneously and hence was used to determine the mode of cell death of the cancer cells upon treatment with TMPyP4. The principle behind the measurement of the first two parameters is the addition of substrates specific to cleavage by certain proteases – a cell permeant fluorogenic substrate to measure the cell viability, a cell-impermeant fluorogenic substrate to measure the activity of proteases released from a dying cell upon loss of membrane integrity. Caspase activity is measured by the addition of a luminogenic Caspase 3/7

substrate. About 2,000 cells were plated in black-welled 96 well plates preferably, and necessary treatments carried out along with media to a final volume of 100 µl. After the time-point, 20 µl of the supplied viability/ cytotoxicity substrates were added together in each well and incubated at 37°C for 30 minutes, followed by reading fluorescence at $400_{Ex}/505_{Em}$ for viability and $485_{Ex}/520_{Em}$ for cytotoxicity. Once the readings are taken, 100 µl of the caspase reagent is added to all the wells and incubated for 30 minutes at room temperature. Luminescence reading was taken, again using Tecan SpectraFluor Plus multi-well plate reader and the values were expressed in terms of absolute units and compared among treatments

2.4.4 Population doubling analysis

The rate of doubling of a cell population is a good index of its proliferative capacity and is a useful measure in long-term treatment studies. At the end of every week, the cells were passaged again and the count was taken before re-seeding the same number of cells as at the beginning. Upon detachment from the

$$PDN = \log_2 (\text{No. of cells harvested/ No. of cells plated initially})$$

flasks by trypsin incubation, the cell suspension was mixed with an equal volume of trypan blue, a dye that permeates and stains a cell blue upon membrane compromise during cell death. This enables counting of the viable cells only, using a haemocytometer. Population doubling number (PDN) was calculated as:

The cumulative PDN was then calculated and used as the representation of the number of divisions an average cell in a population has undergone.

2.4.5 Flow cytometry analysis of cell cycle profiles

The cells were seeded in a 10 cm culture plate, treated appropriately, and harvested at the end of the time-point. Harvesting was done by washing the cells in 0.1% BSA in PBS, spun at 900 rpm for 4 minutes at room temperature, fixed overnight using 70% ethanol, and stained with propidium iodide (Sigma, USA)/ Triton X-100 (Biorad, USA) for 30 minutes at 37°C. Samples were run in a flow cytometer (FACSCalibur^{TM}, Becton Dickinson, USA) at wavelengths $488_{Ex}/610_{Em}$. Data from a total of 10,000 cells were recorded for each sample and analysis performed using WINMDI software.

2.5 Assays to study genome integrity and DNA damage

The study employed techniques to assess the extent of total DNA damage, the activity od DSB repair response, and genome integrity upon treatment.

2.5.1 Alkaline single-cell gel electrophoresis (Comet assay)

Comet assay so named because of the resemblance of a cell with a damaged genome to that of a comet, upon electrophoresis under DNA secondary-structure denaturing alkaline conditions. Findings of the comet assay, a standard one for detecting total DNA damage in a cell is represented by the measurement of the displacement of damaged DNA molecules (the tail) from the integral DNA molecule (the head) as tail moment and the evaluation of the staining intensities of tail versus the head as the percentage of DNA damage.

Cells, after the treatment period are detached, washed and resuspended in ice cold PBS. The resuspension should allow enough cells for analysis (at least 50), nor

should it be too concentrated. Meanwhile, a solution of 0.7% low melting point agarose (Conda Laboratories, Spain) was prepared in PBS, and melted at 70°C and cooled to 39°C until further use. 50 µl of agarose solution was mixed with 6 µl of cell suspension and laid down flat on specially coated comet slides (Trevigen, USA), and allowed to polymerise at 4°C for about ten minutes. The cells were then lysed in a solution containing 2.5 M NaCl, 100 mM pH 8.0 EDTA, 10 mM Tris-HCL, 1% Triton –X at 4°C for 1 hour, following which denaturation of DNA secondary structures including disruption of inter-strand hydrogen bonding was carried out for 40 minutes, in cold alkaline electrophoresis buffer (pH 13.0–13.7). Electrophoresis was subsequently carried out for 20 minutes. Slides were immersed in neutralization buffer (0.5 M Tris-HCL, pH 7.4), dehydrated, air-dried and stained with SYBR Green dye (Trevigen). Images were captured using Zeiss Axioplan 2 imaging fluorescence microscope (Carl Zeiss, Germany) equipped with triple band filter. At least fifty comets per sample were randomly selected and analysed using Comet Analysis Software (Metasystems, Germany) and represented as mean tail moment and percentage of DNA damage.

2.5.2 Immunofluorescent visualisation of γ-H2AX

Cells were seeded in six-well plates on 22x22 mm coverslips, and treated until the end of the desired time-point. Harvesting was carried out by washing the wells with ice-cold PBS, and fixing in 4% formaldehyde, followed by cell permeation using Triton X/100. This facilitates the entry of the antibodies into the cell. This was followed by incubations with blocking agent, 0.5% BSA, primary antibody mouse anti-H2AX (Merck Millipore, USA), and secondary antibody goat anti-

mouse IgG TR (Invitrogen) for an hour each, with the antibody incubations interspersed with three washing steps of five minutes each using 0.1% BSA. The coverslips were then dehydrated, dried and mounted onto slides with DAPI (Vectashield, Vector Labs, USA) as the counterstain. Imaging was done using a Zeiss Axioplan 2 imaging fluorescence microscope (Carl Zeiss, Germany) equipped with triple band filter. At least 50 cells were analysed at x63 magnification or more cells at x20 magnification. Cells were analysed for their semi-quantitative signals of red γ-H2AX foci against the blue-stained nuclei.

2.5.3 Cytokinesis-block Micronucleus (CBMN) assay

CBMN assay gives another measure of genome integrity and an indirect measure of the extent of irreparable DNA damage in a cell. Cytochalasin B, a chemical that can arrest cells at cytokinesis phase is made use of, in order to visualise the cells at the binucleated stage so as to detect any damaged DNA ejected from the macronucleus during cell division.

Cells were seeded in six-well plates and treated with TMPyP4 to the appropriate time-point, then arrested at cytokinesis by treatment with 4 µg/ml Cytochalasin B for a further 22 hours. The cells were then detached, washed and treated with ice-cold 0.075 M KCl, hypotonic solution for 15 minutes and spun at 180 g for 8 minutes at room temperature, following which they were fixed in fixative containing 90% of 3:1 mixture of methanol and acetic acid and 10% of formaldehyde. The fixed cells were then laid onto dry and clean slides and dried overnight, followed by staining with 20 µl of 30 µg/ml Acridine Orange (Sigma Aldrich). At least 1000 binucleated cells with/without the presence of micronuclei

were scored under an Axioplan 2 imaging fluorescence microscope (Carl Zeiss, Germany) with an appropriate triple band filter.

2.6 Western blotting

Western blotting is a technique that was used extensively in the study to assess the status of DNA repair, cell cycle checkpoint-activation, and telomere-interacting proteins among others.

Cells were seeded in six-well plates and washed with PBS, once. Whole cell protein extraction was facilitated by using a radio-immunoprecipitation assay (RIPA) buffer containing 50 mM NaCl (NUMI), 1mM EDTA (Sigma Aldrich), 50 mM Tris-HCl (1st Base, Singapore), 1% Triton X-100 (Biorad), 0.05% SDS (NUMI), 1x Proteasome inhibitor (Roche, USA), 1x Phosphatase inhibitor (Roche, USA), and 0.1% Sodium deoxycholate (Sigma Aldrich). Lysates were collected using a cell scraper and transferred onto pre-chilled eppendorf tubes and incubated in ice for 20 minutes with intermittent voxtexing to ensure uniform and efficient lysis. They were then spun at 15,400g at 4°C using a cooling centrifuge (Eppendorf 5417R) for 10 minutes, and the supernatant was carefully collected in a fresh eppendorf tube and stored at -20 °C or -80 °C until further use.

The lysates were then quantified for then protein concentration, as equal loading on all wells is fundamental to comparing the band intensities. Bradford's reagent (Biorad) was reconstituted by diluting it by four-fold with water to obtain workable concentration. Bradford's reagent contains copper-containing reducing agents, which bind to the amino acid residues specifically, resulting in a shift in the absorbance peak to 595 nm. In order to fall in the linear range of detection of

protein concentration by Bradford's assay so as to extrapolate absorbance to concentration, protein lysates were diluted ten-fold with water and mixed with Bradford's reagent. Absorbance was measured using μQuant plate reader (Biotek Instruments, Singapore), and the protein concentration was calculated by the standard procedure.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was the method used to resolve proteins solely based on their molecular weight. The composition of the gel was tweaked based on the size of the protein of interest, in order to obtain optimum resolution for that protein/ group of proteins. Gels were cast using equipment provided by Biorad, and 30% Acrylamide-Bis solution 37.5 : 1 (2.6% C) (Biorad) was used as the monomer. Also, the gels vary in concentration at least twice – initially more loose to facilitate stacking of the protein samples, followed by a tight gel that would facilitate optimal resolution of the proteins. Gel polymerisation was facilitated by crosslinkers ammonium persulfate and tetramethylethylenediamine (Biorad). Meanwhile, protein lysates were mixed with a concoction called loading dye that would facilitate in rendering the proteins some density to sink in the well once loaded, and also stains the samples so as to make it trackable. The samples were then denatured by heating at 95°C for 5 minutes, spun briefly to ensure no wastage, and then loaded onto the wells in the gel. Electrophoresis was facilitated by a running buffer (3.03 g Tris base, 14.4 g Glycine, 10 ml of 10% SDS per litre of solution). Proteins were transferred onto a nitrocellulose membrane (Thermo Fisher Scientific) following electrophoresis, to make post processing steps easier and amenable for handling and protein

visualisation. The so-called wet transfer was done by electroblotting, facilitated by a polarised running buffer (with 25% methanol) for 1-3 hours depending on the size range of the proteins of interest.

The nitrocellulose membrane was then incubated for 1 hour with 5% w/v skimmed milk in 1x PBST (PBS and Tween-20 (Biorad), a non-ionic detergent) to minimise non-specific binding of antibodies. This was followed by incubation with the primary antibody of interest for 1 hour at room temperature or overnight at 4°C, followed by 3 washes with 1x PBST for 5 minutes. The primary antibodies used in the study are anti-p-ATM (Santa Cruz), hTERT (Epitomics, USA), TRF2 (Santa Cruz), TRF1 (Cell Signaling, USA), c-MYC (Santa Cruz), β-Actin (Santa Cruz), Cyclin D1 (Santa Cruz), and p21 (Santa Cruz).

The membrane was then incubated with the appropriate secondary antibody (which are usually tagged with an enzyme horse raddish peroxidase, HRP) for 1 hour at room temperature, and then washed with 1x PBST for thrice for 5 minutes.

The detection of the antibody-bound protein is made easy by the addition of a chemiluminescent substrate of HRP, Femto ECL (Thermo Fisher Scientific, USA), followed by exposing a light-sensitive film to the membrane and developing using an X-ray developer to obtain protein levels commensurate with the band intensity on the film at the expected molecular weight, facilitated by the presence of molecular weight ladders.

2.7 Techniques to study telomere-telomerase functionality

The effect of TMPyP4 on telomere homeostasis is the crux of the study; hence a multi-centric approach was used to study the same, including the assessment of telomerase activity *in vitro,* telomere length, telomere stability and chromosomal integrity.

2.7.1 Telomere Repeat Amplification Protocol (TRAP)

Telomerase activity was assessed using Telomeric Repeat Amplification Protocol (TRAP) using TRAPeze® XL Telomerase Detection Kit (Chemicon International, USA). The principle of this assay is testing the extent of elongation of a synthetic oligonucleotide primer (that resembles the telomeric sequence) by telomerase in the non-denatured protein extract from cells. All steps were done according to the manufacturer's instructions with some modifications. Briefly, total protein was extracted using CHAPS lysis buffer, a non-denaturing buffer, provided and 1.5 µg protein was quantified and used for all samples. Samples were treated with 1 µl/ml RNase inhibitor to preserve the RNA integrity during amplification cycles by PCR. The PCR was initiated by using the telomerase mediated elongation products (i.e. the telomeric DNA repeats) as template, not earlier than 30 minutes to give enough time for *in vitro* elongation of the synthetic oligonucleotide primer by telomerase present in the protein extract. Subsequently normal PCR cycle was performed using forward and reverse primers with quenched fluorescein to amplify the telomeric DNA repeats. Fluorescence signals were generated by unquenching the fluorescein on PCR primers upon amplification of that oligonucleotide molecule, and the fluorescence signals were

measured by fluorescence multi-well plate reader. Fluorescence signals of PCR products were measured using fluorescence plate reader TECAN SpectraFluor Plus.

Negative controls in this experiment were Taq-negative control, CHAPS-only negative control, heat treated sample and sulforhodamine house-keeping control. Fluorescein levels essentially are a measure of relative telomerase activity, provided the negative control which is a measure of sulforhodamine activity remains low. Telomerase activity was represented as percentage of fluorescein to sulforhodamine values with respect to the untreated controls upon subtracting the blanks.

2.7.2 Peptide Nucleic Acid – Fluorescence *in situ* Hybridisation (PNA-FISH)

Cells were treated up to the desired time-point and then incubated with 40 µl of 10 µg/ml Colcemid (Life Technologies) in 10 ml of media for about 12-16 hours to allow quite a few cells at least, to be arrested at metaphase. Cells were then detached by trypsin treatment, followed by washing off the residual trypsin (after inactivating it by adding serum-containing media) by spinning the cells down. The cell pellet was then tapped and resuspended in a hypotonic solution of 75 mM KCl for about 11 minutes at 37°C and spun down at 1000 rpm (270 g) for 8 minutes at room temperature. After discarding the supernatant, the pellet was then tapped gently and mixed agitated vigorously after adding 1 ml of modified Carnoy's fixative (3:1 mixture of methanol & acetic acid) so as to dispense any clumps that may form. Three ml of fixative was added after stopping the agitation and the cells allowed to stand in the fixative for at least 2 hours before washing

with fresh fixative. Cells were dropped onto slides in a way that spreads the metaphases well, but not excessively so as to splatter the chromosomes all over the place.

The following day, the slides were rehydrated with 1x PBS for 15 minutes, fixed with 4% formaldehyde for 2 minutes, washed and then treated with a solution of Pepsin-HCl (pH~2) at 37°C for 1-3 minutes to destroy the chromatin around the chromosomes and to make them more accessible for the probes to bind to. The timing of pepsin treatment depends on the quality of the metaphase spreads; 1 minute was sufficient for well spread metaphases. The cells were fixed again with 4% formaldehyde, washed, dehydrated with a series of 70%, 90%, and 100% ethanol for 5 minutes each and air-dried.

Hybridization mixture containing 70% formamide, 0.5 μg/ml Cy-3-conjugated TelC and 3 μg/ml FITC-conjugated Cent-FAM peptide nucleic acid (PNA) probe (Panagene, Korea), 0.25% (w/v) blocking reagent in 10 mM Tris (pH 7) was added to the slide, a coverslip (24 × 50 mm) was added followed by DNA denaturation (3 minutes at 80°C). After hybridization for 2-2.5 hours at room temperature, slides were washed with 70% formamide/10 mM Tris (pH 7.2) (two times for 15 minutes) and with 0.05 M Tris/0.15 M NaCl (pH 7.5) containing 0.05% Tween-20 (three times for 5 minutes). Slides were dehydrated with successive ethanol steps (70-100%), air-dried, and covered by 20 μl antifade solution (Vectashield; Vector Laboratories) containing 0.2 μg/ml of 4′-6-diamidino-2-phenylindole (DAPI).

Imaging was done using a Zeiss Axioplan 2 imaging fluorescence microscope (Carl Zeiss, Germany) equipped with triple band filter. At least twenty metaphase spreads were captured and analysed wherever possible, using Isis software (Metasystems, Germany).

2.7.3 Telomere Restriction Fragment (TRF) analysis

TRF analysis has the gold standard assay to determine the mean telomere length and the spread of telomere lengths in a given cell population.

Cells were treated for a period of up to eight weeks and were kept frozen until use. DNA was extracted using DNeasy Tissue Kit (Qiagen, USA), according to the manufacturers' protocol. TRF assay was performed using Telo-TAGGG Length Assay Kit (Roche, USA). One and a half micrograms of pure genomic DNA was digested with Hinf1 and Rsa1 which have genome-wide recognition sites for restriction digestion for 2 hours at 37°C, thus potentially sparing only the telomeric and sub-telomeric regions intact owing to the lack of any restriction sites in those regions. The resulting DNA was subjected to gel electrophoresis in 0.8% agarose gel at 60 V for 3 hours, thus leaving the small and digested DNA out of the gel. The DNA in the gel was then transferred overnight by Southern blotting onto a nylon membrane by capillary action across the assembly and cross-linked onto the membrane using a UV cross-linker (Stratagene, USA). Telomere restriction fragments were hybridised with telomere-specific digoxigenin-labelled (DIG-labelled) probe and incubated with anti-DIG alkaline phosphatase and tetramethylbenzidine, according to manufacturer's protocol. The membrane was incubated with a chemiluminescent substrate (supplied with the

kit), specific to alkaline phosphatase for 5 minutes and the exposed X-ray film developed. The films were then scanned by Kodak Gel imaging system (Kodak, USA) and analysed by Kodak MI imaging software (Kodak) to calculate the quantitative measurements of the mean TRF length.

2.8 Genome-wide gene expression microarray

The formation of G-quadruplexes has been demonstrated in regions other than telomeres as well (Bochman et al., NRG, 2012). Although G-quadruplex ligands have demonstrated preferential binding to the telomeres at large, telomere-independent effects of such ligands including that of TMPyP4 have been reported hitherto (Granotier et al., 2005) (Siddiqui-Jain et al., 2002). Hence, profiling of genome-wide gene expression upon TMPyP4 treatment was carried out as well.

Owing to the practical impossibility of isolating the transient and dynamic mRNA from cells to study gene expression, total RNA would first be extracted, as is the standard procedure. Reverse transcription of total RNA in turn, would yield cDNA which is only reflective of mRNA. cDNA is then transcribed to biotin-labelled cRNA, so as to bind with exquisite affinity to streptavidin-labelled probes, and amplified prior to hybridisation onto the probes embedded on a chip.

ONS76 and KNS60 cells were seeded in 10 cm culture dishes, treated with 100 µM TMpyP4 for 48 hours and then detached, washed and pelleted. Treatment was done as duplicates and all subsequent steps were performed on duplicates of the samples, in order to account for any experimental variations. Total RNA was thus first extracted from about a million ONS76 and KNS60 cells using QIAmp RNA Blood Mini Kit (Qiagen, Germany). The concentration and the purity of the

extracted RNA was obtained using Nanodrop 1000 (Thermo Fisher Scientific) and the integrity of RNA using Bioanalyzer (Agilent Technologies, USA). Reverse transcription to cDNA was done with 500 ng of extracted RNA using Superscript™ II Reverse Transcriptase (Invitrogen) primed with T7-(dT)-24 primer. cRNA was then transcribed *in vitro* from cDNA as biotin-labelled, in the presence of T7 RNA polymerase and biotinylated ribonucleotides (Enzo Diagnostics, USA). The cRNA was then amplified using TotalPrep RNA Amplification Kit (Ambion Inc., USA) in order to obtain a workable amount of RNA. Hybridization of the biotin-labelled, amplified cRNA was done onto HumanRef8 V3.0, Human Whole-Genome Expression BeadChips (Illumina) for 16 hours at 58°C. After the incubation period, the arrays were washed and stained with Streptavidin-Cy3 (GE Healthcare), followed by scanning the arrays using Illumina Bead Array Reader. The signal values of cRNA bound to each probe was further translated into gene expression values using Partek® Genomics Suite™ version 6.5 (Partek Inc., USA).

Principal component analysis was performed before analysis of gene expression to ensure quality control. Analysis of variance (ANOVA) was conducted on the complete data set and a list of differentially expressed genes was obtained using FDR (Benjamini Hochberg) of 0.05 with a two-fold cut-off for fold-change. Unsupervised two-dimensional average-linkage hierarchical clustering of the genes, differentially expressed upon the treatment, was performed for both the cell types by using Spearman's correlation as similarity matrix.

Biological significance of the differentially expressed gene list was better realised by classifying the genes based on their biological pathways and molecular functions using Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, USA). Parameters such as probeset ID, gene symbol, Entrez gene ID as clone clone identifier, p-value, and fold-change values obtained from the statistically differentially expressed dataset were uploaded into IPA, and the significance of the connection between the expression data and canonical pathway were calculated by ratio and Fisher's exact test.

2.8 Statistical analyses

Two-tailed, unpaired Student's t-test; single factor and two-factor ANOVA were employed as tests of statistical significance, wherever applicable, using Microsoft Excel. $p < 0.05$ was used as the threshold; and the degree of statistical significance was indicated by asterisks, as follows: * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$. All values were computed against their respective controls unless mentioned otherwise.

Chapter 3

An acute dose response study of TMPyP4

3. AN ACUTE DOSE RESPONSE STUDY OF TMPyP4

3.1 BACKGROUND

TMPyP4 was among the first drugs to be shown to be a specific G-quadruplex ligand *in vitro*. It had been explored in detail to get to know the prerequisites that a chemical must possess, to stabilise G-quadruplexes. However, following some early studies using synthetic oligonucleotides *in vitro* that assessed the affinity and specificity to quadruplex DNA over duplex DNA of a few promising macrocyclic compounds showed that TMPyP4 was among the least specific quadruplex binder, albeit with high affinity (Grand et al., 2002; Han et al., 2001; Izbicka et al., 1999; Kim et al., 2003; Martino et al., 2009; Monchaud et al., 2010; Weisman-Shomer et al., 2003). Over time, other ligands took precedence, in terms of research concentration – both, in understanding more about its effect on the physiology of cells upon binding, and in improving its toxicity profile and therapeutic efficacy by modifying the side chains if need be.

In 2009, a study simulated molecular crowding conditions in solution, which resembled the milieu that would exist *in vivo.* It was revealed that TMPyP4 actually exhibited remarkable quadruplex-specificity (Martino et al., 2009). Taking cue from that, our study tries to delineate the effects of TMPyp4 on cell physiology, downstream of stabilising G-quadruplexes.

This chapter deals with the assessment of proof-of-principle aspects of TMPyP4 as a telomere-interacting molecule. The effect of TMPyP4 on telomere architecture is studied immediately upon treatment; and its effects on cell fate, genome-wide gene expression, and cell motility.

3.2 RESULTS

3.2.1 Determination of dose and time of treatment with TMPyP4

As mentioned earlier, TMPyP4 has been shown to cause a dose reduction of viability of cancer cell lines of a few types. So, the first aspect that was studied was assessing if TMPyP4 affects the gliobalstoma and medulloblastoma cell lines alike in a dose- and time-dependent manner, while sparing normal cells to a great extent. As shown in figures 3.1 A and B., in the viability profiling of KNS60 and ONS76 cells, TMPyP4 exhibited a minimal toxicity at 24 hours over the various doses tested, while showing a gradual reduction in viability following 48 hours of treatment.

Since 100 µM of TMPyP4 for a period of 48 hours is the common IC_{50} dose for the cancer cells, that would be the dose in the following experiments to investigate the effects of TMPyP4 on telomeres and cell fate.

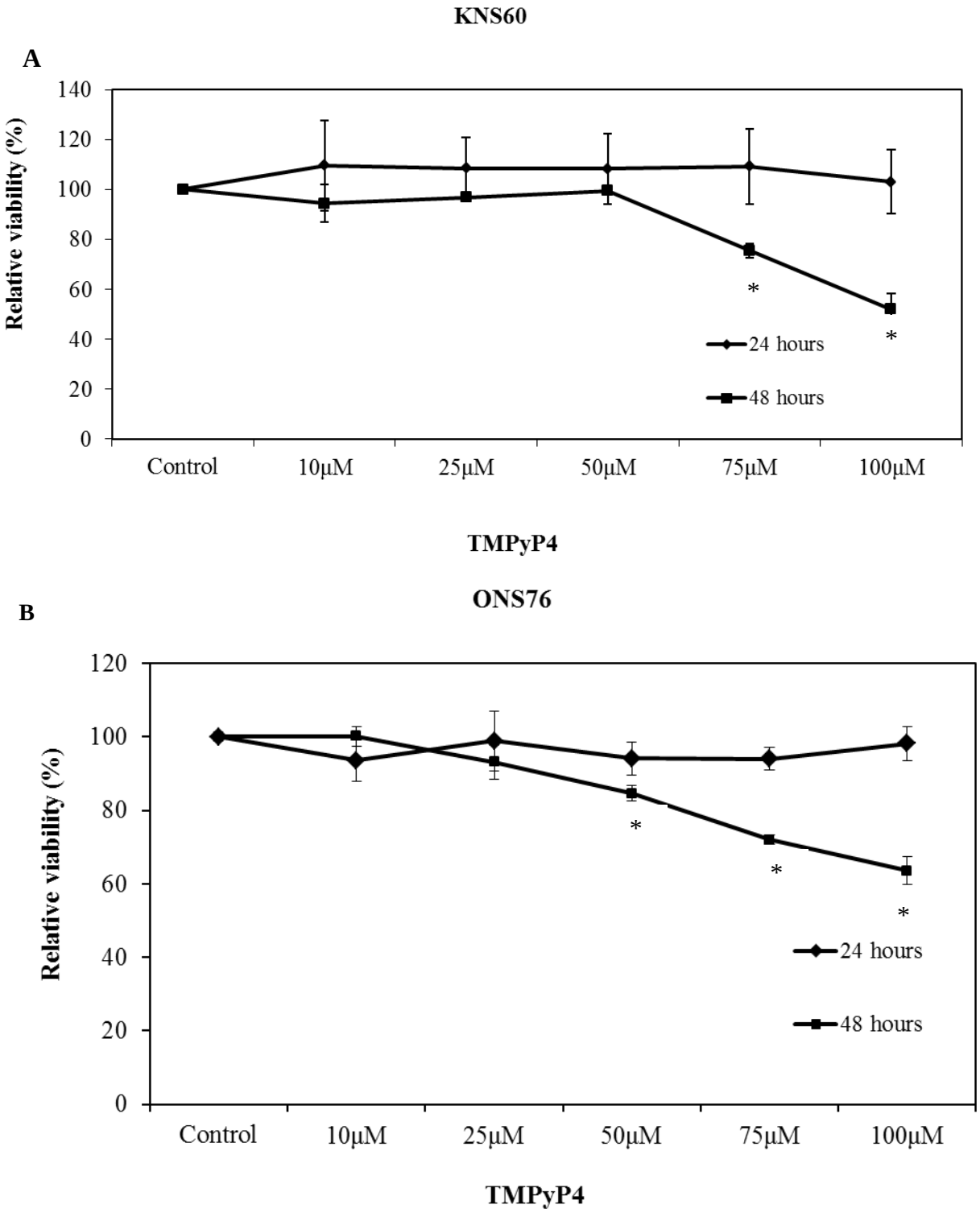


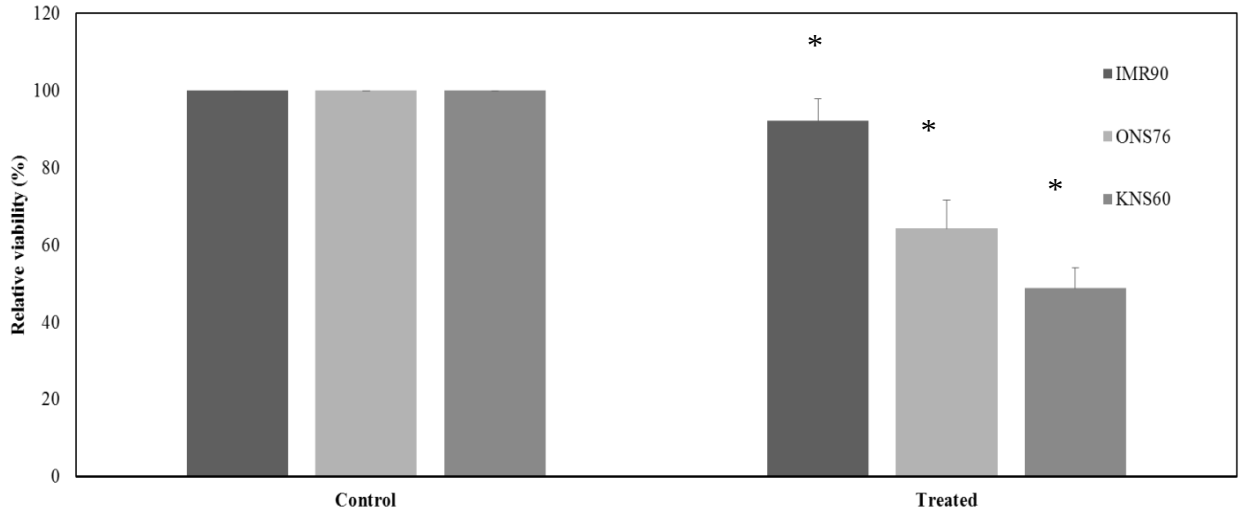
Figure 3.1 **Dose-time response of TMPyP4 on the viability of ONS76 and KNS60 cells.** Data represented as mean of values obtained from three independent repeats ± S.D. A. KNS60 cells; B. ONS76 cells

3.2.2 Evaluation of cytotoxicity of TMPyP4

There have been reports of many promising potential G-quadruplex stabilising ligands being preferentially toxic to cancer cells, while sparing normal cells. Accordingly, it was seen if TMPyP4 also had the same effect. Hence, along with a panel of 2 brain tumour cell types – KNS60 and ONS76, a normal human lung fibroblast, IMR90 was also used as a model for testing the effects of TMPyP4 on their viability, due to the unavailability of a normal human-derived glial cell type.

Notably, while 100 µM of TMPyP4 after 48 hours caused nearly 50% reduction in the viability of KNS60 and ONS76 cells, it reduced merely 8% of that of the normal control used – IMR90 (normal lung fibroblasts). This indicates that TMPyP4 acts preferentially on cancer cells (Figure 3.2A).

A



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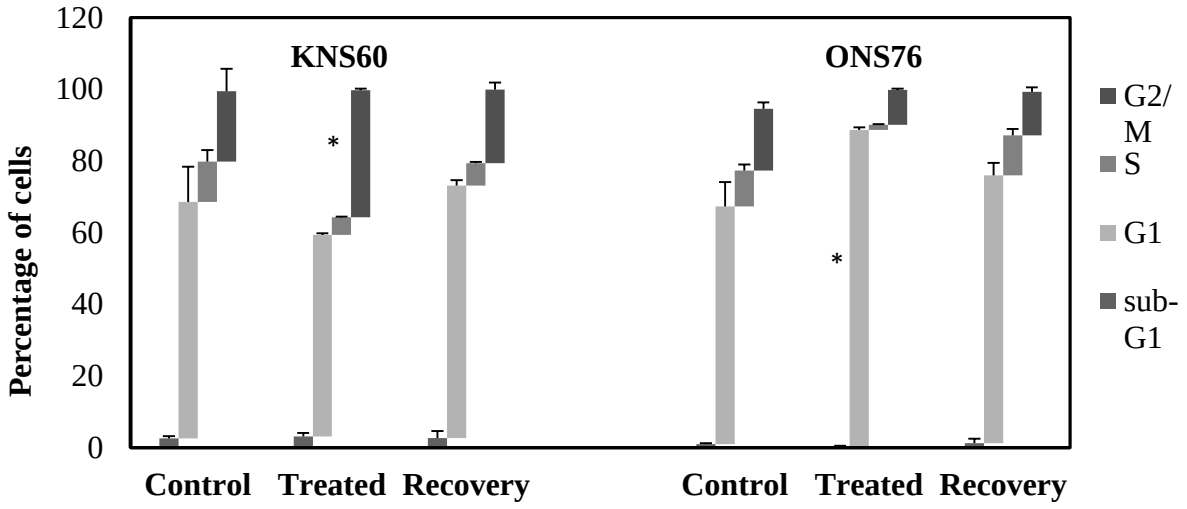

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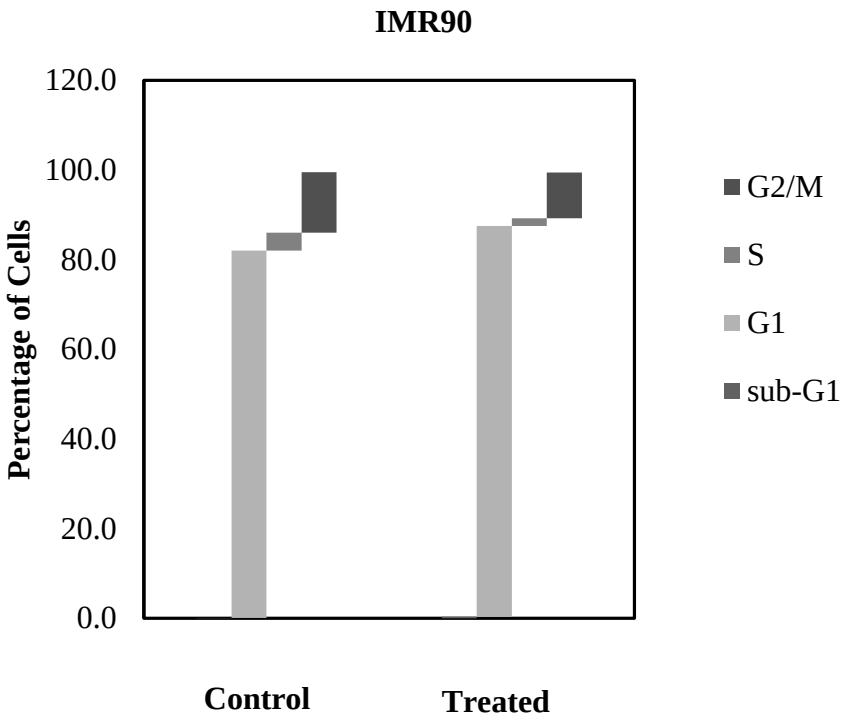


Figure 3.2 **Cytotoxicity of TMPyP4** A. Viability of IMR90, ONS76 and KNS60 upon treatment with 100 μM TMPyP4 for 48 hours. Significance was evaluated by Student's t-test. B. Cell cycle profile of ONS76 and KNS60 upon the same treatment. 'Recovery' indicates 24 hours of recovery period following the treatment. C. Cell cycle profile of IMR90 following 48 hours of treatment. Significance was evaluated by Single factor ANOVA. A and B The values represent Mean ± S.D. of three independent repeats.

Analysis of the effect of TMPyP4 on the cell cycle of the cell types was assessed using flow cytometry following treatment with 100 μM of TMPyP4 for 48 hours. KNS60 and ONS76 cells were significantly arrested after treatment, in G2/M and G1 phases respectively, whereas IMR90 cells displayed a very mild arrest at G1 phase (Figure 3.2).

Moreover, after incubating the cancer cells in TMPyP4-free media for a further 24 hours following treatment, both KNS60 and ONS76 cells showed significant recovery from the arrest induced by TMPyP4. This was corroborated by assessing the protein levels of Cyclin D1, the executioner of progression through G1 checkpoint, by western blotting of whole cell lysates (Figure 3.3). Cyclin D1 levels in ONS76 reduced after treatment, only to increase after the 24-hour recovery period. Cyclin D1 levels were rather low and undetected at the same exposure, in KNS60 cells. This could merely be attributed to the innate differences in the physiology of the two cell types. On similar lines, the levels of p21, a protein that is phosphorylated by p53 to execute cell cycle arrest upon a DNA damage stimulus, was assessed (Figure 3.3). It was observed that p21 levels were promptly upregulated following treatment in ONS76, whereas KNS60 lacked any detectable p21 even after long exposure. This is due to the fact the KNS60 harbours functionally mutant p53, as opposed to WT p53 in ONS76 cells. Such differences in the cancer cell types are to be expected, and only projects what is to be anticipated in a clinical scenario where tumour heterogeneity is the norm, and a successful drug is expected to work largely efficiently, across those

groups. Suitably enough, this variability has been a common strand throughout the study.

Overall, TMPyP4 arrests cancer cells and does not interfere with the cycling and viability of normal IMR90 cells largely in the IC_{50} dose, which is a high dose, as such.

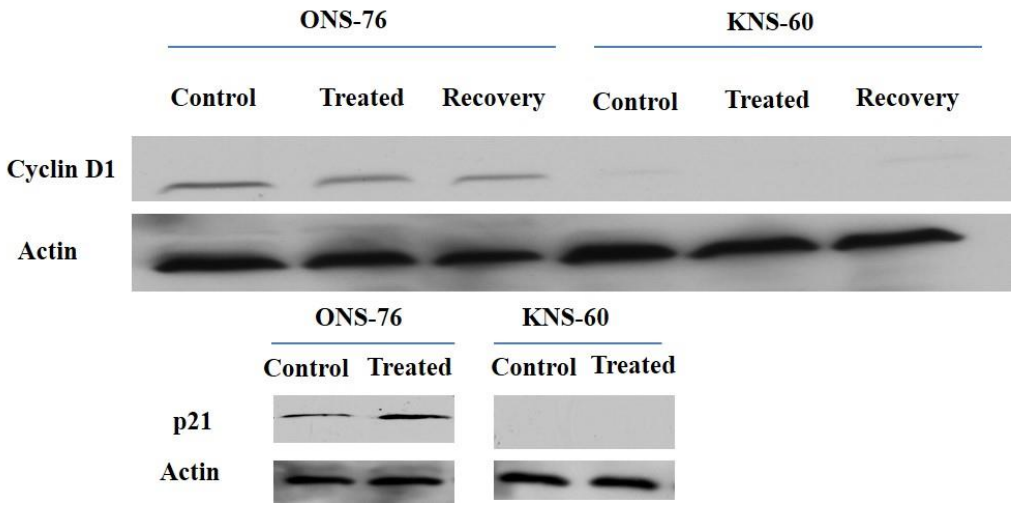


Figure 3.3 **TMPyP4 treatment and checkpoint activation** Western blot analysis of Cyclin D1 and p21 levels in ONS76 and KNS60 cells, following treatment with 100 μM TMPyP4. 'Recovery' indicates 24 hours of incubation in TMPyP4-free media following treatment

3.2.3 Effect of TMPyP4 on telomere maintenance in cancer cells

Since TMPyP4 is a ligand that has shown promise to bind to and stabilise telomeric G-quadruplexes, the next thing that was assessed in the study was its effects on perturbing telomere maintenance in the cancer cells. To this end, the effect of TMPyP4 on telomerase activity was evaluated by measuring telomerase activity using TRAP, following treatment. Not surprisingly, TMPyP4 treatment for 48 hours caused telomerase activity to plummet (Figure 3.4A). Consequently, its effects on hTERT, the catalytic component of telomerase, and TRF2, the most

crucial shelterin protein were assessed by western blotting of whole cell lysates. Indeed, the levels of both hTERT and TRF2, reduced appreciably following 48 hours of treatment (Figure 3.4 B and C). Conversely, after a recovery period of 24 hours in TMPyP4-free media, the levels of hTERT and TRF2 sprang back to normalcy.

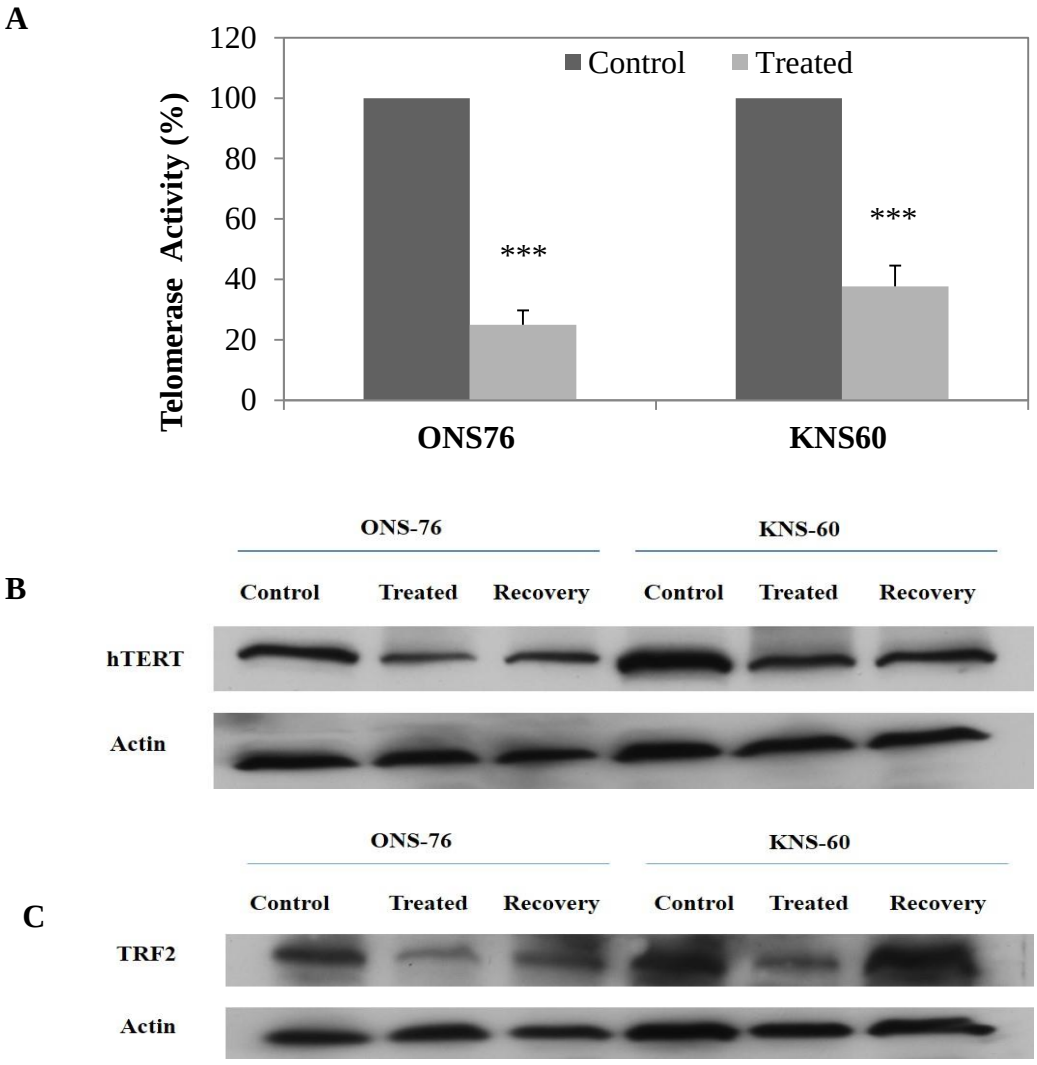


Figure 3.4 **Effect of TMPyP4 on telomeres in ONS76 and KNS60 after 48 hours treatment with 100 µM TMPyP4** A. Telomerase activity, as measured by TRAP after treatment. Error bars indicate S.D. from three independent repeats. Student's t-test was performed to assess significance. B. Levels of hTERT and C. TRF2 determined by western blotting after treatment and recovery, where 'recovery' means incubation in TMPyP4-free media for a further 24 hours after treatment.

It is possible that the effect of TMPyP4 on telomerase activity and TRF2 levels may, at least in part, be a consequence of the toxicity exerted over the 48 hours of treatment. In order to ascertain if the effects of TMPyP4 on telomere maintenance was specific, rather than due to a multitude of other cellular perturbations, the levels of TRF2 and hTERT were assessed intermittently in ONS76 cells over a 72-hour regime, with the first 48 hours being treatment with 100 μM TMPyP4; and the next 24 hours being incubation in normal media following the removal of TMPyP4-containing media. There was a staggering reduction in TRF2 levels from as early as 2 hours after treatment; and notably, the levels did not recover even after incubation in TMPyP4-free media, when assessed after the 72-hour regime (Figure 3.5 A). hTERT levels showed a more gradual reduction though, and also appreciable recovery following incubation in TMPyP4-free media (Figure 3.5 B). This indicates that TMPyP4 is an agent whose effects are specifically telomere-mediated. Thus, clearly, TMPyP4 affects telomere architecture and telomerase activity, and hence telomere maintenance in the cancer cells assessed.

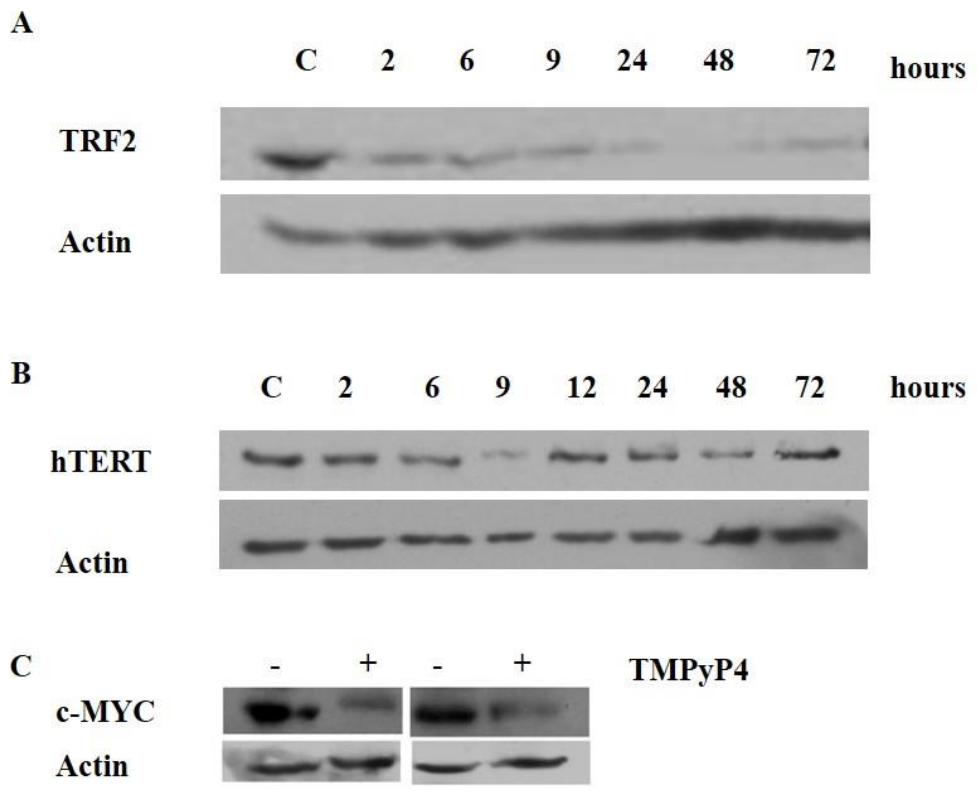


Figure 3.5 **The immediate effect of TMPyP4 at the telomeres** Western blot time-course analysis of the following in ONS76 cells: A. TRF2 B. hTERT upon treatment with 100 µM TMPyP4. '72 hours' means 48 hours of TMPyP4 treatment, followed by 24 hours of growth in TMPyP4-free media. C. c-MYC levels after treatment with 100 µM TMPyP4 for 48 hours.

If TMPyP4 mediates its effects specifically at the telomeres, then the reduction in hTERT protein levels which would normally be a repercussion of that of telomerase activity; but this occurs too soon upon TMpyP4 treatment. To seek an explanation for this reduction in hTERT, the levels of c-MYC, a major transcription factor of hTERT, was assessed amidst reports that c-MYC also harbours potential G-quadruplex forming regions at its promoter sites, and that TMPyP4 can stabilise those structures as well. Indeed, at least following 48 hours of treatment, c-MYC levels went down appreciably in both – KNS60 and ONS76

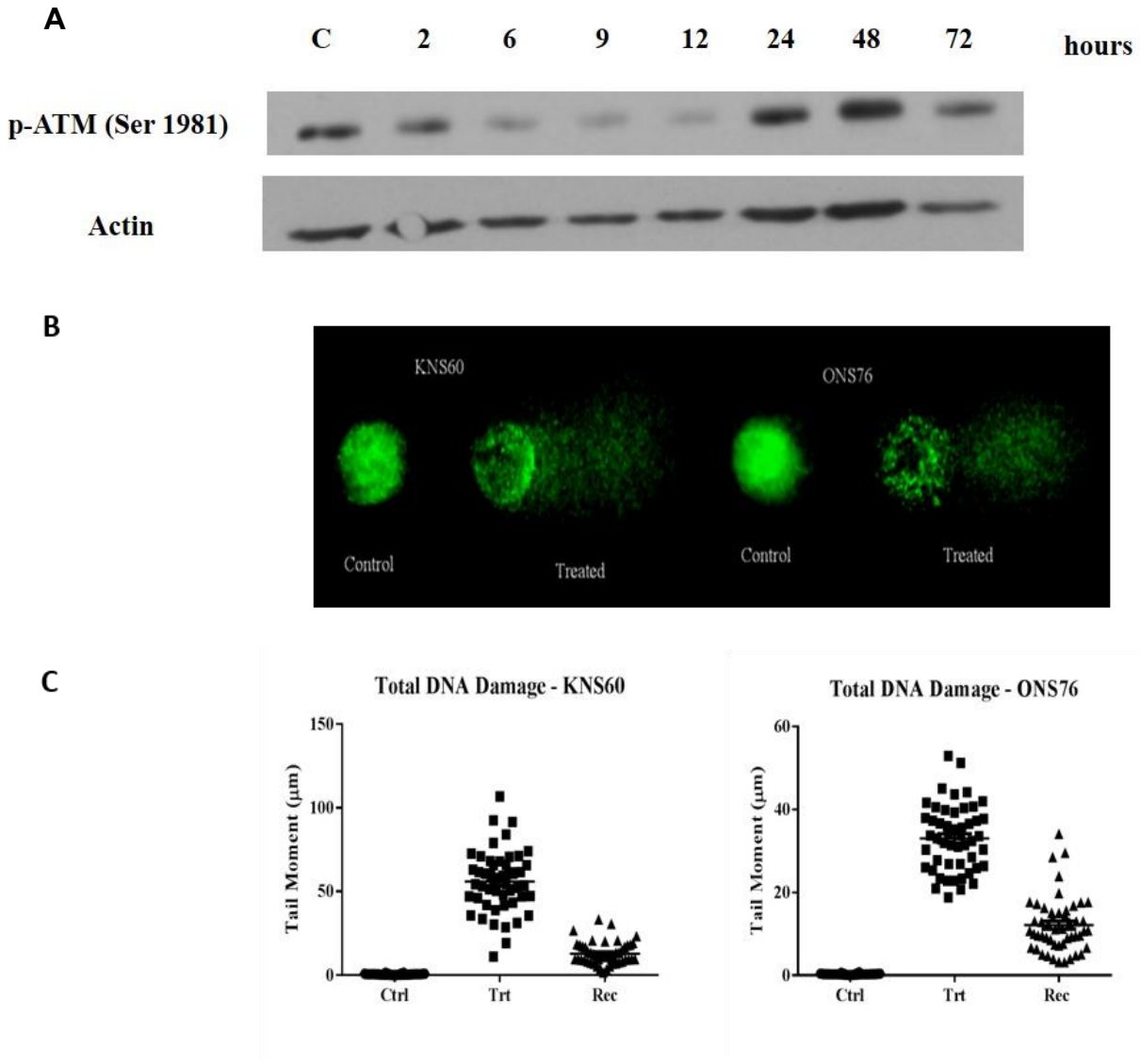
cells. This could, at least in part, account for the reduction in hTERT that was observed (Figure 3.5 C).

3.2.4 TMPyP4 treatment and the DNA damage response in cancer cells

Following the observation of telomere-damage by TMPyP4 in ONS76 and KNS60, a telomere-mediated DNA damage response was anticipated. ATM kinase-mediated DNA damage response at the telomeres is known to be suppressed by intact TRF2 in normal cells (de Lange, 2009). In the cancer cells studied, since TRF2 levels were reduced upon TMPyP4 treatment, ATM kinase activation may be the reason for the potent checkpoint activation leading to cell arrest. Indeed, a time-course analysis of phosphor-ATM (Ser1981) using western blotting revealed the activation of ATM kinase after 24 hours of TMPyP4 treatment in ONS76 (Figure 3.6A).

Since an IC_{50} dose of TMPyP4 was used in these experiments, stochastic DNA damage across the genome, apart from just at the telomeres cannot be ruled out. Hence, alkaline single cell gel electrophoresis (COMET assay) was resorted to, to get the levels of total DNA damage upon TMPyP4 treatment. Indeed, TMPyP4 triggered enormous DNA damage in both KNS60 and ONS76 alike. However, the observed damage after the recovery period of 24 hours in TMPyP4-free media was of significantly smaller magnitude when compared with the levels right after treatment (Figures 3.6 B and C). The appreciable release from arrest during the recovery period suggests that the cells either managed to repair the damaged DNA, or have ejected the irreparable DNA from the nucleus and evade checkpoints to continue proliferating. To this end, we assessed genome stability

by blocking the cells in cytokinesis, following treatment, and then looking for ejected DNA in the form of micronuclei using cytokinesis-blocked micronucleus assay (CBMN). CBMN showed that both – KNS60 and ONS76 had highly elevated levels of micronuclei following treatment with 100 µM treatment with TMPyP4 for 48 hours. Surely enough, the cancer cells must possess highly efficient ways to deal with genomic instability (Figures 3.6 D and E).



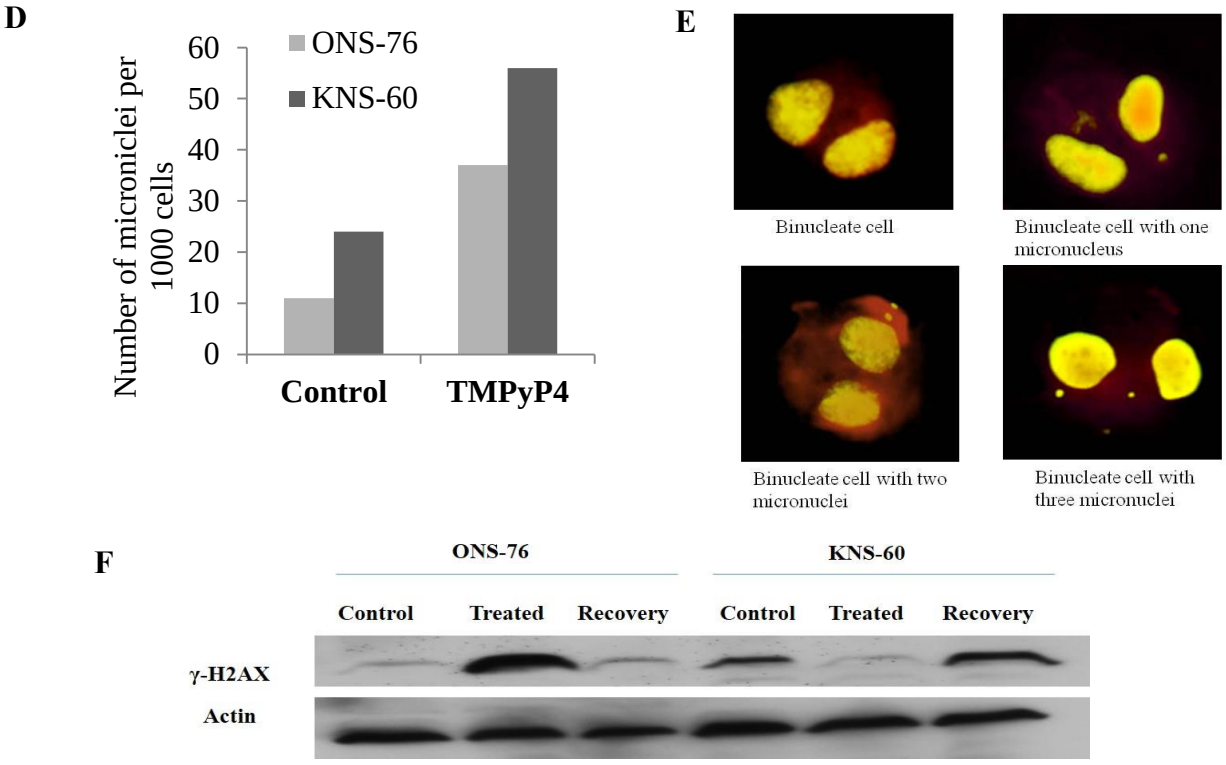


Figure 3.6 **DNA damage induced by TMPyP4** A. Time-course western blot analysis of p-ATM in ONS76 (72 hours indicate the recovery period) B. Representative images of undamaged and damaged ONS76 and KNS60 nuclei by alkaline COMET assay. The intact nucleus is brighter than the tail (damaged DNA) C. Dot-Scatter plot of the intensity of total DNA damage represented as tail moment. Each dot represents one cell; Mean and S.E. of mean of at least 50 cells are represented in dashes. D. Evaluation of micronuclei as a measure of genome stability after treatment. E Representative images of cells with and without micronuclei (smaller bright dots) F. Western blot analysis of phospho-H2AX levels after treatment and 'recovery'. All the experiments were performed in ONS76 and KNS60 cells. Treated refers to 100 µM TMPyP4 for 48 hours; and Recovery – growth in TMPyP4-free emdia for a further 24 hours.

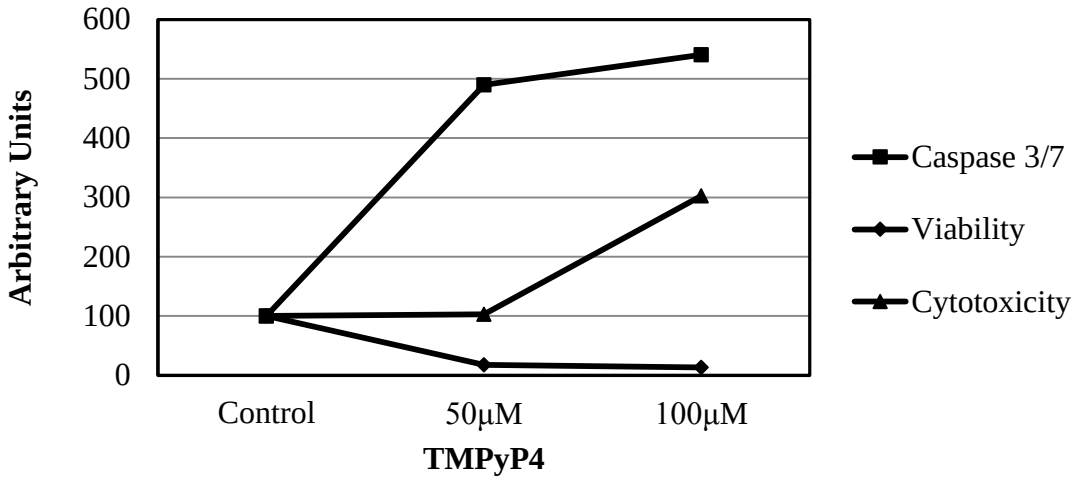
Consequently, the levels of ɣ-H2AX, the protein that makes the chromatin of the damaged DNA, amenable to binding by various DNA repair factors during double-strand breaks, was obtained upon TMPyP4 treatment, by western blotting. Although ONS76 showed a canonical DNA damage response profile, with ɣ-H2AX highly amplified following treatment and appreciable reduction following

recovery, KNS60 showed an unexpected profile. Although, COMET results show that damage occurs upon treatment and repair takes place in KNS60 cells upon removal of TMPyP4 from the medium, it did not reflect in ɣ-H2AX profile (Figure 3.6F). This underscores the variation that cancer cells tend to exhibit often. It is possible that DNA repair is orchestrated in a different way in KNS60 cells.

3.2.5 The mode of cell death following TMPyP4 treatment

Previous studies have shown that G-quadruplex ligands, in general, induce senescence or apoptosis eventually. In the study, TMPyP4 led to cell death as seen earlier in the viability assay. In order to assess the underlying mechanism, a kit that evaluates viability, cytotoxicity, and caspase 3/7 activity at the same time, was made use of and the profile of ONS76 and KNS60 were determined. Caspase 3/7 activity, which translates to the execution of apoptosis, usually is accompanied by a concomitant reduction in the viability and mild increase in cytotoxicity. ONS76, to an extent, revealed signs of classical apoptosis when observed after 48 hours of treatment in a dose-dependent manner (Figure 3.8 A). KNS60, however, exhibited a reduction in caspase activity, with a reduction in both viability and cytotoxicity (Figure 3.8B). This may seem paradoxical; but it could mean that the initiation of apoptosis in these cells has taken place at an earlier time-point. The cells may have undergone fast-acting apoptosis.

A



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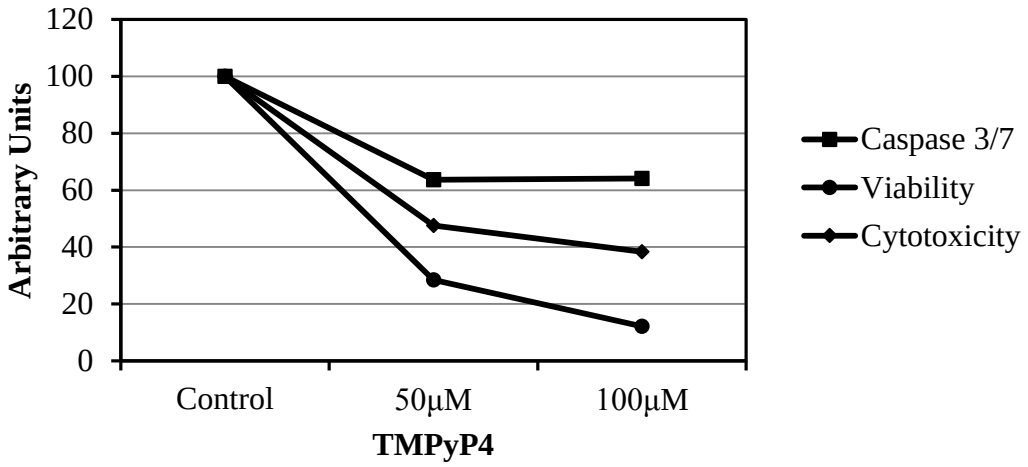


Figure 3.8 **Mode of cancer cell death induced by TMPyP4** A. ONS76 B. KNS60 by virtue of a simultaneous measure of viability, cytotoxicity, and caspase 3/7 activity using Apotox Glo Triplex assay kit.

3.2.6 Genome-wide gene expression profiling of TMPyP4 in the cancer cells

Apart from assessing the above specific effects of TMPyP4 on the cancer cells, in order have a comprehensive understanding of other pathways by which it eventually causes death, a genome-wide gene expression microarray was performed in KNS60 and ONS76 cells. cDNA from cells were hybridised onto a human genomic probes on an Illumina chip, following reverse transcription from total RNA extracts from the cells. Microarray was then performed in duplicates of all categories, chips scanned for their resulting fluorescent ability, data imported in to Partek Genomics Suite, then analysed.

At a time point where about 50% of the cells were killed, a profound effect on genome-wide gene expression is to be expected, and that was indeed the case. Plotting commonly deregulated genes between control and treated of both cell types revealed that 1471 genes were significantly deregulated in ONS76, whereas it was 1860 in KNS60 (ANOVA, FDR $p<0.05$, fold change > 1.5). Notably, about 20-25% of the differentially regulated genes in either cell type following treatment was common between the two cancer cell types (Figure 3.9). Corroborating with the previously quoted experimental observations, genes involved in DNA damage and repair, cell survival, cell cycle, and stress response were deregulated following 48 hours of treatment in both the cancer cells alike. In addition, many transcriptional factors were repressed too, thus possibly indicating a transcriptional shutdown, consistent with the intense cell cycle arrest following 48 hours of treatment (Figure 3.10)

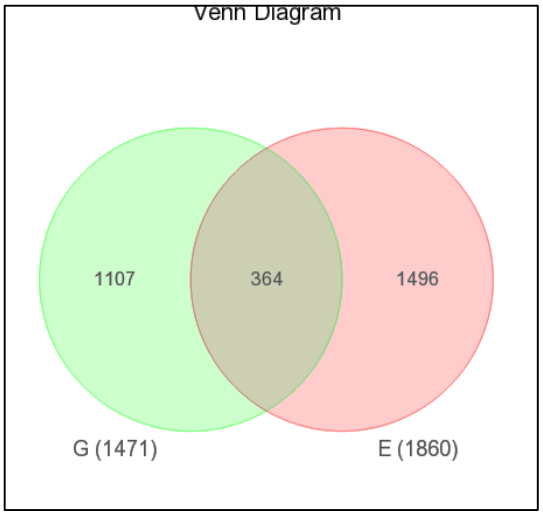


Figure 3.9 **Venn Diagram of deregulated genes** Stringent analysis with $p<0.05$ with FDR, and FC>2 (fold change) performed using Partek Genomics Suite revealed 364 genes common to both, G. ONS76 and E. KNS60, following treatment with 100 µM TMPyP4 for 48 hours.

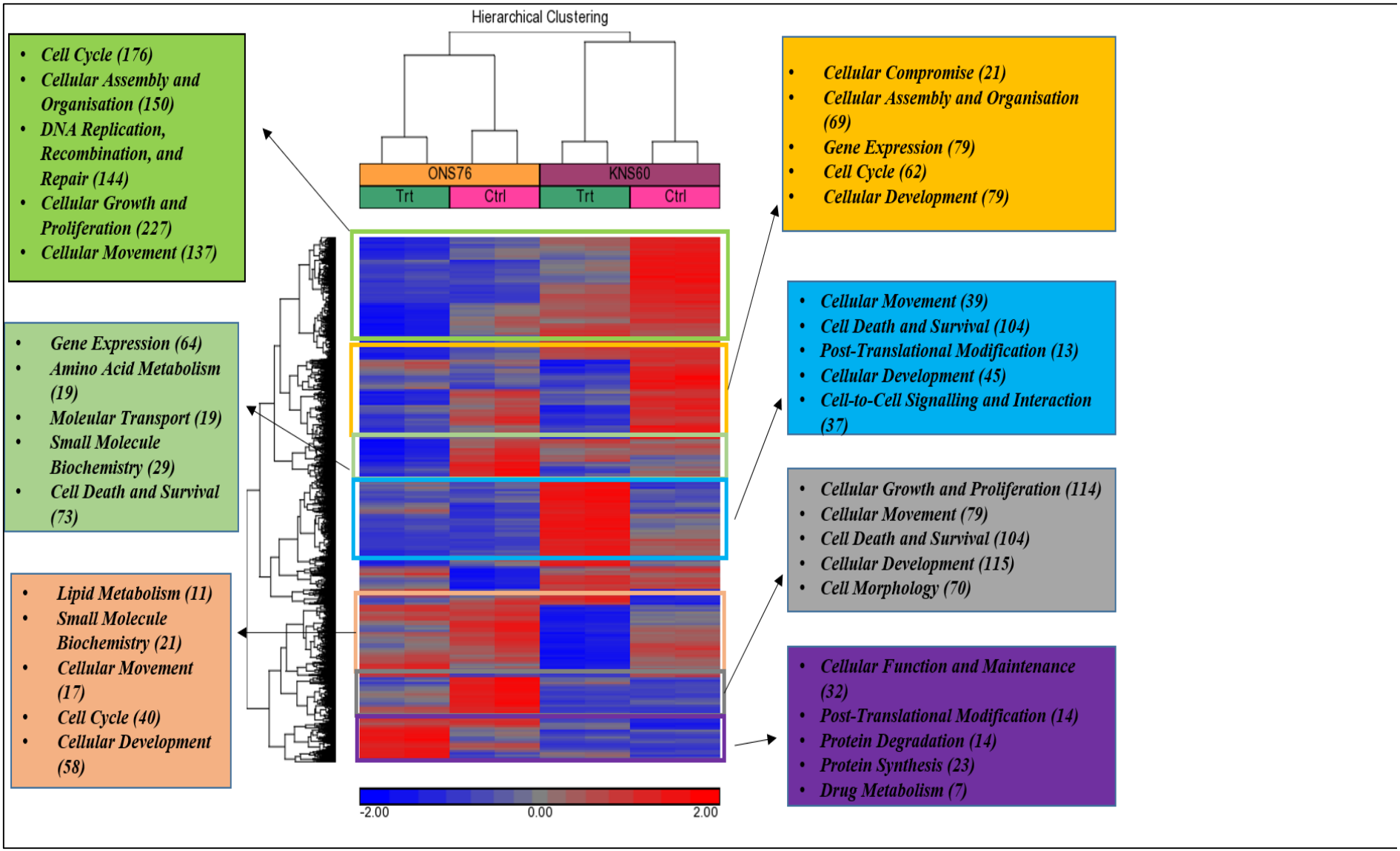


Figure 3.10 **Unsupervised clustering of significantly deregulated genes.** Seven patterns of concentrated deregulation were picked out using Partek Genomics Suite and plotted along with their associated molecular and cellular functions usinig Inguinuity Pathway Analysis, following treatment of ONS76 and KNS60 cells with 100 µM TMPyP4 for 48 hrs. Numbers of genes involved in the differential regulation of the molecular and cellular functions are mentioned within brackets; the order of the functions mentioned in each cluster is based on the significance of the differential regulation; the colour gradient represents fold change.

3.2.7 TMPyP4 and cancer cell migration

Genes from other interesting pathways were deregulated too, as revealed by pathway analysis using Ingenuity Pathway Analysis, following treatment. Interestingly, three out of the seven deregulated clusters showed 'cellular movement' deregulated with 245 genes involved in the process; and with 'cellular movement' being among the top two affected functions in two of the three clusters (Figure 3.9). The other affected pathways are largely related to cell fate, which is expected to happen with the IC_{50} dose used. However, deregulation of cellular movement was an unanticipated observation.

This study therefore looked into the ability of TMPyP4 to affect cell motility of ONS76 and KNS60 cells. To this end, a scratch wound assay was performed. Cells were plated in a confluent manner in culture dishes and a scratch was done using a micro-pipette tip. The closure of the 'wound' or the gap created, gives an indirect assessment of cell motility Accordingly, TMPyP4 hampered wound closure in both the cell types alike, as seen after 48 hours of treatment (Figure 3.11A). Probing at molecular players could be more confirmatory of such functional assays, and hence the levels of one class of integrins – the cell surface proteins in charge of cell adhesion – was measured using flow cytometry- based detection of integrin β1. An appreciable reduction in the cell surface expression of integrin β1 was observed in both, KNS60 and ONS76 after TMPyP4 treatment (Figure 3.11B).

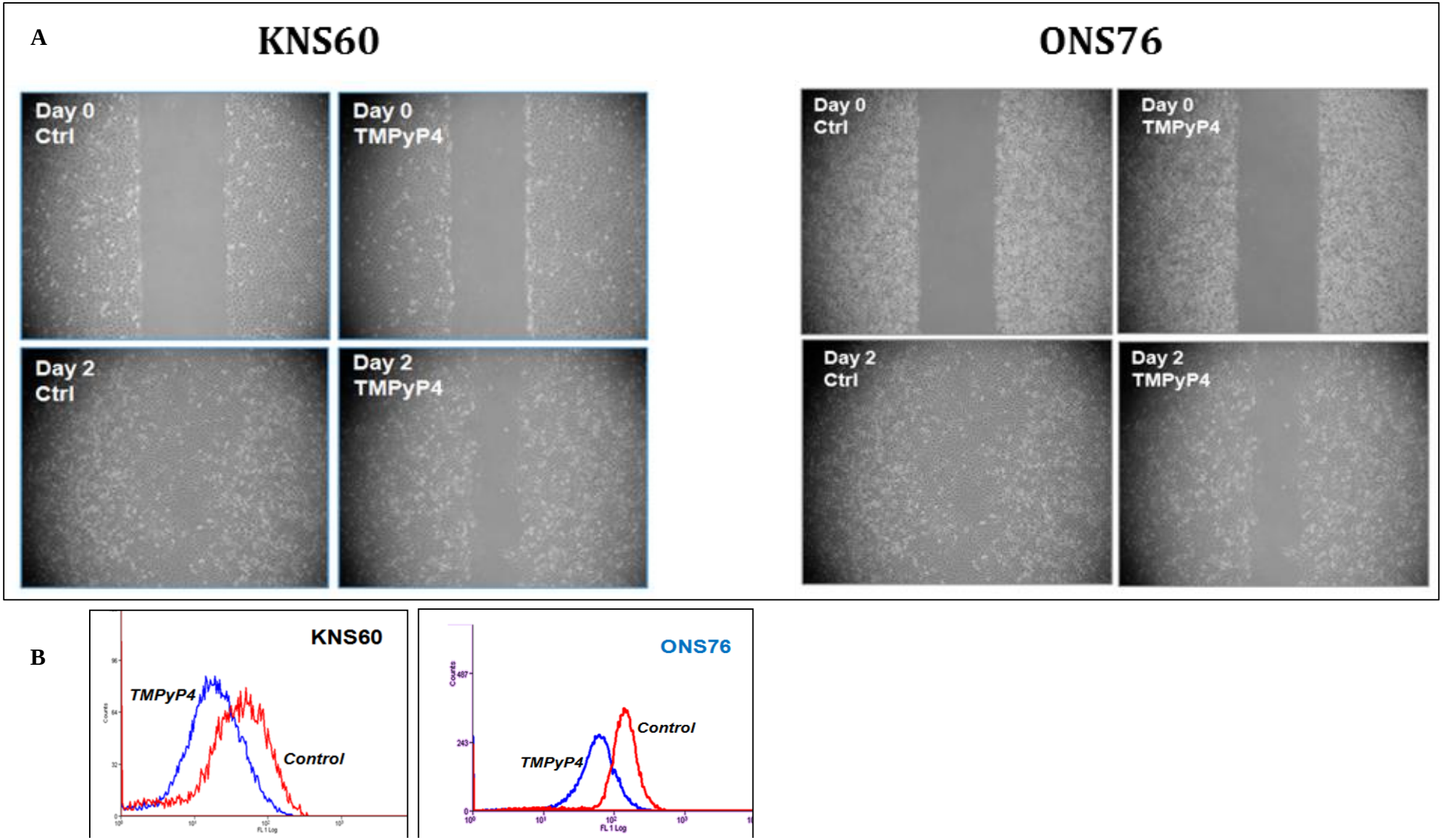


Figure 3.11 **Effect of TMPyP4 on cancer cell metastasis** A. Scratch wound assay revealed TMPyP4 treatment hampered wound closure in both, KNS60 and ONS76 cells. B. Reduction of cell surface expression of integrin β1, as measured by flow cytometry.

3.3 DISCUSSION

This is the first study of TMPyP4 in glioblastoma and medulloblastoma cells. Previous studies on TMPyP4 were performed in leukemia, myeloma, retinoblastoma, breast cancer, and prostate cancer cell lines and breast and prostate tumour xenograft mouse models (Table 1.1). All the studies, hitherto, have shown a largely cancer-specific effect of TMPyP4, compared to normal cell types tested.

In this study, short term treatment with TMPyP4 caused a reduction in telomerase activity in the cancer cells, much like what has been documented previously. Also its effect on c-MYC has been observed in earlier studies with TMPyP4. However, the effect of TMPyP4 on TRF2 levels in the cancer cells as early as 2 hours after treatment, has not been shown before. The observation that TRF2 levels do not recover even after growth in TMPyP4-free media elucidates the telling, yet specific effect of TMPyP4 on telomeres. TRF2, which has been shown to be the linchpin of telomere integrity, is essential for cancer cells' survival in a stressful and challenging microenvironment. The mechanism of the plummeting of TRF2 levels after treatment could be attributed to cytoplasmic trafficking of TRF2, followed by ubiquitin-mediated degradation. Telomeres consist of G-rich hexameric tandem repeats and is prone to forming many secondary structures like the hairpin, t-loops, and quadruplexes. It is known that the preference for a particular conformation depends on the microenvironment, the competition between various ligands/ conducive ions like Na^+ and K^+, and the stabilisation energy of the reaction (Zahler et al., 1991). Shelterin proteins dictate terms at the

telomeres by binding strongly to the telomeres and inducing the formation of t-loops and d-loops, in order to regulate legitimate access to the telomeres (de Lange, 2009). A majority of original G-quadruplex ligands exert their anticancer effect by causing telomere uncapping and hence, telomere dysfunction. A study with RHPS4, a promising G-quadruplex ligand on melanoma cells revealed telomere uncapping as the mechanism behind the cancer-specific effects observed (Salvati et al., 2007). A study of neuroblastoma cell lines on the effects of telomestatin, one of the first G-quadruplex drugs to enter clinical trials, showed disruption of telomere maintaenance over short term of treatment (Binz et al., 2005). Another study, on uterine carcinoma cells *in vitro* and xenograft models showed telomere uncapping as one of the mechanisms attributable to the anticancer effect of BRACO-19, the first drug synthesised based on computational modelling analyses for optimal G-quadruplex stabilisation(Burger et al., 2005). The current study strongly supports an uncapping mechanism at the telomeres upon TMPyP4 treatment, much like other promising G-quadruplex ligands like RHPS4, Telomestatin, and BRACO-19. It is highly likely that TMPyP4 is able to dislodge shelterins from the telomeres, by binding more strongly to the telomeres. This though, would mean a reduction in the amount of other shelterin proteins as well; but that is yet to be assessed.

In ONS76 cells, appreciable reduction in hTERT levels occurs at a later time point though, around 12 hours unlike TRF2, which was spontaneous. Nonetheless, the reduction in hTERT happens too soon for it to get affected by the c-MYC reduction which was only assessed after 48 hours. It is noteworthy that TMPyP4

has been shown to directly bind to and stabilise G-quadruplexes at a c-MYC promoter (Siddiqui-Jain et al., 2002). Notwithstanding this ability of TMPyP4, the effects observed here are at least majorly attributable to the direct binding of TMPyP4 to the telomeres and presumably by stabilising G-quadruplex structures there due to the fact that it resulted in a drastic reduction in TRF2 levels as early as two hours after treatment, and also because as shown in earlier studies, TMPyP4 is able to block telomerase from accessing the telomeres. The observed reduction in hTERT levels may also be due to trafficking of hTERT out of the nucleus and subsequent degradation by the proteasome machinery as demonstrated upon treatment with another G-quadruplex compound, BRACO-19 (Burger et al., 2005).

The enormous amounts of DNA damage observed upon TMPyP4 treatment could well be a result of telomere-mediated genomic instability. However, since the concentration of TMPyP4 used was an IC_{50} dose, non-specific effects is understandable and may well cause stochastic DNA damage throughout. The observation that the extent of damage observed after the 24-hour recovery period is significantly lower as compared to the treated category, there are two speculations about the nature of the DNA damage. One possibility is that, the nature of DNA damage induced is mild, though extensive, and hence, is reparable largely. Alternatively, the nature of the inflicted damage is severe and irreparable, and is rejected from the nucleus in the form of micronuclei during the next round of cell division. Another aspect to note is that the cells get back to cycling at a nearly normal rate after 24 hours of recovery, as compared to that after 48 hours

of treatment. Therefore, both the possibilities exist: TMPyP4 induces mild DNA damage that is reparable and also severe, irreparable damage that is often ejected in the form of micronuclei. The observation that IMR90, a normal cell line with proficient arrest checkpoints is largely not arrested upon TMPyP4 treatment, as opposed to the cancer cells, explains the cancer-specific nature of TMPyP4, at least in terms of stochastic DNA damage. This is intriguing, but possibly indicates that the cancer cells are inherently, genomically unstable. Hence, it is may be easier to induce catastrophic DNA damage specifically in the cancer cells, similar to the rationale behind radiotherapy for cancers. The cytotoxicity of short term treatment with TMPyP4 is conceivable, keeping the specific effects in mind and also given that other studies on G-quadruplex ligands like RHPS4 also reported the same (Salvati et al., 2007).

Cells after TMPyP4 treatment seem destined for cell death, and not senescence, consistent with the massive DNA damage observed. The mode of cell death seems to be apoptosis, as evidenced by caspase 3/7 levels after treatment along with the reduction of viability and an increase in cytotoxicity in ONS76 cells. KNS690 and A172 also seem to undergo apoptosis, a profile of decreasing cytotoxicity and viability, with decreasing caspase activity with dose is consistent with the possibility of fast-acting apoptosis in the cells. Perhaps the 48-hour time point is too late to look for signs of apoptosis in KNS60 and A172 cells.

Once again, this dissonance in the timing of initiation of cell death only reiterates the heterogeneous nature of cancer cells, even those derived from the same tissue. The other notable differences in the behaviour of KNS60 are the non-canonical

regulation of ɣ-H2AX in response to massive DNA damage, which at least in part, would account for DSBs; and the execution of arrest in the p53-lacking KNS60 similar to the extent in ONS76 bearing WT p53, among others. The use of such model systems with a variety of cell types would in fact, be helpful in screening for agents that are effective against a broad range of such cells.

The gene expression profiling corroborated largely with the observation in other experiments, in terms of deregulation of similar pathways, if not genes. In both, KNS60 and ONS76, TMPyP4 exhibited changes in the expression of quite a few oncogenes as well. Overall, genes of telomere maintenance, DNA damage and repair response factors, cell arrest checkpoint genes, oxidative stress response genes, among others. An assessment of gene expression profile at an earlier time-point would give a more specific profile of the hit by TMPyP4 in the cancer cells. Nonetheless, it should not be ignored that the gene expression profiling was carried out after the execution of cell cycle checkpoints, and so, apart from the canonical specific pathways, the rest may well be repercussions, largely. Since the study demonstrated telomere-mediated effects TMPyP4 at early time-points itself, which was then followed by the induction of DNA damage *en masse* in the cancer cells, the gene expression profile obtained, perhaps reiterates the repercussions of telomere dysfunction in cancer cells. Profiling of genome-wide gene expression was also done upon short term treatment with 100 μM TMPyP4 after 48 hours in a leukemia cell type, K562. While the analysis was not as extensive as that in our study, it showed the deregulation of genes of a similar spectrum of cellular pathways, predominantly that of cell signalling and transcriptional regulation

(Mikami-Terao et al., 2009). A smaller subset of genes in that study was a result of a more stringent cut-off set for deregulated genes, which may have led to the loss of detection of some key genes(Mikami-Terao et al., 2009) .

This is not the first time that antitelomerase treatment comes along with that of metastasis as well. RHPS4, a G-quadruplex stabilising agent and GRN163L, a telomerase inhibitor have been shown to exhibit anti-metastatic ability in mouse models (Leonetti et al., Clin Can Res, 2008; Jackson, Zhu and Paulson, Cancer Res, 2007). Therapeutic intervention by causing telomere shortening leads to the inhibition of migration of malignant tumours (Uziel et al., PLoS One, 2010). Also G-quadruplex ligands have been observed to affect promoter regions of a growing list of oncogenes like MYC and the angiogenesis inducer VEGF, which can affect metastasis(Siddiqui-Jain et al., 2002; Sun et al., 2005; Sun et al., 2011) Also, malignant gliomas are known to exhibit intracranial and extracranial metastases in advanced cases, and hence is of interest. In addition, targeting integrins in glioma therapy has been shown to be rewarding (Sathornsumetee et al., 2007).

Overall, TMPyP4 during short term treatment, shows telomere-mediated effects in glioblastoma and medulloblastoma cells, sparing normal cells IMR90. Telomere dysfunction, telomerase inhibition, and DNA damage induction together resulted in cell cycle arrest and apoptotic cell death in the cancer cells, in addition to exhibiting slightly uncharacteristic role in the inhibition of cell migration as well.

Chapter 4

Investigation into potential for DNA repair inhibition and radiation along with TMPyP4 treatment

4. INVESTIGATION INTO POTENTIAL FOR DNA REPAIR INHIBITION AND RADIATION ALONG WITH TMPyP4 TREATMENT

4.1 TESTING THE POTENTIAL OF TMPyP4 AS A RADIOSENSITISER

4.1.1 BACKGROUND

Radiotherapy has been the mainstay of cancer management in general, and more so for malignant brain tumours, for which treatment options are lacking and survival rate despite treatment remains dismal (Yabroff et al., 2012). Ionising radiation – while it affects cancer cells largely, by inducing massive DNA damage – also affects somatic cells of fast proliferative nature. Consequently, quite often, the dose of irradiation is limited to ensure that the benefit outweighs the side effects considerably at least. Hence, radiotherapy though in principle can eradicate much of the tumour, is often unable to be used to its full potential. To circumvent this, pre-treatment with a few drugs have been resorted to with the aim of sensitising cells to irradiation, and hence to achieve a higher therapeutic efficacy with a lower dose of IR. Combining treatment with cytotoxic drugs and irradiation has been employed in the past, but although it efficiently reduced tumour bulk, was often limited by toxicity to normal somatic cells and also lacked a definitive rationale (Tannock, 1996). Potential targets for radiosensitisation that have been studied hitherto include mediators of DNA repair, modulators of apoptotic response, transcription factors, growth factor receptors, cytoplasmic signal transduction (Tofilon and Camphausen, 2009). These strategies are not surprising ones, given that cellular response to IR is mediated by all of the above. Even so, there are not many studies on these lines *in vivo* (Raleigh and Haas-

Kogan, 2013). However, there is one concept that has not been given importance that is commensurate with the promise it holds – telomere maintenance.

Targeting telomere maintenance in combination with conventional therapy has a strong mechanistic backing, owing to the limiting of cancer cell proliferation by the former strategy, as explained in Figure 4.1 (Shay and Wright, 2002).

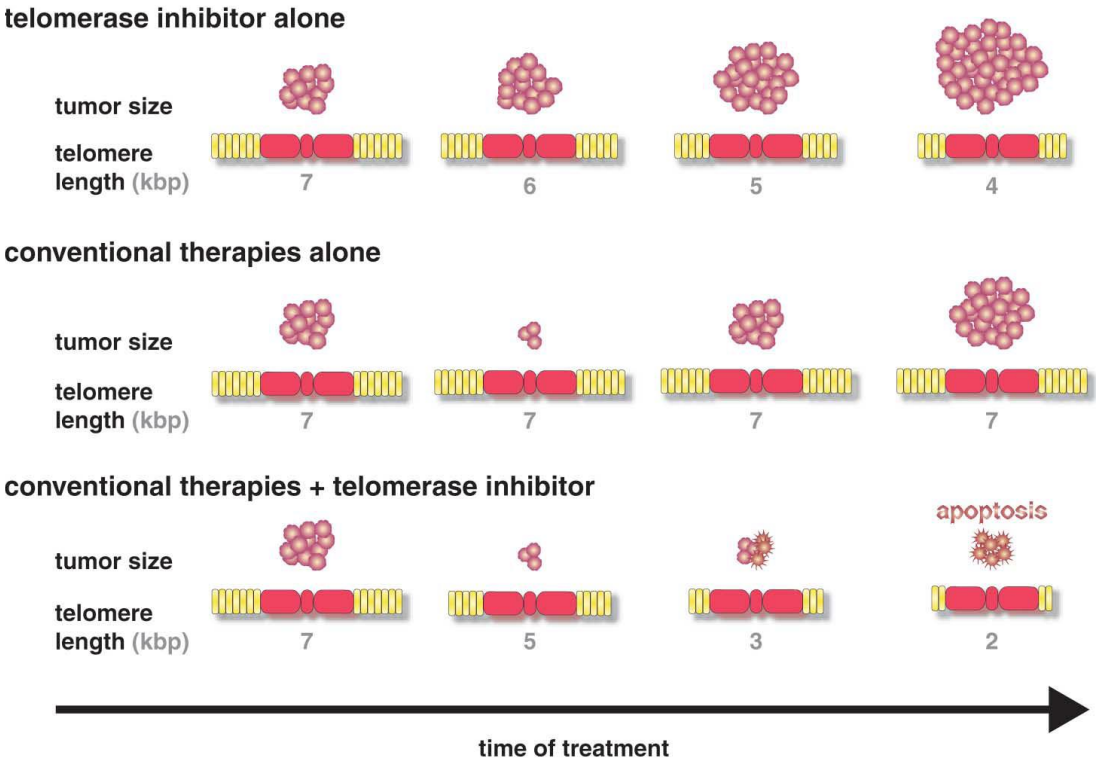


Figure 4.1 **Combining telomerase inhibition or telomere-targeting along with standard-of-care therapies in cancer.** Conventional therapies like chemotherapy or radiotherapy typically counter the tumour mass by eliminating the fast-proliferating cells by way of DNA damage-induced cell death. While this results in tumour debulking, the risk of tumour relapse is still intact. Targeting telomere-telomerase pathway would invariably limit the proliferation of cancer cells with high specificity as cancer cells predominantly have near-critical telomeres, unlike normal somatic cells and germline cells. Image source: (Shay and Wright, 2002).

Working model of the above hypothesis is illustrated in the table below, that shows a compilation of studies employing standard-of-care treatments for various cancers showing promising synergism or increase in sensitivity when combined

with a treatment affecting telomere biosynthesis (Figure 4.2)(Cunningham et al., 2006).

Inhibitor/Sensitizer	Standard Therapeutic Agent	Outcome
2'-*O*-methoxyethyl RNA	Cisplatin/carboplatin	Synergistic effect
Antisense-hTR	Paclitaxel	Significant increase in sensitivity
Antisense-hTR	Cisplatin	Increase in sensitivity
DN-hTERT	Cisplatin/taxanes/etoposide	Increase in induction of apoptosis
DN-hTERT	Daunorubicin	Increase in apoptosis
Ribozyme-hTERT	Doxorubicin	Increase in sensitivity
RNAi-hTERT	Topoisomerase inhibitors/bleomycin/radiation	Increase in sensitivity
hTR-NAT	[^{131}I]MIBG	Induced uptake
AZT	Paclitaxel	Increased activity and effect

NAT = noradrenaline transporter gene; MIBG = [^{131}I]meta-iodobenzylguanidine; AZT = azidothymidine.

Figure 4.2 **Sensitisation of cancer cells to standard therapeutic agents by telomerase inhibitors** Image source: (Cunningham et al., 2006).

The link between telomere functionality and radiosensitivity is one that has been demonstrated in a handful of studies. Direct evidence of telomere dysfunction rendering sensitivity to ionising radiation in a mouse model was shown a decade ago (Wong et al., 2000). A study of 181 individuals uncovered an inverse correlation between telomere length and *in vitro* radiosensitivity as evidenced by an increase in micronuclei (irreparable damaged DNA ejected from the nucleus, a mark of genome instability) induced by ionising radiation (Castella et al., 2007). Also, telomerase activity was shown to negatively correlate with radiosensitivity independent of telomere length in neuroblastoma cell types (Wesbuer et al., 2010). Coherently, telomerase recruitment has been speculated to be necessary in the repair of ionising radiation-induced DSBs from a wealth of studies on *in vivo*

models and *in vitro* cell lines (Ayouaz et al., 2008). Disruption of telomere maintenance has been postulated to be a hallmark of irradiation(Ayouaz et al., 2008). Moreover, the observation of accelerated telomere shortening and telomere dysfunction in radiosensitive cell types, points to the possibility that defective telomere maintenance mechanisms may lead to radiosensitivity (Cabuy et al., 2005).

Given the realisation that cancer cells have a certain vulnerability to perturbation of telomere maintenance, this trend in the lack of studies and clinical application of telomere-related radiosensitivity is alarming, and is clearly a lacuna in literature on the repertoire of strategies to eradicate cancer cells with maximum efficacy. Investigations on the radiosensitivity of G4 ligands are particularly lacking, with only one study till date on the effects of a G4 ligand, TAC in glioblastoma cell types (Merle et al., 2011). To this end, the current study investigates the potential of TMPyP4 pre-treatment in sensitising the glioblastoma cells, KNS60 and A172 cells to irradiation.

4.1.2 RESULTS

4.1.2.1 Determination of dose of the combination treatment

To determine the dose of γ-radiation to be used in the study, KNS60 and A172 cells were irradiated with doses 2 and 4 Gray (Gy) of γ-radiation and the viability assessed using Cell Titer Glo kit after 24 hours. While 2 Gy γ-radiation did not affect the viability of A172, it reduced that of KNS60 meagrely (Figure 4.3 A). However, 4 Gy γ-radiation led reduced the viability of both – KNS60 and A172 cells considerably (Figure 4.3 A). Hence, 4 Gy γ-radiation was chosen as the dose for this study. Since the question for study is on the ability of TMPyP4 to merely sensitise the cancer cells to irradiation than to eradicate the cells by itself, the dose of TMPyP4 had to be lower than that used in studies in chapter 3 (100 µM). Assessment of viability of KNS60 and A172 cells revealed that a dose of 50 µM TMPyP4 is not as cytotoxic as 100 µM over 48 hours (Figure 4.3 B). Moreover, evaluation of telomerase activity of 50 µM TMPyP4 after 48 hours of treatment performed using TRAP assay showed extensive inhibition of the same, and hence was the chosen dose (Figure 4.3 E).

4.1.2.2 Effect of TMPyP4 pre-treatment on the viability of the irradiated cancer cells

With the doses of the individual treatments optimised, the effect of pre-treatment with 50 µM TMPyP4 for 24 hours, followed by incubation of the irradiated cells for a further 24 hours with TMPyP4 on the viability of KNS60 and A172 cells

was assessed using Cell Titer Glo kit. Signs of radiosensitisation of both – KNS60 and A172 cells by TMPyP4 was witnessed, as seen by more than a 10% and 27% reduction respectively, in the viability between cells that were exposed to 4 Gy γ-radiation, with and without TMPyP4 pre-treatment (Figures 4.3 C and D). Following this observation, a possible activation of cell cycle checkpoints was assessed.

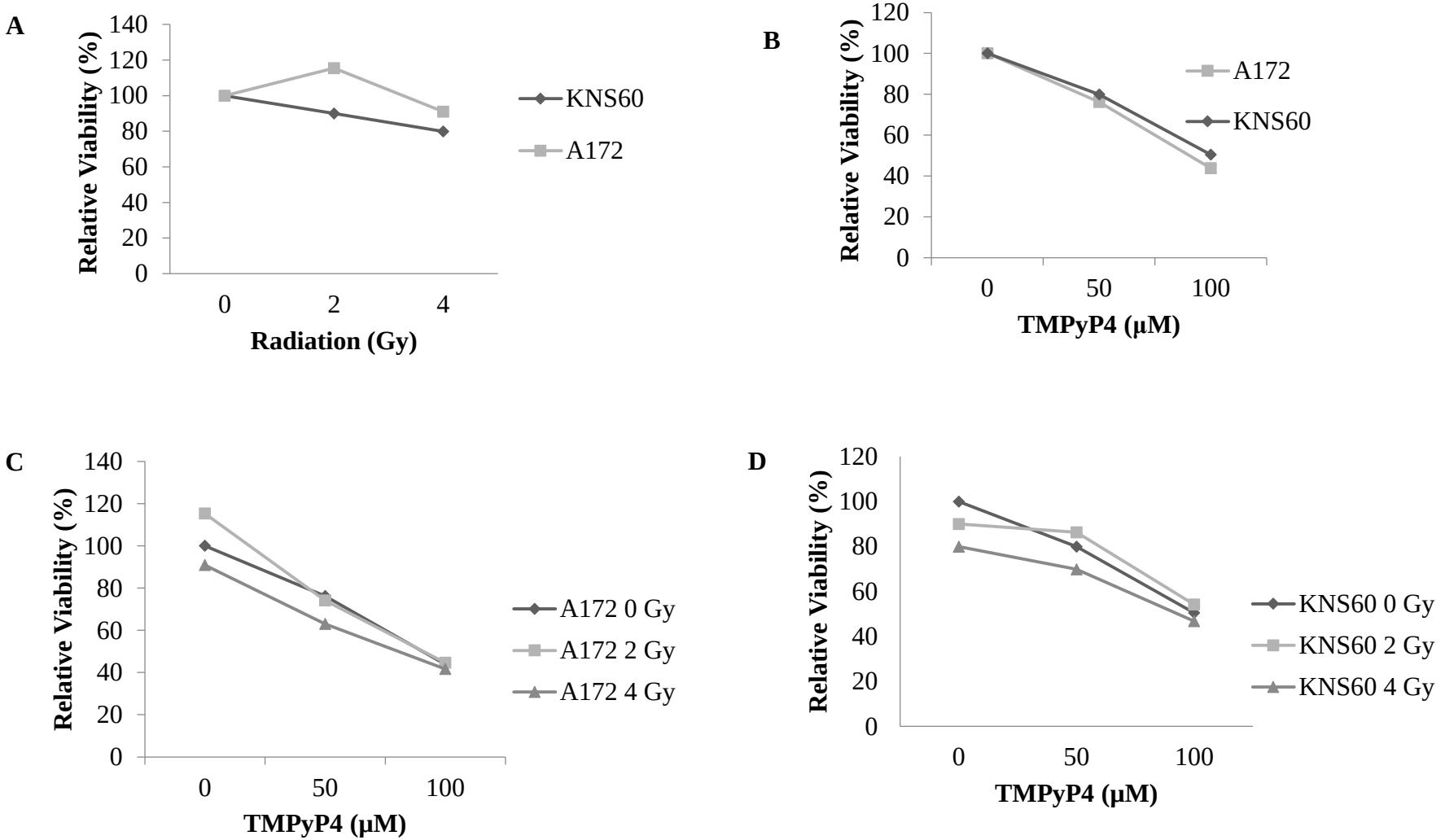
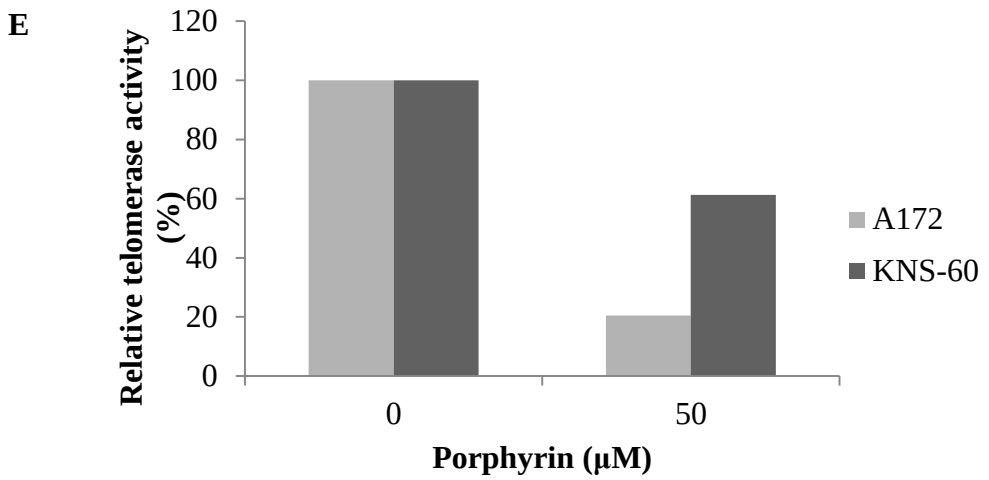


Figure 4.3 **Dose determination of TMPyP4 and γ-radiation and study of the combination treatment on the viability of the cancer cells.** The viability of cells as a percentage of the control cells, as measured by the Cell Titer Glo kit upon treatment with different doses of **A.** γ-radiation; **B.** TMPyP4; **C & D.** pre-treatment with TMPyP4 for 24 hours, followed by irradiation and further incubation in TMPyP4-containing media for another 24 hours in A172 and KNS60, respectively. **E.** Evaluation of relative telomerase activity of A172 and KNS60 extracts, following 50 µM TMPyP4 treatment for 48 hours, as measured by the TRAP assay.



To check if the additive reduction in viability of the combination treatment is due to the enforcement of cell cycle arrest, the cell cycle profile of KNS60 and A172 cells were obtained using staining of the cellular DNA by Propidium Iodide (a nucleic acid intercalator) following RNase treatment and fixing the cells.

4.1.2.3 Determination of a possible cell arrest induced by TMPyP4 treatment prior to irradiation

Flow cytometric analysis of the fixed cells indeed revealed some striking patterns of cell arrest. Fifty micromolar of TMPyP4 treatment for 48 hours induced a significant G2/M arrest ($p < 0.05$, ANOVA) in KNS60 cells, while A172 cells did not seem to be arrested significantly (Figure 4.4). It is noteworthy that this pattern of arrest was similar to that observed earlier with 100 µM TMPyP4 in KNS60 cells (Chapter 3, Figure 3.2 B). A172 cells were arrested in G1 phase ($p < 0.05$, ANOVA), as observed 24 hours after irradiation, while KNS60 cells were not significantly arrested (Figure 4.4). These variations notwithstanding, the TMPyP4 pre-treatment, followed by irradiation resulted in a robust G2/M arrest of both

KNS60 and A172 cells (Figure 4.4). Given the existence of considerable body of literature on the nature of anti-telomere agents and also on the *modus operandi* of ionising radiation, an assessment of direct/ indirect infliction of DNA damage was a natural consequence of this experimental outcome.

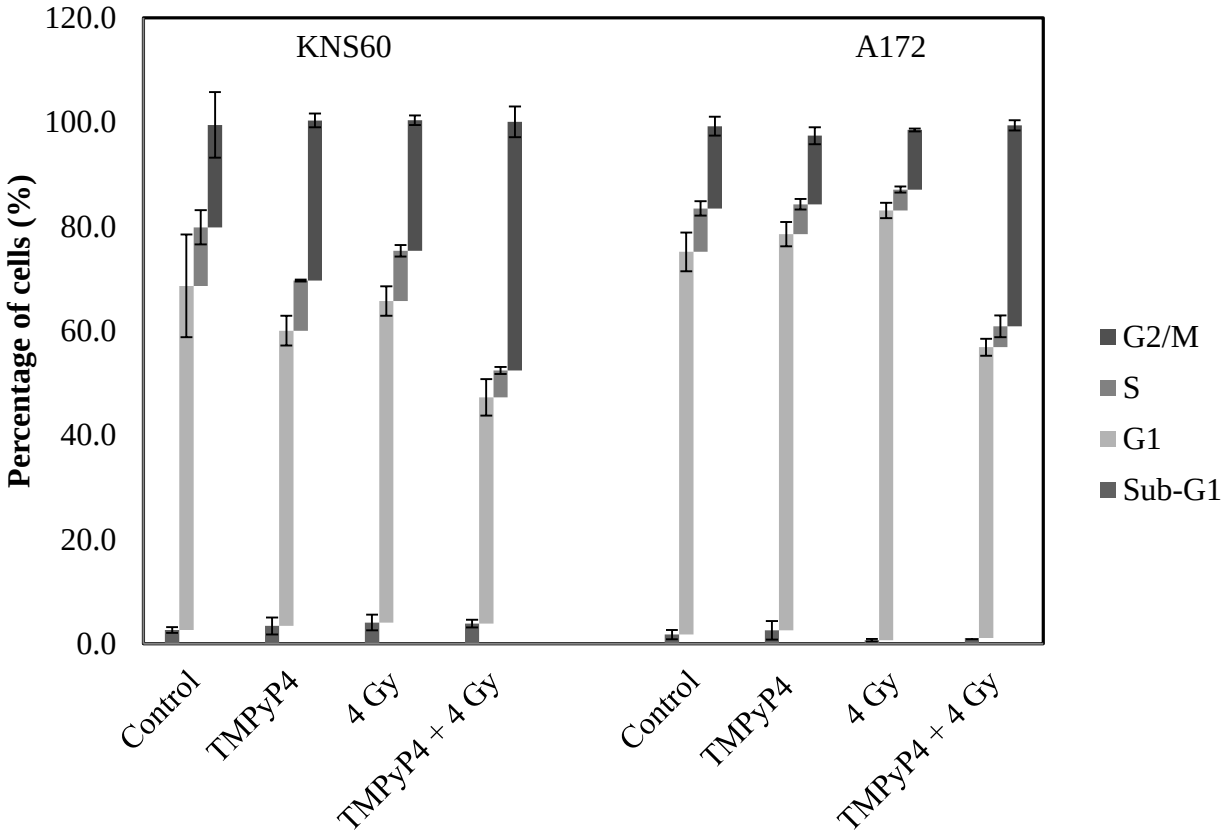


Figure 4.4 **Assessment of the contribution cell cycle arrest towards the radiosensitising ability of TMPyP4.** The cells were stained with Propidium Iodide (PI), following treatment with RNase, and fixation using ethanol. Flow cytometry was used to obtain the cell cycle profile of KNS60 and A172 cells following the combination treatment, alongside appropriate controls. The combination – 'TMPyP4 + 4 Gy' represents pre-treatment with TMPyP4 for 24 hours, followed by irradiation and further incubation in TMPyP4-containing media for another 24 hours. Single factor ANOVA was performed to assess significance.

4.1.2.3 Evaluation of total DNA damage induced by the combination treatment

Total DNA damage present in a population of cells was assessed by alkaline single cell gel electrophoresis assay. The intensity of damage was expressed as mean tail moment, a measure of the distance moved away from the integral DNA by fragmented DNA upon electrophoresis. This showed while the cells that were just irradiated had a higher level of damage intensity than the controls, the combination treatment resulted in a similar spread of tail moments as that of TMPyP4-treated cells – an apparently much higher intensity of damage (Figures 4.5 A & B).

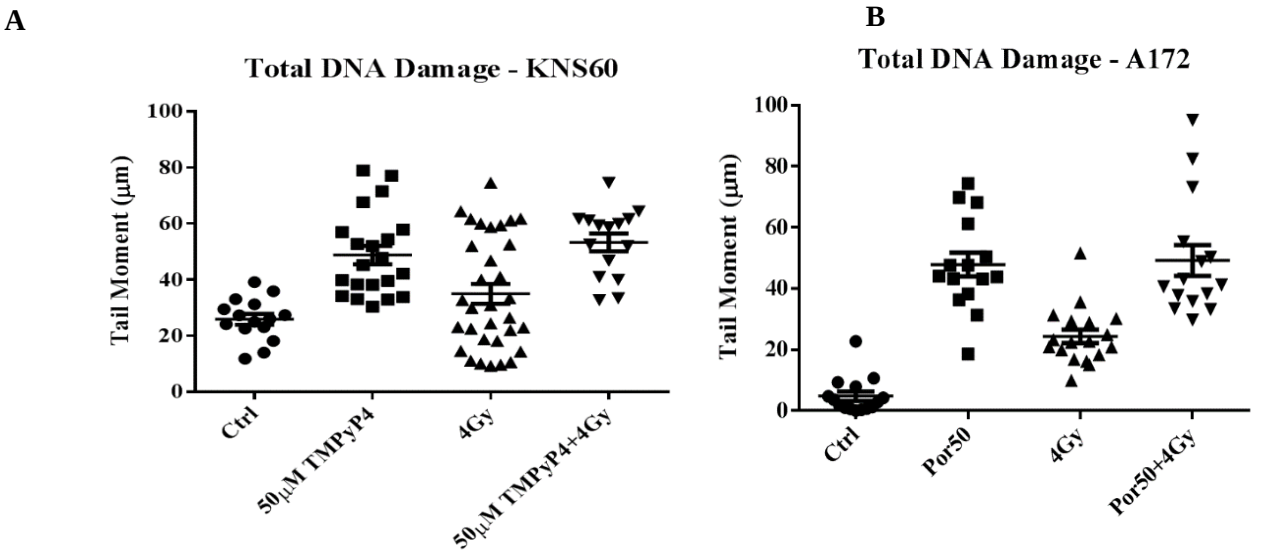


Figure 4.5 **Intensity of total DNA damage induced by TMPyP4 treatment and irradiation in the cancer cells.** Total DNA damage assessment was done by performing single-cell gel electrophoresis under denaturing (alkaline) conditions and its intensity plotted as tail moment of **A.** KNS60 and **B.** A172 cells. 'Ctrl' refers to the untreated controls; '50 μM TMPyP4' and 'Por 50', both to treatment with 50 μM TMPyP4 for 48 hours; '4 Gy' to the dose of γ-radiation; and ''50 μM TMPyP4 + 4 Gy' and 'Por 50 + 4 Gy' to the combination treatment, i.e. pre-treatment with TMPyP4 for 24 hours, followed by irradiation and further incubation in TMPyP4-containing media for another 24 hours. The nuclei were stained with SYBR Green dye and images captured using COMET Imager software. At least 15 nuclei were analysed for each category.

4.1.3 DISCUSSION

Treatment with radiosensitising agents renders cells more susceptible to a given dose of radiation, than they would be without the treatment. Interestingly, the formation of G-quadruplexes has been implicated previously with regard to rendering cells susceptible to radiation-induced DNA damage: a study of the effects of treatment with telomere-sequence mimicking oligonucleotide (T-oligo) on mammary carcinoma cells *in vitro* and xenograft model attributes the radiosensitivity conferred by the treatment to the possible formation of intermolecular G-quadruplexes with the telomeric single-stranded overhang (Weng et al., 2010). There has been only one G-quadruplex stabilising (G4) ligand though, TAC, that has been evaluated for and that has demonstrated radiosensitising ability, also in glioma cells (Merle et al., 2011). In this study, TMPyP4 clearly accentuated the effect of γ-radiation on cell death (Figure 4.3 C & D). This study also corroborated with the one on TAC in terms of induction of G2/M arrest in the combination (Figure 4.4) (Merle et al., 2011). The sheer proportion of cells arrested in G2 phase in strongly suggestive of precedence of an extensive DNA damage checkpoint. Thus, it follows that although the intensity of DNA damage as measured by alkaline single-cell gel electrophoresis was similar in both – TMPyP4 treated and the combination categories in KNS60 and A172, the more robust enforcement of the damage checkpoint in the combination is reflective of the actual extent of DNA damage suffered by the cells of that category. The study on the G4 ligand, TAC, demonstrated a slow decay of γ-H2AX foci (indicative of DNA damage response activation) in the combination

category as opposed to that with just irradiation, indicating that pre-treatment with TAC, the G-quadruplex ligand, resulted in the persistence of DNA damage induced by irradiation.

Remarkably, a study on a small molecule inhibitor of survivin, YM155, in a model of non-small cell lung cancer cell lines showed the radiosensitivity conferred was due to the attenuation of DNA repair response (Iwasa et al., 2008). Moreover, a study on the assessment of the strategy of combining a histone deacetylase inhibitor, NVP-LAQ824, with irradiation in a mouse model of non-small cell lung cancer showed decrease in DNA repair efficiency before the onset of the observed radiosensitivity (Cuneo et al., 2007). Another study in a tumour xenograft model, also of non-small cell lung cancer, evaluated the radiosensitising effects of a small molecule inhibitor of insulin growth factor 1 receptor, CO-751,871, and speculated the inhibition of DNA repair response as one of the main factors behind the observed radiosensitivity (Iwasa et al., 2009). Thus, it emerges that studies investigating the radiosensitivity of small molecules with other mechanisms have also implicated the hampering of DNA damage response, either directly or indirectly as the underlying mechanism.

With the mechanistic insight behind impairment of DNA damage response in case of telomere dysfunction-induced radiosensitivity being largely elusive, a study of normal human fibroblasts revealed that short telomeres in the late passage cells invoked localised chromatin modifications that hampered the activation of ATM-mediated DNA damage response, thereby giving rise to the observed radiosensitivity (Drissi et al., 2011). Hence, it is possible that the telomere

dysfunction induced by TMPyP4, as observed in Chapter 3, may have led to the radiosensitivity of the TMPyP4-treated cancer cells. Although it remains to be seen if 50 μM TMPyP4 induced telomere dysfunction, it led to effective suppression of telomerase activity (Figure 4.3 E) and potent infliction of DNA damage (Figure 4.5 A & B), much like 100 μM TMPyP4 did (as seen in Chapter 3, Figure 3.4 A) albeit with lesser cytotoxicity. Therefore, it is highly likely these effects of 50 μM TMPyP4 is initiated by telomere dysfunction in the cancer cells assessed.

4.2 TESTING THE IMPACT OF HAMPERING DNA REPAIR UPON TMPyP4 TREATMENT ON THE FATE OF CANCER CELLS

4.2.1 BACKGROUND

As mentioned in chapter 1 (Section 1.3.2.2.5), it is now known that key DDR proteins are involved in telomere maintenance. Recent work suggests differential regulation of DDR pathways following damage at the telomeric region, as opposed to that at interstitial regions of a chromosome (Section 1.3.3). Cancer genomes, despite being prone to a certain level of threat to its stability deploy efficient DDR pathways to the rescue. Moreover, cancer cells tend to be over-reliant on a limited set of DNA repair pathways, owing to the genomic alterations that take place during carcinogenesis (Helleday et al., 2008). Hence, inhibiting key DNA repair proteins in combination with the induction of telomeric instability not only supresses DNA repair response by the cell, but also hampers the activation back up telomere maintenance mechanisms by those proteins. A classic example of such a strategy is the inhibition of PARP-1 (a DNA repair protein implicated in telomere maintenance as well) along with telomere uncapping by RHPS4, a G-quadruplex stabilising ligand *in vitro* and in a xenograft mouse model of cancer (Gomez et al., 2006) (Salvati et al., 2010).

To reiterate, DNA repair is orchestrated by a range of pathways depending on the type of damage, the cell cycle, and the intracellular milieu (Figure 1.4) (Hoeijmakers, 2009). Insults to the DNA range from base adduct formations to base modifications to single and double strand breaks. Although any kind of damage can be mutagenic, double strand breaks are potentially catastrophic, more

often than not, if not repaired meticulously. The commonest inducer of direct DSBs is IR of high linear energy transfer (LET). Double strand breaks can be a result of direct break at both strands of DNA at a particular locus, or indirectly by conversion from SSBs, either by replication of a DNA segment with a break or upon conversion of base modifications by DNA-metabolising enzymes (Figure 4.6).

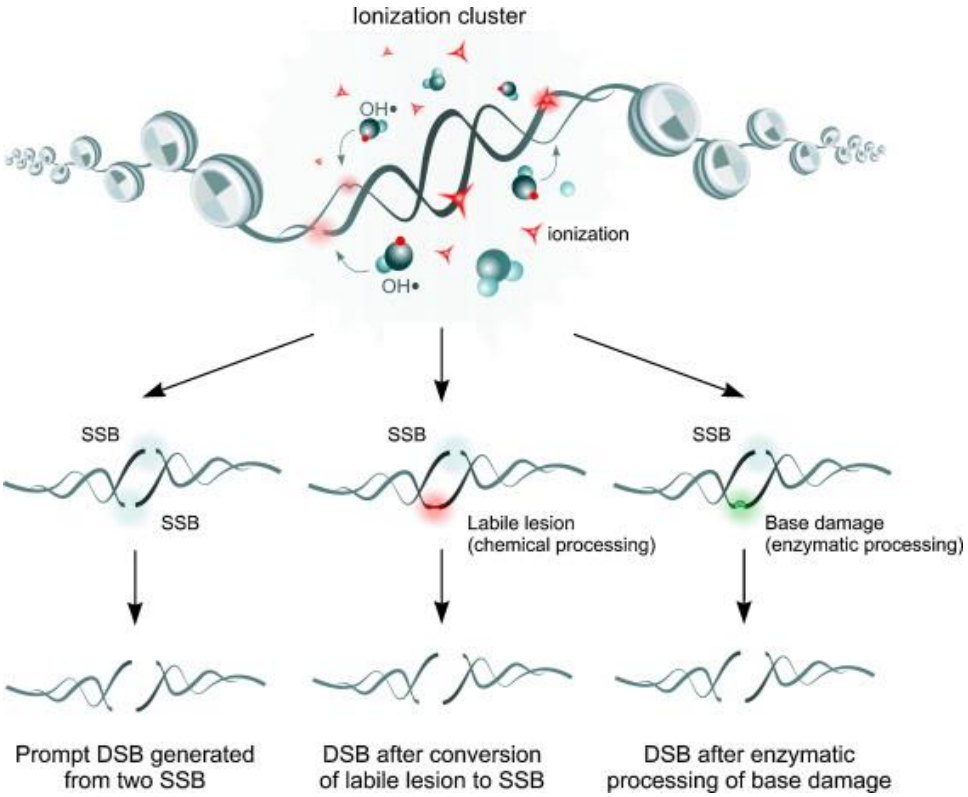


Figure 4.6 **Formation of double-strand breaks (DSBs).** DSBs can form either by direct insults to DNA or by conversion of SSBs (single-strand breaks) to DSBs upon replication, or by conversion of base damages to DSBs by stochastic enzymatic processing.
Image source: (Mladenov and Iliakis, Mutat Res, 2011).

As mentioned earlier in chapter 1, DSB repair is typically executed by two major pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) (Figure 4.7) (Khanna and Jackson, 2001). HR mediates error-free repair of DSBs by using available homologous DNA as template, while NHEJ operates by involving ligases to join the broken segments in an error-prone, homology-independent manner (Hoeijmakers, 2001).

Repair by HR takes place by a sequence of steps, viz. autophosphorylation-based activation of ATM (Ser1981), leading to the phosphorylation and activation of histone protein, γ-H2AX followed by recognition of DSBs and binding to the vicinity of damage, making it conducive binding by DNA processing enzymes and recombination-mediators including the MRN complex, and also checkpoint activators like MDC1 and Chk2 to ensure cell arrest until efficient repai(Hoeijmakers, 2001). Upon recombination-mediated strand fill-in, DNA ligases seal the gaps in the DNA, while resolvases ensure disentanglement of Holliday junctions formed during invasion of homologous strand so as to serve as template for homologous recombination (Hoeijmakers, 2001). The importance of HR in telomere maintenance has been demonstrated in studies involving the inhibition of MRN complex, Rad54 knock-out cells among others, which show telomere trimming and uncapping in these scenarios (Jaco et al., 2003) (Chai et al., 2006). Moreover, the requirement of Rad51 and MRN complex in facilitating telomere recombination-mediated alternative lengthening of telomeres have also been brought to light (Tarsounas, 2013) (Cesare and Reddel, 2010). ATM kinase has been previously implicated in positive regulation of telomere length by

affecting the association of TRF1 from telomeric DNA (Wu et al., 2006). Recnt evidence unravels the direct role of ATM in telomere maintenance. ATM was shown to phosphorylate TRF1, thereby coating it for proteosomal degradation, thereby promoting the access of telomerase to telomeres for elongation (McKerlie et al., 2012). ATM is important to efficiently arrest cells displaying telomere dysfunction and attempt to repair the damage so as to preserve genome stability (Thanasoula et al., 2012).

While HR requires the presence of sequence homology between the template and the damaged NDA, NHEJ involves a less cumbersome mode of DSB repair, without the need for a homologous DNA template for repair. NHEJ, though more error-prone, restores genomic integrity nonetheless, and ensures resuming of cell cycling and proliferation (Hoeijmakers, 2001). A few NHEJ pathways have been recently uncovered to take place when the major NHEJ pathway, DNA-PK-dependent NHEJ (D-NHEJ) is unable to be activated. D-NHEJ involves recognition of and binding to DNA breaks by Ku, which facilitates recruitment of DNA-PK, (the major kinase responsible for orchestrating the repair) to the site of damage. DNA-PK, by phosphorylating various proteins, facilitates processing of the damages ends by nuclease Artemis to make it conducive for subsequent sealing of the breaks by DNA ligase, LigIV, and recombination proteins XRCC4/XLF complex (Mladenov and Iliakis, 2011).

A study of the telomeric state of primary mouse embryonic fibroblasts that lacked Ku uncovered the accumulation of telomere end to end fusions, and revealed the interaction of Ku with the telomere-binding shelterin protein, TRF1 (Hsu et al.,

2000). Another pioneering study on immunodeficient (SCID) mice that exhibited the suppression of DNA-PK activity to a great extent due to a loss-of-function mutation in DNA-PKcs (the catalytic subunit of the enzyme DNA-PK) unveiled unusually long telomeres as opposed to that of normal mice, pointing to the role of DNA-PK in telomere capping (Hande et al., 1999b). These studies along with more others reviewed in detail elsewhere, underscore the role of NHEJ factors in telomere maintenance too (Ayouaz et al., 2008)

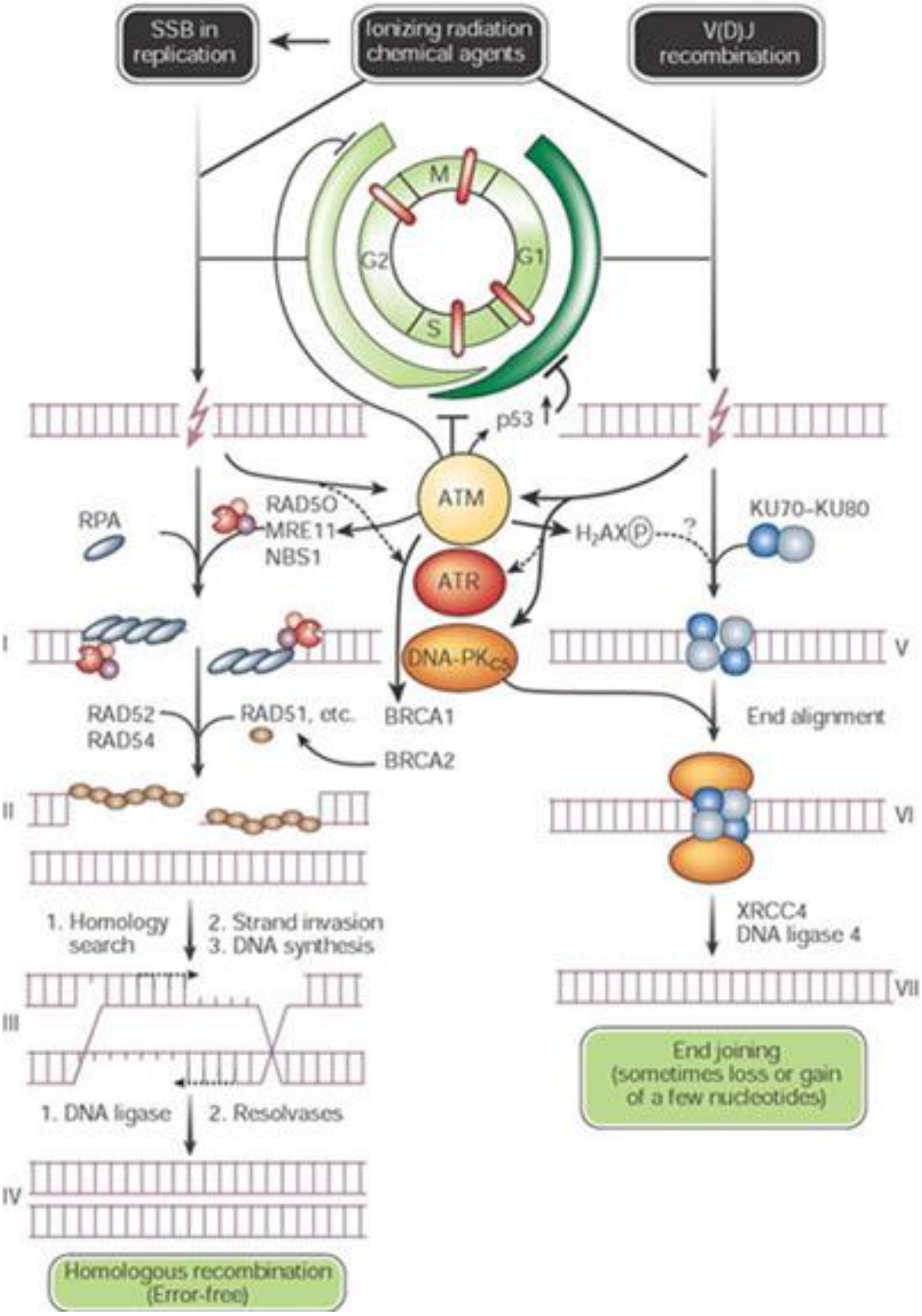


Figure 4.7 **Repair of DSBs in mammalian cells.** DSB repair in cells take place by a meticulous, error-free, homology-dependent pathway - homologous recombination (HR), and an error-prone non-homologous end joining (NHEJ) pathway. The choice of their deployment is dictated by factors such as the phase of cell cycle, availability of repair proteins (especially in cancer cells), and intracellular milieu. Image source: (Hoeijmakers, 2001).

4.2.2 RESULTS

4.2.2.1 Evaluation of the effect of TMPyP4 in glioblastoma cells upon inactivation of ATM kinase

Although ATM activation in ONS76 cells coincided with the observation of efficient DNA repair in the cancer cells following TMPyP4 treatment, it still was not clear if the activation of ATM in the cells led to the DDR (Chapter 3; Figure 3.6 A). To answer this, 3 μM (dose chosen based on preliminary results) a chemical inhibitor Ku60019 that would prevent the activation of ATM, was used during the recovery period following 48 hours of TMPyP4 treatment in the two glioblastoma cells used extensively throughout the course of the entire study, KNS60 and A172 cells. Western blotting showed that ATM was indeed activated in both ONS76 and KNS60 following TMPyP4 treatment, and that, 3 μM Ku600019 in TMPyP4-free medium over 24 hours, following 48 hours of 100 μM TMPyP4, successfully inhibited ATM kinase that was activated upon TMPyP4 treatment to a great extent (Figure 4.8 A). The effect of this unavailability of ATM kinase on DNA repair was assessed by performing alkaline single cell gel electrophoresis (COMET analysis). It showed that while Ku600019 alone and TMPyP4 treatment alone led to an increase in the total DNA damage, the recovery period led to a reduction in the observed DNA damage, corroborating with those obtained earlier in ONS76 cells. However, ATM inhibition (Ku600019 treatment) during the recovery period caused a staggering increase in the observed total DNA damage (Figure 4.8 B). This not only showed that ATM kinase is the

main orchestrator of TMPyP4-induced DNA damage in the cancer cells, but also that the cancer cells had been actively involved in repair process during TMPyP4 treatment itself.

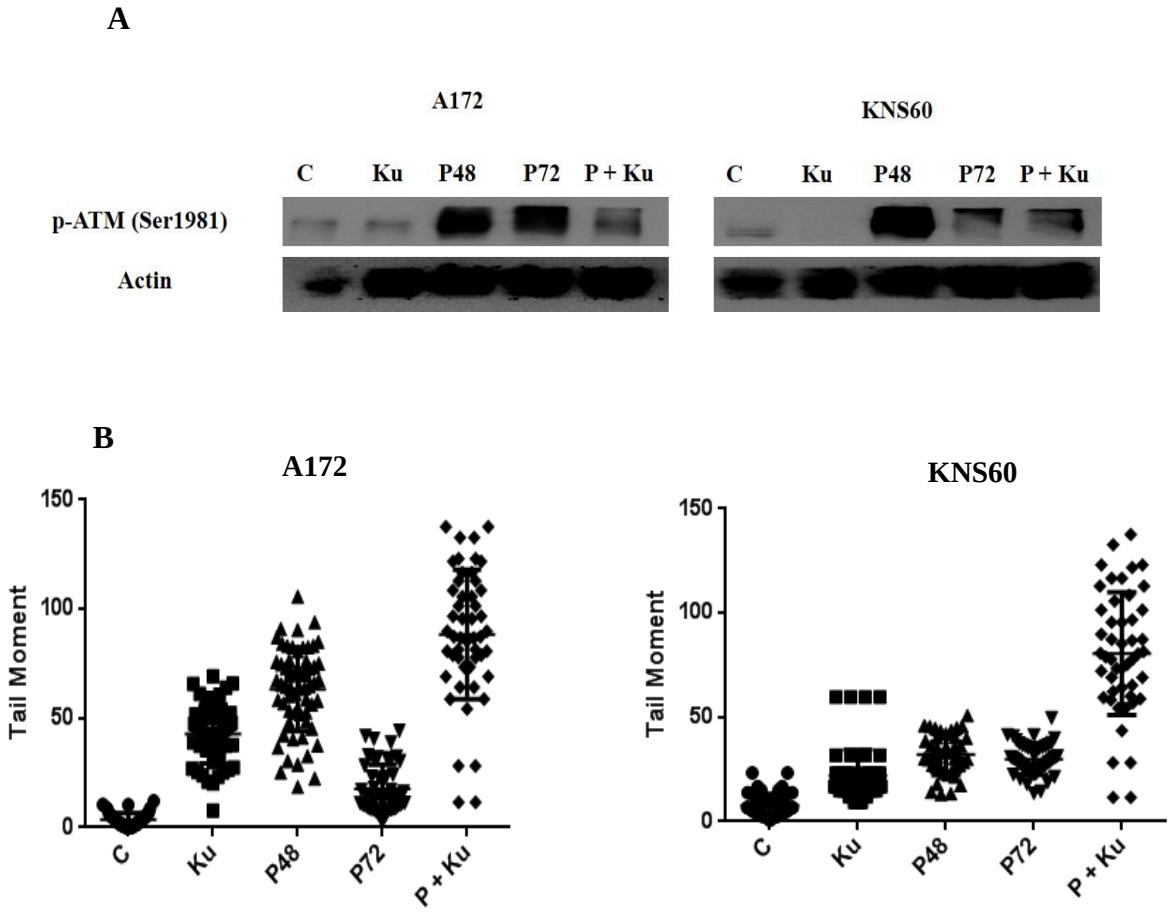


Figure 4.8 **ATM-mediated repair of DNA damage induced by TMPyP4.** A. Western blot analysis of activated ATM kinase (p-ATM) B. Dot-scatter of the intensity of DNA damage (tail moment in µm) by alkaline COMET; each cell represented as a dot. Mean and S.E.M. of at least 50 cells represented as dashes. Legend: C- Control; Ku- 3 µM Ku60019 for 24 hours; P48- 100 µM TMPyP4 for 48 hours; P72- 100 µM TMPyP4 for 48 hours followed by no treatment for 24 hours; P+Ku-100 µM TMPyP4 for 48 hours, followed by 3 µM Ku60019 for 24 hours in fresh media. At least 50 cells were analysed for each category.

4.2.2.2 Evaluation of the effect of TMPyP4 in glioblastoma cells upon inactivation of DNA-PKcs

As mentioned in the introduction to DNA repair, earlier in section 4.2.1, the repair of DSBs are essential for cell survival. Clearly, given that the inhibition of ATM kinase potentiated the DNA damage-mediated cell death of glioblastoma cells, the inhibition of the NHEJ orchestrator – DNA-PKcs in combination with TMPyP4 treatment in glioblastoma cells is a worthwhile strategy. Owing to the availability of two glioma cell lines derived from the same patient, one with DNA-PK deficiency attributed to spontaneous point mutations (M059J) and the other with normally functional DNA-PK (M059K), they were the models of this investigation.

A small molecule inhibitor of DNA-PKcs (the catalytic subunit of DNA-PK holoenzyme), NU7026 was used in studying the effects of abolition of NHEJ in M059K cells. The dose of NU7026, 10 μM was chosen by pre-determined studies in the laboratory (Data not shown). Cells were also allowed 24 hours after treatment to repair the inflicted DNA damage by the treatment modalities. M059K cells showed remarkable ability to repair the damage induced by 48 hours of 100 μM TMPyP4 treatment. Strikingly, upon NU7026 pre-treatment, the cells displayed an evident inability to repair the damage (Figure 4.9 A). This profile was mimicked by M059J cells (that inherently lack DNA-PKcs) upon 48 hours of TMPyP4 treatment followed by recovery in TMPyP4-free media (Figure 4.9 B).

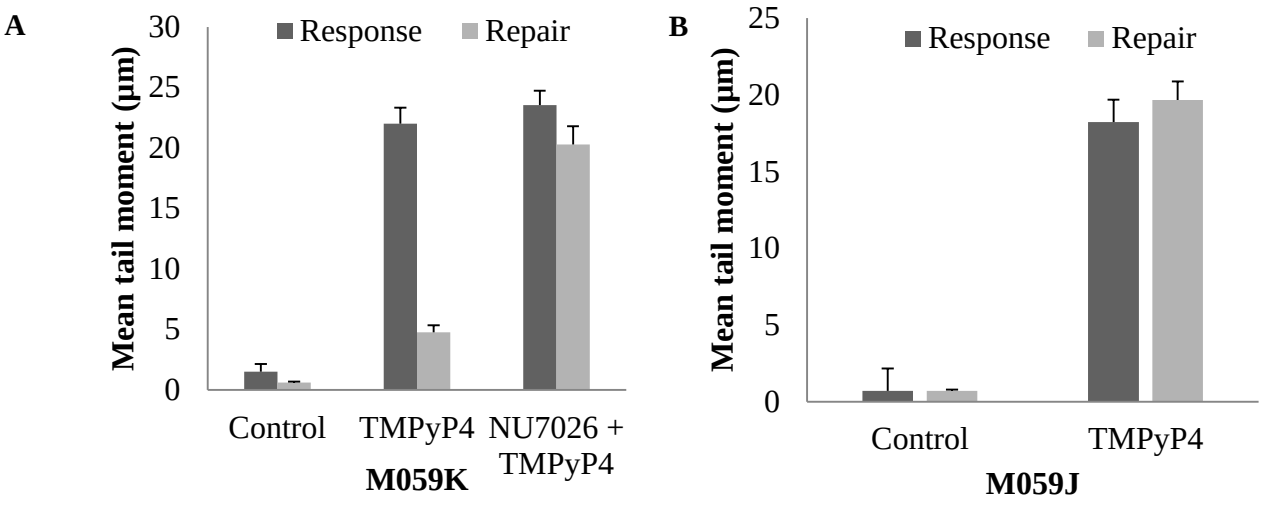


Figure 4.9 **Effect of combination of TMPyP4 treatment and DNA-PKcs inhibition on the intensity of DNA damage in the cancer cells.** Alkaline single-cell gel electrophoresis of **A.** M059K cells upon DNA-PKcs inhibition and TMPyP4 treatment and **B.** M059J cells upon TMPyP4 treatment. Legend: 'Response' means treatment for 48 hours; 'Repair' means incubation for a further 24 hours in media devoid of any treatment agents, following the 'Response' period. 'TMPyP4' means treatment with 100 µM TMPyP4; 'NU7026 + TMPyP4' means pre-treatment with 10 µM NU7026 for 2 hours, followed by incubation along with 100 µM TMPyP4 for a further 48 hours. At least 50 cells were analysed for each category.

The effect of absence of DNA-PKcs upon TMPyP4 treatment on double-strand break repair was assessed by immunofluorescent visualisation of γ-H2AX foci, the regions of DSB damage signalling. Snapshots of representative images show the persistence of the foci even after the 24 hour response period in M059K (NU7026 + TMPyP4 repair) and M059J (TMPyP4 repair) as opposed to that without DNA-PK inhibition in M059K cells (TMPyP4 repair) (Figure 4.10).

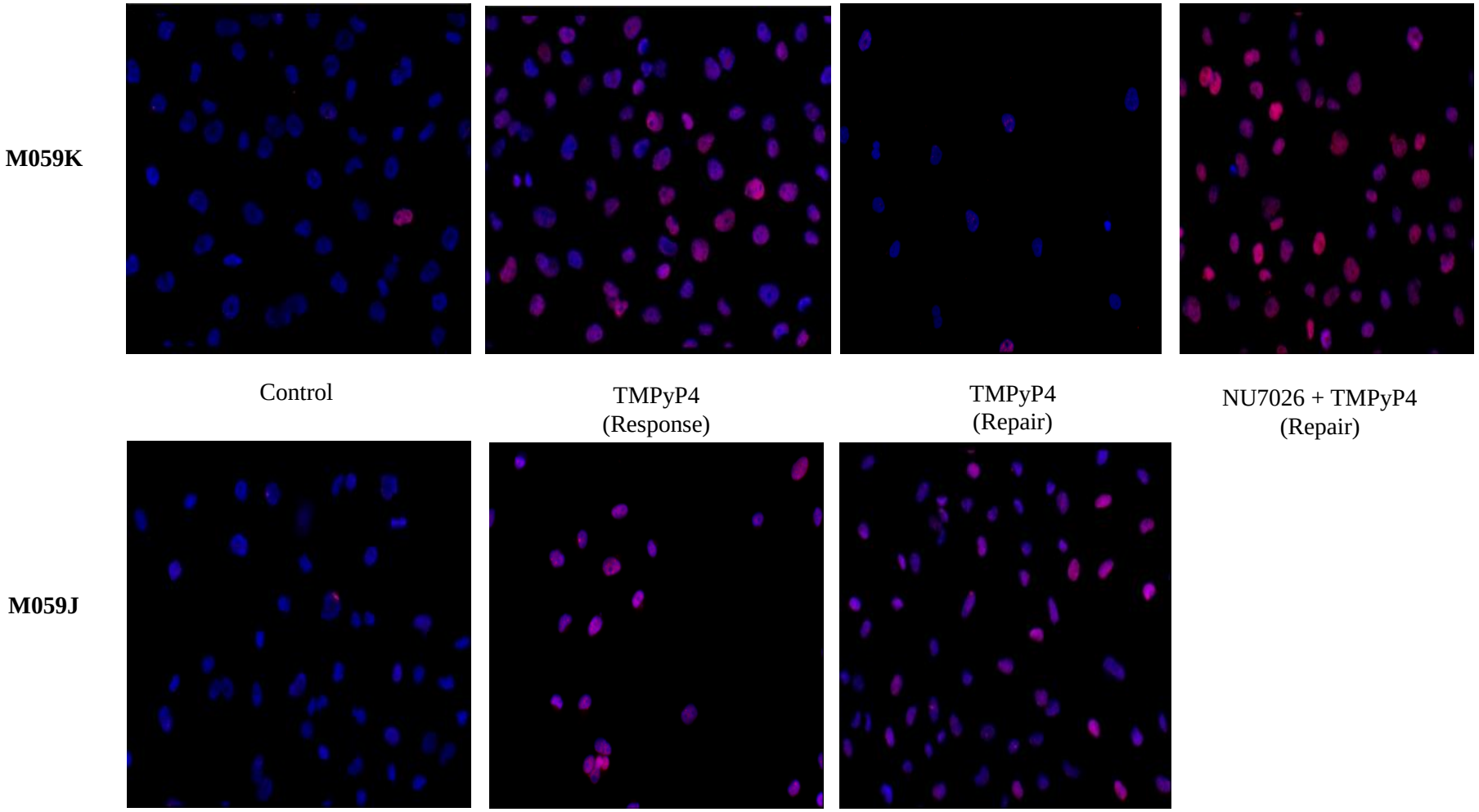


Figure 4.10 **Effect of TMPyP4 treatment following DNA-PKcs deficiency on double-strand break repair in glioblastoma cells.** Semi-quantitative analysis of γ-H2AX foci by immunofluorescence staining of γ-H2AX upon TMPyP4 and NU7026 treatment in M059K cells; and just TMPyP4 in M059J cells. Legend: 'Response' means treatment for 48 hours; 'Repair' means incubation for a further 24 hours in media devoid of any treatment agents, following the 'Response' period. 'TMPyP4' means treatment with 100 µM TMPyP4; 'NU7026 + TMPyP4' means pre-treatment with 10 µM NU7026 for 2 hours, followed by incubation along with 100 µM TMPyP4 for a further 48 hours. DAPI – nuclear staining; TR (Texas Red) – γ-H2AX staining. Images captured using MetaSystems isis software at 200X magnification.

Cell cycle arrest, as we have seen earlier in this study, has been a natural consequence of persistent DNA damage response. Both, TMPyP4 alone and in combination with NU7026 induced a G2/M arrest in M059K cells, with the latter treatment inducing a slightly more robust arrest ($p < 0.05$, ANOVA) (Figure 4.11). Thus, DNA-PKcs absence proved fatal to the glioblastoma cells treated with TMPyP4 too.

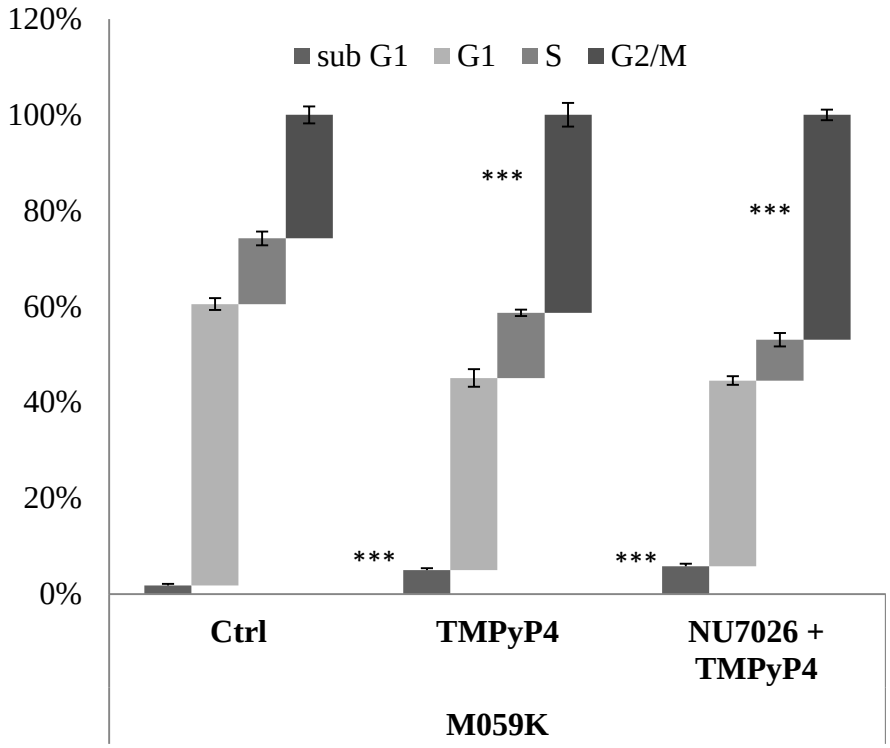


Figure 4.11 **DNA-PKcs inhibition, TMPyP4 treatment and cell arrest.** Cell cycle profile of TMPyP4-treated M059K cells upon DNA-PKcs inhibition was assessed using flow cytometry of a population of at least 10,000 cells. The experiment was repeated with three independent repeats of cells. Legend: 'TMPyP4' means treatment with 100 µM TMPyP4; 'NU7026 + TMPyP4' means pre-treatment with 10 µM NU7026 for 2 hours, followed by incubation along with 100 µM TMPyP4 for a further 48 hours. Single factor ANOVA was performed to assess statistical significance.

4.2.3 DISCUSSION

The strategy of inhibiting DNA repair-orchestrating enzymes along with the induction of telomere-mediated genomic instability has been made use of in a study with another promising G-quadruplex interacting ligand, RHPS4 (Salvati et al., 2009). RHPS4 induced telomere dysfunction *in vivo* and PARP inhibition led to the irreparability of the telomere uncapping effect of RHPS4 (Salvati et al., 2010). Although RHPS4 was shown to activate an ATR-dependent ATM pathway of cell arrest following telomeric replication stress, the possibility of potentiation of RHPS4 upon ATM inhibition was not explored (Salvati et al., 2010). The development of highly specific inhibitors to PARP and DNA-PKcs for the clinic envisages more studies using DNA repair perturbation as combination to the primary treatment (Riabinska et al., 2013); (Tinoco et al., 2013). ATM was shown to be essential in repairing the telomeric insult by a G-quadruplex ligand, 360A, in a model of normal and AT (lacking ATM) lymphocyte cell lines (Pennarun et al., 2008)

Since the cell has evolved only two major DSB repair pathways, it should follow that a cell that is defective in one of the two canonical DSB repair pathways – HR or NHEJ, must be proficient enough in repairing spontaneous DSBs by the other pathway in order to survive. While studies on the mechanism of most small molecules, including G-quadruplex ligands implicate one major DNA repair pathway, a recent study on 360A implicated the role of both – ATM kinase and DNA-PK in repairing the telomeric damage induced by 360A in a panel of human cancer cell lines (Gauthier et al., 2012). This is not surprising, given that the two

major pathways of DSB repair are interlinked, that too in response to telomeric damage. Although an early, pioneering study on the delineation of the role of DNA-PK at mammalian telomeres suggested that ATM and DNA-PK pathways operate through separate pathways following telomeric damage, this view has been overturned by recent work (Bailey and Murnane, 2006). Recent studies have shown that ATM kinase exercises greater control over DDRs corresponding to the telomeres, than that over interstitial DNA in human cells (Miller et al., 2011). A very recent study in human clinical samples has concluded just that – that disabling ATM mutations occur in a fraction of human lung and haematological malignancies among others, and that although these cancers seem to be refractory to conventional chemotherapeutics, they appear to be over-reliant on DNA-PKcs mediated DDR (Riabinska et al., 2013). All this put together brings out the picture of inter-dependence of HR and NHEJ pathways; and even more so in response to telomere dysfunction.

In this study, combining either ATM inhibition or DNA-PK inhibition, with TMPyP4 treatment showed glimpses of promise as a combination modality for the efficient reduction of tumour mass and prevention of recurrences. Similar to the radiosensitisation study in Section 4.1, this study also seems to underscore the importance of inducing telomere dysfunction, leading to persistence of DNA damage signalling, and eventually to weakened cellular defences.

Chapter 5

Chronic low dose study of TMPyP4

5. CHRONIC LOW DOSE STUDY OF TMPyP4

5.1. BACKGROUND

It is customary for telomere-telomerase targeting agents to be evaluated over long-term, as telomere shortening takes a few population doublings before eliciting an apparent effect on cell physiology. Also, long-term treatment over a few weeks is more clinically relevant, given that it takes some time for the treatment to take effect on the heterogeneous cell populations within a tumour.

Some G-quadruplex ligands that have entered clinical trials in the past have exhibited telling effects on telomere maintenance over long-term treatment in various cancer models including xenograft models (Burger et al., 2005; Kim et al., 2002; Leonetti et al., 2004). TMPyP4 has shown promise as a telomere-specific agent that could induce telomere dysfunction and DNA damage over short-term treatment, as seen in chapter 3. In order to evaluate the potential for TMPyP4 to become a cancer drug, it is essential to investigate the effects of TMPyP4 on telomere maintenance over a few weeks of treatment in glioblastoma cells owing to the lack of studies on the same. Hence, human-derived glioblastoma multiforme cell types, KNS60 and A172 will be the model of this study.

5.2 RESULTS

5.2.1 Dose response with long-term treatment with TMPyP4

Various concentrations of TMPyP4 (1-10 µM) were tried out on the cancer cells KNS60 and A172, over long term in an attempt to use a therapeutically relevant dose-time regime using chronic, low doses. While 1 µM was ineffective in causing massive cell death at least until eight weeks of treatment, the rest of the doses eliminated the cancer cells within six weeks, with 4, 5 and 10 µM being potentially cytotoxic, all inflicting massive cell death within two weeks.

TMPyP4 (µM)	**Cell survival (weeks)**	
	KNS60	**A172**
10	1	1
5	2	2
4	2	2
3	4	3
2	6	5
1	>9	8

Table 2:Survival of cancer cells in long-term chronic doses of TMPyP4

Since TMPyP4 inflicted accelerated death at higher concentrations, their effect on cyclins and hence on cell cycle arrest was evaluated for G1/S checkpoint activation by assessing the levels of cyclin D1 (which gets diminished upon G1 arrest) in A172 cells using western blotting after treatment with 1, 5 and 10 µM TMPyP4 over a few weeks or until complete cell death was achieved. It was observed that 5 and 10 µM TMPyP4 significantly reduced cyclin D1 and hence prominently arrested cell proliferation after just 1 week of treatment, while 1 µM

TMPyP4 only mildly reduced cyclin D1 levels and largely permitted cell proliferation, at least in the initial few weeks (Figure 5.1). This corroborated with the observation of cell death in A172 cells over a range of doses of TMPyP4 (Table 5.1). While the effect of this treatment of TMPyP4 on inducing arrest in KNS60 cells was not evaluated, the gradual reduction in the viability of these cells over weeks of treatment, similar to that of A172 cells point out to the possibility of the same. Subsequently, lower doses viz. 1, 2 and 3 µM of TMpyP4 were used to potentially hamper telomere maintenance with minimal toxicity, so as to affect the cancer cells largely.

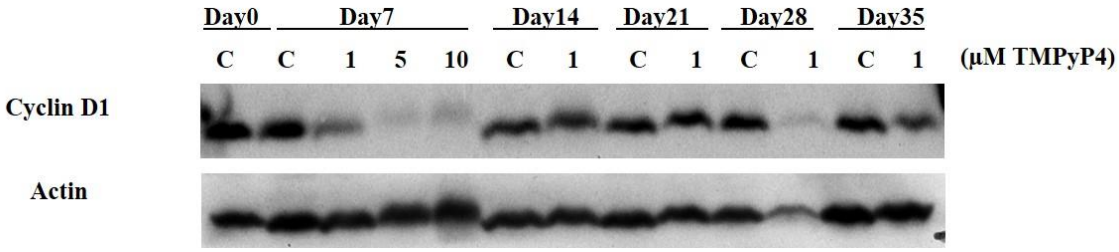


Figure 5.1 **Effect of long-term chronic doses of TMPyP4 on cell proliferation** Cyclin D1 levels were obtained by western blotting after the various dose-time treatments with TMPyP4 in A172, as a measure of cell cycle progression.

Cells were counted using Trypan Blue dye exclusion at the end of every week before seeding for the subsequent week. A detailed population doubling analysis of 1 and 2 µM TMPyP4 in A172 cells at the end of every week of treatment showed that TMPyP4 reduced the viability of the cells in a dose- and time-dependent manner (Figure 5.2).

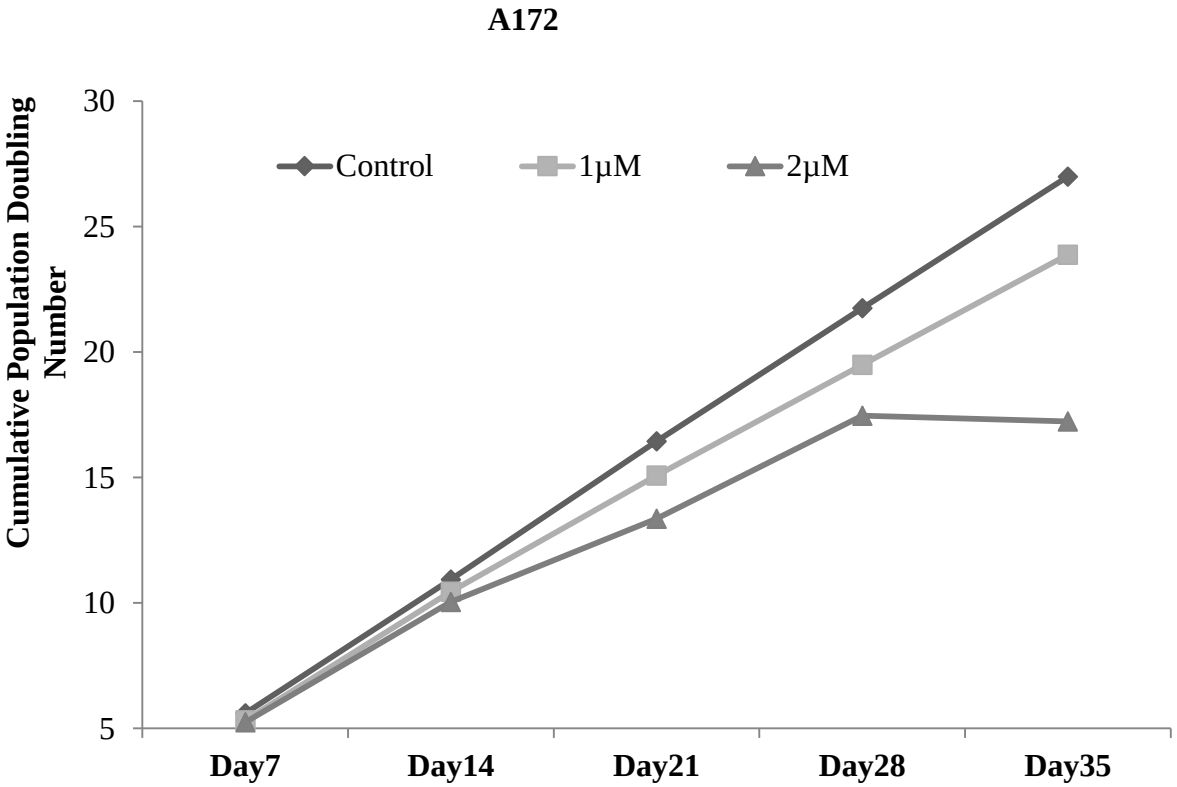


Figure 5.2 **TMPyP4 and long-term doubling of cancer cells** Population doubling analysis of A172 cells with 1&2 µM TMPyP4 over 5 weeks of treatment.

5.2.2 Deregulation of telomere-associated proteins by TMPyP4

TMPyP4 showed prominent telomere-specific binding as seen by its immediate effect on TRF2 levels as shown earlier (Figure 3.5). Hence, the effect of long term treatment with TMPyP4 on telomere maintenance was assessed by probing for the most important shelterin protein, TRF2 and the catalytic subunit of telomerase, hTERT in KNS60 and A172 cells. TMPyP4 has been noted to exhibit drastic effects on telomere maintenance at high doses as seen after 48 hours of treatment with 100 µM TMPyP4 on KNS60 and ONS76 cells (Figure 3.4). Not surprisingly, the higher doses 5 and 10 µM exerted a strong reduction on the associated proteins at the telomeres, following treatment for one week, in the case of A172

cells and for two weeks, in the case of KNS60 cells (Figure 5.3 A). While, treatment with low dose of 1 µM TMPyP4 led to a slight reduction in hTERT levels in the early weeks and a pronounced reduction in the later weeks, TRF2 levels reduced drastically just before the onset of massive cell death, in both KNS60 and A172 (Figure 5.3 A). To see if this is the case with other shelterin proteins too and other low-range doses of TMPyP4 too, the levels of another telomeric dsDNA binding protein, TRF1 was assessed after treatment with 1 and 3 µM TMPyP4 in KNS60 and A172 cells for up to 3 weeks which was when massive cell death was induced by 3 µM TMPyP4 (Table 5.1). While TRF1 levels remained largely unaffected by 1 µM TMPyP4 within the three-week time period except in the case of KNS60 cells treated for three weeks, 3 µM led to plummeting of TRF1 levels in two weeks in A172 cells and three weeks in KNS60 cells (Figure 5.3 B). To reiterate, A172 cells were eliminated within three weeks; and KNS60 cells in four weeks upon treatment with 3 µM TMPyP4 (Table 5.1). As shown in chapter 3 (Figure 3.5), TMPyP4 treatment could lead to a staggering reduction in the levels of shelterin proteins, almost immediately after treatment. Taking this together with the observation of reduction in TRF1 and TRF2 levels upon long term treatment with TMPyP4, it is convincing that telomere uncapping is the *modus operandi* of TMPyP4.

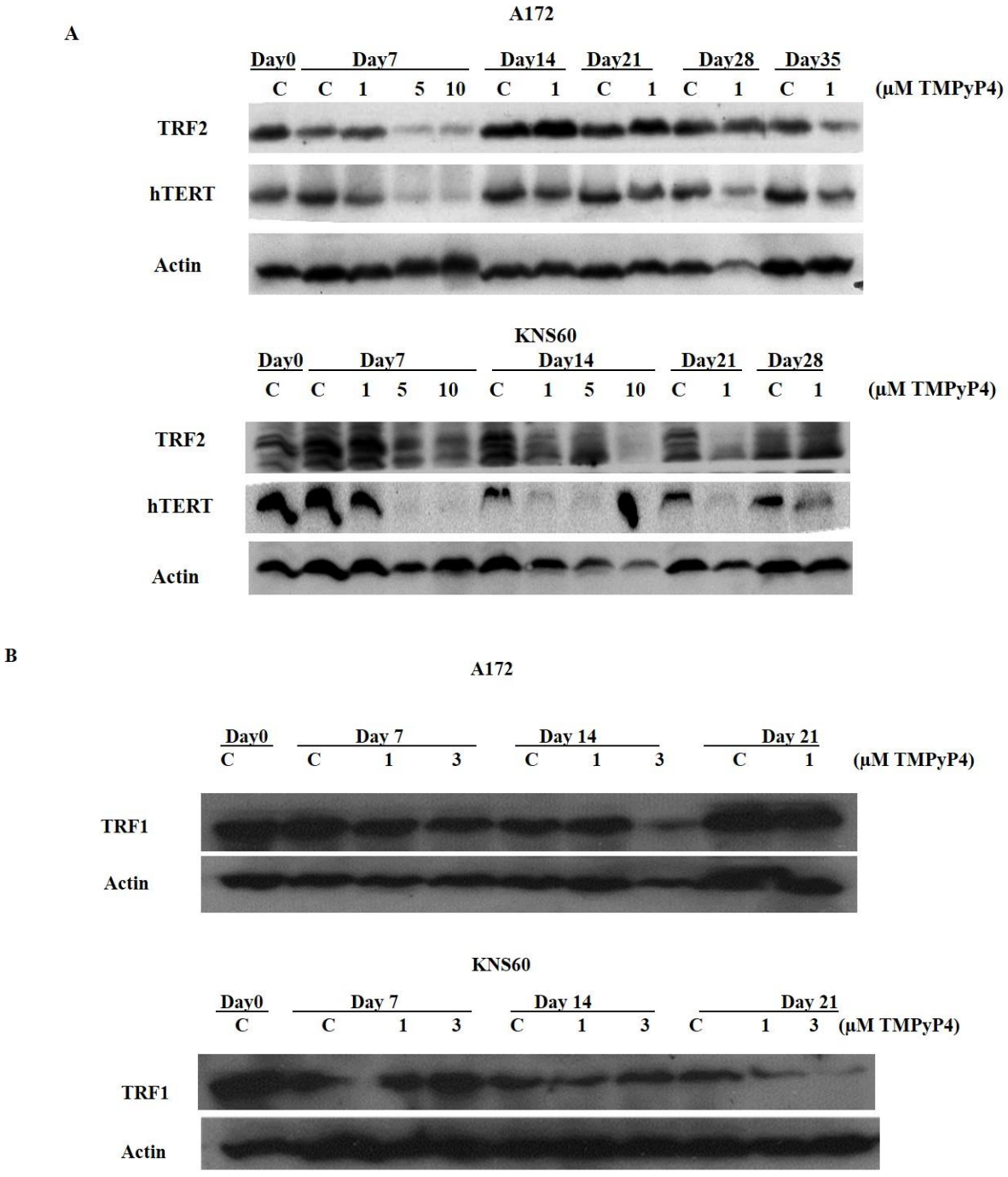


Figure 5.3 **Deregulation of telomeric proteins by chronic treatment with low doses of TMPyP4.** Effect of A. 1, 5, and 10 μM TMPyP4 on TRF2 and hTERT B. 1 and 3 μM TMPyP4 on TRF1, as assessed by western blotting of whole cell proteins.

5.2.3. Telomere shortening caused by TMPyP4

TMPyP4 treatment as observed in the earlier section (5.2.2) led to substantial reduction in the levels of TRF1, TRF2 and hTERT, at least in the later weeks, before massive cell death. As hTERT, the catalytic subunit of telomerase, is necessary for telomerase activity; and shelterin integrity for telomerase recruitment to the telomeres, TMPyP4 may induce telomere shortening. Accordingly, KNS60 and A172 cells were treated with 1, 2, 5, and 10 μM of TMPyP4 until massive cell death was observed, and their telomere lengths measured using Telomere Restriction Fragment analysis (TRF) following southern blotting of the DNA digested using pan-restriction endonucleases.

Corroborating with the earlier observations on the effect of TMPyP4 on TRF2 and hTERT, the higher doses, 5 and 10 μM caused appreciable reduction in mean telomere length by a few hundred base pairs within a week of treatment in KNS60 cells, a week before massive cell death was observed (Figure 5.4 B). The same doses effected massive cell death in a week in A172 cells and hence, telomere length measurement was not attempted within that time period.

The lower and less toxic doses displayed an interesting profile. On the one hand, low dose of 1 μM TMPyP4 did not induce any significant telomere shortening in A172 cells (Figure 5.4 A), as observed until eight weeks of treatment and a mild shortening of a few hundred base pairs reduction in KNS60 in the sixth week of treatment (Figure 5.4 B). On the other hand, while a dose of 2 μM TMPyP4 did not affect telomere length for the first two weeks of treatment, it led to shortening of a few hundred base pairs after three weeks; and a staggering profile of

unprecedented increase in the mean telomere length by as much as seven times, and a homogenous smear, characteristic of a very wide range of telomere lengths, suggestive of acute changes to telomere architecture at that time-point (Figure 5.4 C). Remarkably, massive cell death ensued in the fifth week, resulting in a wipe-out of the entire cell population (Table 5.1). Thus, piecing information from telomere-associated protein levels and telomere length analyses, the induction of telomere dysfunction by TMPyP4 is highly likely.

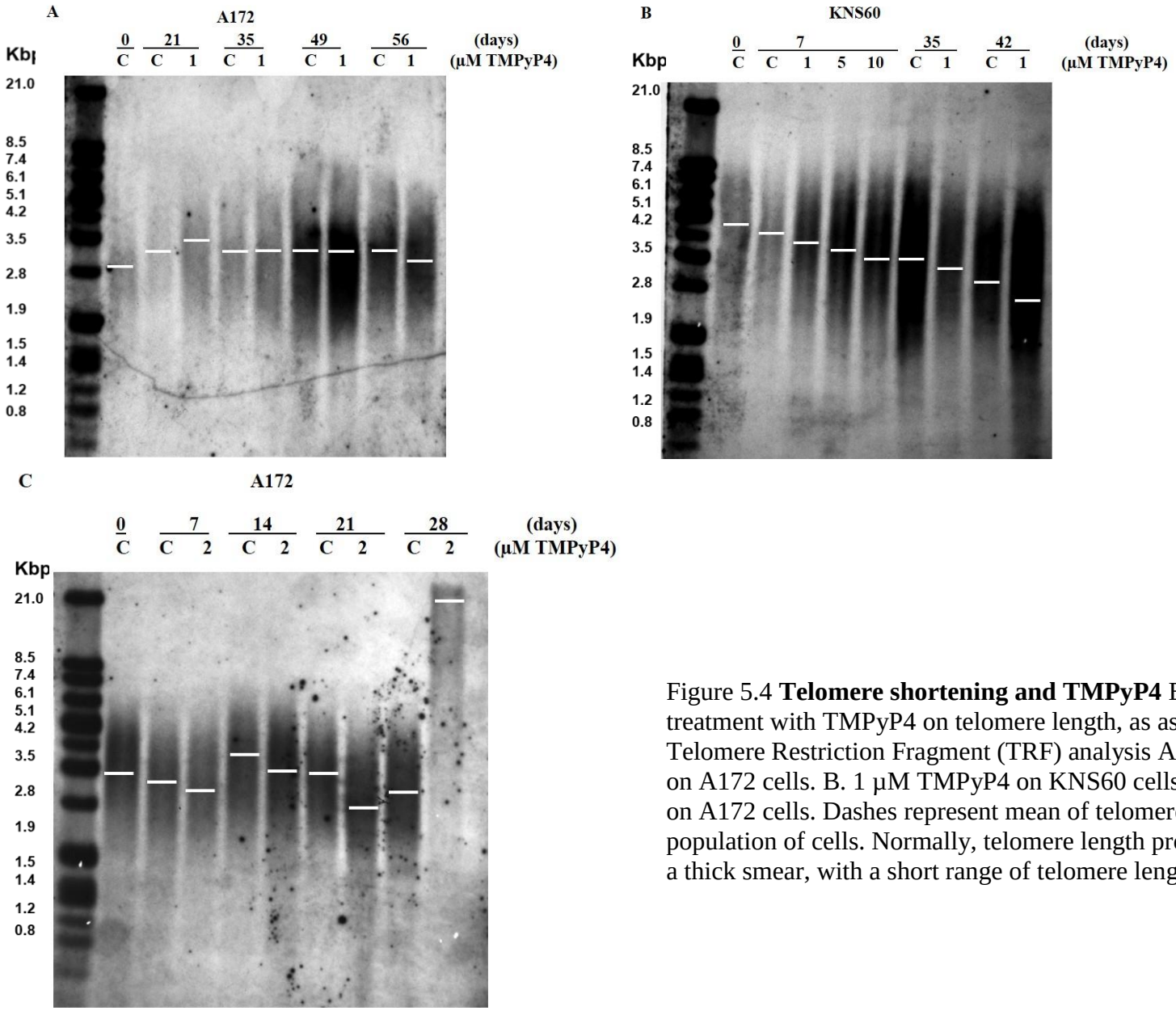


Figure 5.4 **Telomere shortening and TMPyP4** Effect of long term treatment with TMPyP4 on telomere length, as assessed by Telomere Restriction Fragment (TRF) analysis A. 1 μM TMPyP4 on A172 cells. B. 1 μM TMPyP4 on KNS60 cells C. 2 μM TMPyP4 on A172 cells. Dashes represent mean of telomere lengths of a population of cells. Normally, telomere length profiles of cells bear a thick smear, with a short range of telomere lengths.

5.2.4. Chromosome instability (CIN) induced by TMPyP4

CIN is the major contributor of genomic instability, and cancer cells that already have shorter telomeres and deregulated cellular checkpoints, have a certain level of persisting genomic instability and are highly prone to triggers of telomere dysfunction. To check the extent of CIN upon TMPyP4 treatment, KNS60 and A172 cells were subjected to 1 µM TMPyP4 for a few weeks until massive cell death, and cells harvested at the end of every week and analysed for telomere and chromosomal aberrations using telomere-FISH, that makes use of fluorescently labelled peptide nucleic acid probes against telomeres and centromeres, along with whole chromosome staining using DAPI. Telomere dysfunction was indeed induced by TMPyP4 treatment, as evidenced by the observation that missing telomere signals were more frequent in TMPyP4-treated cells than in untreated controls; and dicentric chromosomes also increased with treatment, progressively over weeks, in both – KNS60 and A172 cells (Table 5.2). It is noteworthy that while treatment with 1 µM TMPyP4 resulted in an increase in missing telomere signals and dicentric chromosomes to an extent, it still was incapable of inducing genomic instability *en masse* as evidenced by the lack of spontaneous chromatid breaks and DNA fragments upon treatment (Table 5.2). Figure 5.5 shows the various aberrations found in the treated cells. This is understandable, given that 1 µM TMPyP4 was not good enough to induce significant telomere shortening, nor drastic telomere uncapping as seen in the results of earlier experiments. Nonetheless, these results show that TMPyP4 acts by uncapping of telomeres, telomere shortening and telomere dysfunction.

Cell Type	*Duration*	*Condition*	*No. of cells*	*Signal Free Ends*	*DNA Fragments*	*Dicentric & Tricentric fusions*	*Chromatid Breaks*
A172	Day7	Control	17	24 **(141.1)**	0 **(0.00)**	1 **(5.88)**	0
		1 µM	6	34 **(570.0)**	1 **(16.67)**	2 **(33.33)**	0
	Day 28	Control	18	78 **(433.0)**	0 **(0.00)**	8 **(44.44)**	1
		1 µM	11	110 **(1000.0)**	1 **(9.09)**	14 **(127.27)**	0
	Day 49	Control	18	112 **(620.0)**	0 **(0.00)**	17 **(94.44)**	1
		1µM	12	128 **(1070.0)**	1 **(8.33)**	20 **(166.67)**	1
KNS60	Day 14	Control	20	59 **(295.0)**	6 **(30.00)**	1 **(5.00)**	0
		1 µM	20	143 **(715.0)**	7 **(35.00)**	3 **(15.00)**	3
	Day 28	Control	15	47 **(313.0)**	10 **(66.67)**	0 **(0.00)**	1
		1 µM	20	150 **(750.0)**	11 **(55.00)**	2 **(10.00)**	1
	Day 42	Control	5	23 **(460.0)**	3 **(60.00)**	1 **(20.00)**	0
		1 µM	16	147 **(920.0)**	3 **(18.75)**	6 **(37.50)**	0

Table 3 Chromosomal instability induced by TMPyP4 Telomere-FISH was used to evaluate chromosomal instability in KNS60 and A172 cells upon treatment with 1 µM TMPyP4 for up to seven weeks. Number of aberrations per 100 cells is given within parenthesis and in bold. Two-factor ANOVA (without replication) for signal free ends: in A172 between control and treated of days 28 and 49 with $p < 0.05$; in KNS60 between control and treated of days 14 and 28 with $p < 0.01$. Note: Day 7 in A172 and Day 42 in KNS60 were omitted from statistical analysis due to the lack of a sizeable number of cells analysed.

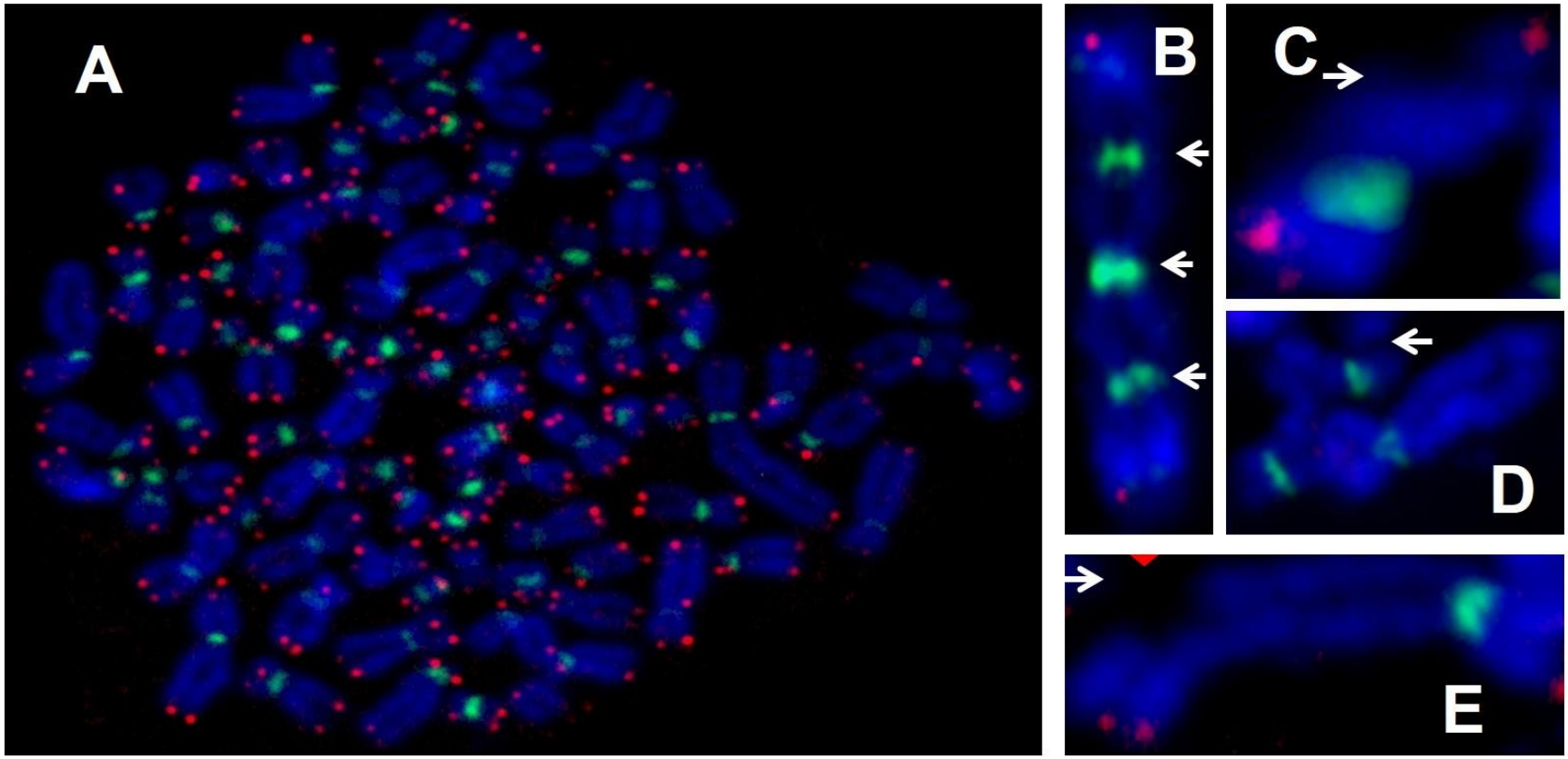


Figure 5.5 **Chromosome instability induced by TMPyP4.** Telomere PNA-FISH was used to assess chromosome instability by detecting telomere dysfunction and chromosomal aberrations; telomeres were stained by a Cy3-Telomere PNA probe (red); centromere by FITC-Centromere PNA probe (green); and DNA counter-stained by DAPI (blue). Representative images of: A. a typical metaphase from untreated A172 cells; B. a tricentric chromosome (presumably fused due to loss of telomeres); C. a terminal deletion (accompanied by loss of telomeres); D. a triradial-like structure (complex fusion); E. a chromatid break. All aberrations are using white arrows.

5.2.5. DNA damage inflicted by TMPyP4

To summarise, long term treatment of KNS60 and A172 cells with 2 μM or more of TMPyP4 induced telomere uncapping, telomere dysfunction, genome instability, cell cycle arrest, and cell death as seen earlier in this chapter. Hence, it is highly likely the observed genomic instability upon TMPyP4 treatment could be due to triggering massive DNA damage, including that at the telomeres. To test this notion, alkaline single cell gel electrophoresis was resorted to, upon 1 and 2 μM TMPyP4 treatment in A172 cells, for up to five weeks when massive cell death was occurred. Analysis of the tail moment of nuclei subjected to electrophoresis in alkaline buffer, a measure of the total DNA damage, shows an obvious DNA damaging effect of TMPyP4 which increases with dose (Figure 5.6).

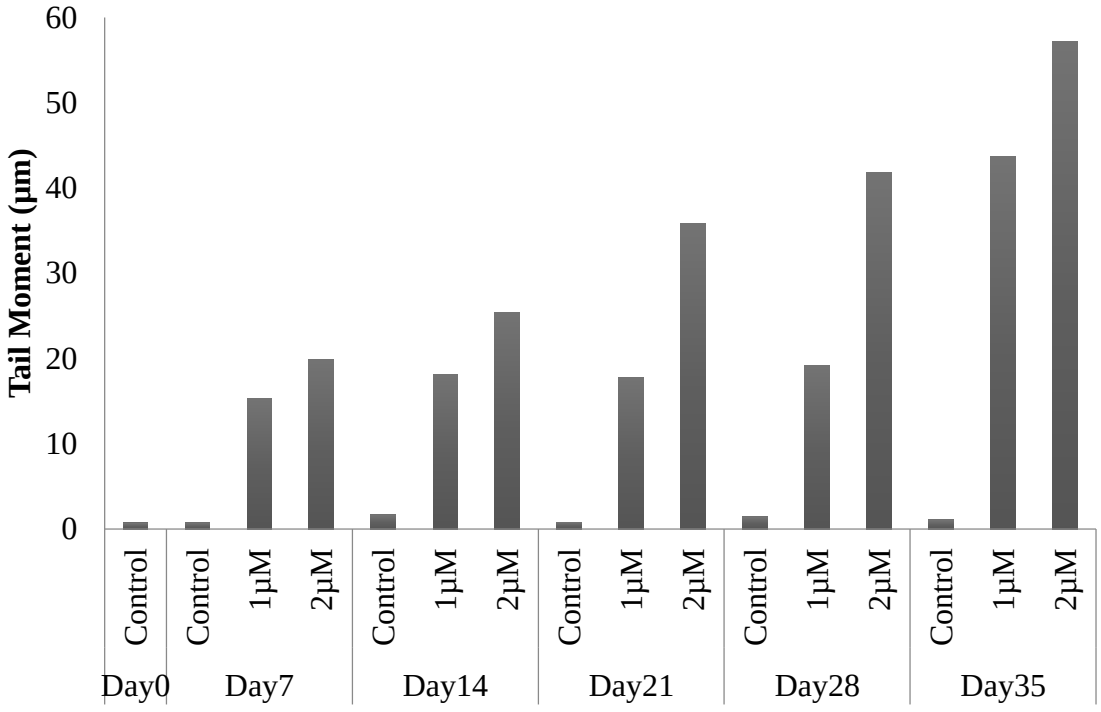


Figure 5.6 **DNA damage induced by TMPyP4.** Total DNA damage inflicted by TMPyP4 in A172 cells was evaluated by subjecting nuclei to electrophoresis in alkaline conditions and staining the nuclei using SYBR Green, followed by measurement of tail moment, i.e. the length of the non-integral DNA as an indicator of the intensity of damage.

It is noteworthy that the cells (A172) undergo massive cell death after five weeks of treatment with 2 µM TMPyP4. It was earlier seen that, upon treatment with 100 µM TMPyP4 over short term, ATM kinase gets efficiently activated in ONS76 cells (Figures 3.6A and 3.7). To check if the same is the case with long term treatment as well, the whole cell levels of phosphrylated ATM (Ser1981) (that constitutes active ATM kinase) was probed and analysed using western blotting. It revealed that, indeed, there is progressive activation of ATM kinase over three weeks of treatment with 2 µM TMPyP4 in A172 cells (Figure 5.7).

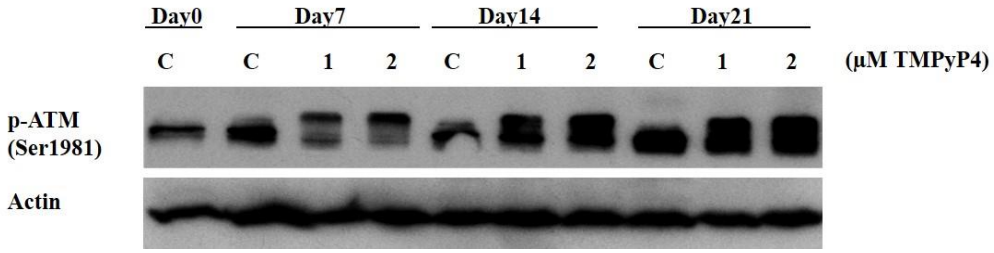


Figure 5.7 **Activation of DDR upon TMPyP4 treatment.** Western blotting was employed to assess the activation of ATM kinase (p-ATM Ser1981) upon 1 and 2 µM TMPyP4 treatment for up to three weeks on A172 cells.

5.3. DISCUSSION

The higher doses, 5 and 10 µM of TMPyP4 induced extensive telomere uncapping and telomere shortening in one week of treatment (Figures 5.3 A and 5.4 B respectively). However, as seen by the thorough depletion of cyclin D1 levels (Figure 5.1) and rapid cell death *en masse* in a week of treatment (Table 5.1), it is highly likely that those doses work by inducing extensive genomic DNA damage, not restricted to the telomeres alone. A low dose of 1 µM TMPyP4, though not cytotoxic like the higher doses, was not effective either in inducing telomere dysfunction and hence, cell death in the cancer cells (Tables 5.2 and 5.1 respectively). Though it reduced the proliferation of the cancer cells, it was not effective enough in inducing massive cell death even after eight weeks of treatment in the cancer cells studied. It resulted in an increase in missing telomeric signals, but could not induce chromosomal instability of note. It did not lead to significant telomere shortening, either (Figures 5.4 A and B). A dose of 2 µM TMPyP4 was found to be ideal for long term treatment of A172 cells as it resulted in telomere shortening of a few hundred base pairs after three weeks of treatment and in extensive alteration of telomere architecture after four weeks (Figure 5.4 C). The intensity of total DNA damage was very high too, following four and five weeks of treatment (Figure 5.6). Consequently, it also resulted in the extermination of the entire cell population after five weeks (Table 5.1 and Figure 5.2).

The effect of TMPyP4 on shelterin levels is telling. TMPyP4 causes extensive reduction of TRF1 and TRF2 (the other shelterin proteins were not assessed), hinting at telomere uncapping, at least before massive cell death was observed (Figures 5.3 A and B). The delocalisation of some shelterin proteins from the

telomeres has been demonstrated before, upon treatment with G-quadruplex ligands. Treatment with a chronic low dose of a reputed G-quadruplex ligand, RHPS4, in human transformed fibroblasts and melanoma cells *in vitro*, led to rapid delocalisation of POT1 and TRF2 from the telomeres before the onset of cell death (Salvati et al., 2007). Another study on the effects of treatment of cancer cells with telomestatin (the first G-quadruplex drug to enter clinical trials) revealed the delocalisation of TRF2 as the main mechanism behind the resulting telomere deprotection and the onset of apoptosis (Tahara et al., 2006). Although the levels of TRF1 upon treatment with 1 µM TMPyP4 and that of TRF2 upon treatment with 3 µM TMPyP4 were not assessed, the reduction in telling TRF2 upon 1 µM TMPyP4 and that of TRF1 with 3 µM TMPyP4 in A172 cells indicated the possibility that a particular dose of TMPyP4 could lead to a robust suppression of both TRF1 and TRF2 levels. Remarkably, none of the previous studies have implicated the reduction in the levels of both TRF1 and TRF2 in the same study of any particular G-quadruplex ligand. To reiterate, TRF1 and TRF2 are cogs of the shelterin machinery and also the so-called telosome – the interactome of telomeres (de Lange, 2009). Thus, a reduction in the levels of both TRF1 and TRF2 would mean telomere destabilisation-mediated catastrophe.

TMPyP4 also exhibited effects on telomere stability and hence, chromosome stability, as visualised by telomere-FISH. Although the treated cells exhibited fairly uniform chromosome stability, this could be attributed to the lack of potency of 1 µM TMPyP4 on the cancer cells. Similar effects of the higher doses were not assessed. Nonetheless, signal-free ends, a measure of missing telomeres were more frequent upon 1 µM TMPyP4 treatment in both, KNS60

and A172 cells (Table 5.2). Moreover, extensive DNA damage was observed upon treatment with 1 and 2 µM TMPyP4, progressively over time (Figure 5.6). Although the presence of DNA damage at the telomeres was not assessed in this study, it is highly likely that a sizeable proportion of DNA damage observed could be at the telomeres, given the nature of TMPyP4 and the FISH result that shows highly abundant signal-free ends following treatment (Table 5.2). A specific G-quadruplex ligand, 360A, induced telomere aberrations including signal-fee ends, telomere sister-chromatid exchanges, and fragile telomeres in HeLa cells after 8 days of treatment with a non-cytotoxic dose (Pennarun et al., 2008). RHPS4, which exhibited its anticancer effects by virtue of its ability to stabilise G-quadruplexes, was shown to inflict telomeric damage as soon as 8 hours after treatment, in human melanoma cells but not in primary cells or even transformed fibroblasts (Salvati et al., 2007).

Apart from eliciting telomere de-protection and telomere damaging effects, TMPyP4 induces appreciable telomere shortening, presumably to below the crisis levels, in addition to reducing hTERT levels significantly, in the telomerase-positive cancer cells before the onset of massive cell death (Figures 5.3 A and 5.4 C respectively). Other G-quadruplex ligands have induced telomere shortening as well in various cancer models. Of note, treatment with a low dose of BRACO-19, in a study of prostate a cancer cell type exhibited progressive telomere shortening over three weeks as seen indirectly by an increase in the number of telomere end to end fusion events (Incles et al., 2004). To reiterate our study, telomere length pattern after four weeks of treatment with 2 µM TMPyP4 exhibited a largely homogenous smear, which portrays a wide range of telomere lengths, unlike in a typically

normal cell. The pattern is a hallmark of cells undergoing massive telomere recombination, suggestive of an ALT (Alternate Lengthening of Telomeres) phenotype (Cesare and Reddel, 2010). Given that the effect of 2 µM of TMPyP4 on telomere shortening and the intensity of DNA damage got exacerbated with time, it is possible that extensive telomere dysfunction and telomere shortening in A172 cells, leading to a surge in telomere recombinatorial events, in an attempt to survive the telomere crisis, only to go in vain, as observed by their elimination within a week after the observation. Interestingly, a metaphase spread of adenocarcinoma cells Du-145 after three weeks of BRACO-19 treatment showed numerous end to end fusions, reflecting the fact that the telomeres have shortened to critical levels (Incles et al., 2004). A dose of 3 µM, while it proved cytotoxic to A172 cells (that underwent massive cell death within 3 weeks), it bore well with KNS60, as seen by its gradual reduction of the shelterin protein TRF1 with time, and induction of massive cell death not before 4 weeks of treatment (Figure 5.3 B and Table 5.1 respectively). Hence, 3 µM TMPyP4 in KNS60 cells may have an effect, equivalent to that of 2 µM TMPyP4 in A172 cells.

Although the effect of 2 µM TMPyP4 in A172 cells on telomerase and hTERT was not evaluated, it is highly likely that telomerase activity may have been diminished largely, following the effects of 1, 5 and 10 µM TMPyP4 in these cells, as seen earlier.

Given the involvement of ATM kinase in response to telomere dysfunction and DNA damage inflicted by 2 µM TMPyP4 over four weeks (Figure 5.7), it is intriguing to note that an apparent attempt at the activation of ALT by A172 cells after four weeks was overhauled, resulting in their elimination, instead.

Although ATM kinase is the main orchestrator of homologous recombination, another complex of proteins – the MRN complex has been shown to be required for the execution of ALT (Cesare and Reddel, 2010). MRN complex also seems to bind to sites of DSBs first and functions beyond ATM-mediated HR pathway (Lamarche et al., 2010). Moreover, it was observed that TMPyP4 induces massive DNA damage. Hence, it is likely that along with telomeric DNA, interstitial DNA is damaged as well, though to unknown extent. Also, telomere elongation can occur by mechanisms independent of homologous recombination in ALT (Figure 1.11) (Cesare and Reddel, 2010). Putting these together, it is possible that TMPyP4, along with the induction of telomeric damage, also inflicts interstitial DNA damage which may explain the occupation of some of the activated ATM at least. Consequently, since the levels of MRN complex in the treated cells remain unassessed, it is possible that activated ATM, that is available for the repair of telomeric DNA, is insufficient to mediate ALT and rescue these cells from telomere crisis. It is remarkable to note that a study on sea urchin eggs as the model has shown the formation of anaphase bridges upon treatment with TMPyP4 (Kim et al., 2003). Mechanistically, TMPyP4 predominantly stabilises intermolecular G-quadruplexes, like telomeres of sister chromatids for instance, unlike telomestatin which likely stabilises intramolecular G-quadruplexes (Kim et al., 2003). This could potentially initiate breakage-fusion-bridge cycles, leading to mitotic catastrophe. Although it would be worthwhile to look for the formation of anaphase-bridges upon TMPyP4 treatment over long-term and upon cytokinesis arrest, it follows that TMPyP4 is highly likely to be effective against ALT-positive, telomerase-negative cancer cells as well.

Promising telomere-targeting G-quadruplex ligands such as RHPS4 and BRACO-19 have been shown to cause telomere dysfunction in colon, prostrate and uterus carcinoma cell types over a few weeks of treatment (Burger et al., 2005) (Salvati et al., 2007) G-quadruplex ligands have exerted an intriguing range of functions depending on the dose and time period of treatment. RHPS4, telomestatin and 360A– all cause telomere uncapping over short-term treatment, while acting as a telomerase inhibitor over long-term treatment with a non-cytotoxic dose (Tahara et al., 2006) (Pennarun et al., 2008; Salvati et al., 2007). The effects of BRACO-19 (while not as well characterised as it TMPyP4 has been in this study) have been attributed to both telomere uncapping as well as telomerase inhibition over long-term treatment (Burger et al., 2005). The subtle variations in such physiological behaviour of cells in response to G-quadruplex stabilising ligands have been attributed to the physical interaction between the ligand and the DNA (Kim et al., 2003). The present study strongly indicates that TMPyP4 induces a combination of telomerase inhibition and disruption of telomere maintenance over long-term treatment, resulting in telomere dysfunction as well.

CONCLUSION

G-quadruplexes has been realised to be a hugely promising target for cancer therapy because of their tendency to form at the telomeres to a great extent. Strategies to stabilise G-quadruplexes could pave way to triggering DNA damage signalling and telomerase inhibition at the same time, thereby promising relatively immediate effects on cancer cells, with enhanced specificity (Neidle and Read, 2000). Two attributes of the promising G-quadruplex ligands studied are baffling. Firstly, G-quadruplex ligands have exhibited minimal toxicity at large, in both *in vitro* and *in vivo* models of study, while effectively eliminating cancer cell populations and greatly reducing tumour mass (Hurley et al., 2000; Monchaud et al., 2010). Secondly, while it is known that G-quadruplexes have a tendency to form at regions other than the telomeres, G-quadruplex ligands have elicited telomere-specific effects on the cells (Bochman et al., 2012). Somehow, by mechanisms not greatly understood, G-quadruplex ligands have been shown to preferentially localise to the telomeres instead of other chromosomal regions (Granotier et al., 2005). Interestingly, TMPyP4 has been shown to preferentially localise inside the nucleus of cancer cells (Izbicka et al., 1999).

Porphyrins are the first family of ligands that were tested extensively for quadruplex-stabilising ability of small molecules; and TMPyP4 was the first ligand to show early promise in that regard (Hurley et al., 2000; Izbicka et al., 1999). TMPyP4 is still used extensively in studies as a tool for understanding telomere dynamics amidst the presence of secondary structures like the quadruplexes (Monchaud et al., 2010).While a number of ligands have shown promise in stabilising G-quadruplexes, it is noteworthy that TMPyP4 has been

a template for structure of a potential G-quadruplex ligand (Dixon et al., 2007). Early studies on the preferential binding of TMPyP4 to quadruplexes using G-rich oligonucleotides *in vitro* demonstrated high affinity but low selectivity of binding quadruplexes over duplexes (De Cian et al., 2005). This coincided with the advent of *in silico* modelling of small molecules that has given rise to a deluge of potential G-quadruplex ligands (Bochman et al., 2012; Monchaud et al., 2010). However, owing to the nature of molecular biology research, laboratory-based research has not been able to catch up with the pace at which *in silico* approaches spew out promising molecules. Hence, amidst all this, TMPyP4, a harbinger of sorts, has been ignored, largely (Martino et al., 2009; Monchaud et al., 2010). With few studies existing on the anticancer effects of TMPyP4 that lay down the sequence of events quadruplex stabilisation to arrest or death of cancer cells (Table 1.1, Chapter 1), this study on the mechanism of TMPyP4 has filled that knowledge gap considerably.

TMPyP4 has been shown by studies on various synthetic oligonucleotides, to bind to telomeric sequence with remarkable specificity *in vitro* (Han et al., 2001; Neidle and Read, 2000; Read et al., 2001). The current study shows that TMPyP4 treatment almost instantly led to the plummeting of the levels of shelterin protein, TRF2; and hTERT – soon after, in the cancer cells. At non-cytotoxic doses of treatment with TMPyP4 over a few weeks, the brain tumour cells exhibited signs of telomere shortening, telomere aberrations, and telomere dysfunction progressively. The current study has thus, demonstrated the specificity of TMPyP4 to the telomeres in the glioblastoma and medulloblastoma cells.

One of the main hurdles for promising drug candidates and one of the main advantages of modest, yet safe candidates is their toxicity profile. Telomestatin, a promising drug that entered clinical trials exhibited a remarkably low toxicity profile (Monchaud et al., 2010). In the current study, TMPyP4 induced a dose-dependent reduction of the cancer cell viability and also the induction of cell arrest, whereas the normal control cell type, IMR90 was neither affected in the viability nor in its cell cycle profile. While it would be worthwhile to use a normal glial cell type as a control, or to assess the long-term effects of TMPyP4 on IMR90, and also to assess the DNA damage levels in IMR90, the safety of TMPyP4 is nevertheless, convincing, Another advantage with TMPyP4 is that the mechanism of binding to the telomeres and stabilisation of G-quadruplex has been thoroughly investigated, understood and endorsed (Monchaud et al., 2010). Moreover, porphyrins have been used as photosensitisers in the treatment of malignant solid tumours and are well known for their preferential tumour-localisation (Dougherty et al., 1998). Hence, it is highly likely that the promise shown by TMPyP4 would hold good in studies *in vivo* too.

The efficacy of TMPyP4 as a drug is thus left to be examined, by xenograft models and genetic mouse models of cancers, particularly malignant glioma as that was the model of this study. It is intriguing though to note that, despite there being a report on TMPyP4 in malignant liver xenograft mouse showing nearly complete abrogation of tumour mass and metastatic potential, TMPyP4 has been left untouched, largely (Aviezer et al., 2000). One main concern for drug delivery to brain tumours is the passage across blood-brain-barrier (BBB). It would be great to investigate the potential of TMPyP4 to cross BBB.

Even if a side-chain modification is needed to improve the delivery, it is possible to find one, given that TMPyP4 has been a template for design of many candidate drugs *in silico* (Monchaud et al., 2010). More remarkable is the fact that TMPyP4 has given rise to a modified compound with nearly 10,000 fold specificity *in vitro* for quadruplex DNA over duplex DNA (Dixon et al., 2007).

Most importantly, the findings on the mechanism of TMPyP4 action in this study – which is perhaps the most comprehensive one on TMPyP4 yet, among studies in cancer cell types – can be extrapolated to any modified analogue of TMPyP4, if needed, during the drug development process.

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