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1. Introduction

Telomeres are essential for chromosome replication and genome integrity. The 3' single-stranded overhang of human telomere may adopt particular conformations such as T-loops and G-quadruplexes. Reactivated in most tumors, telomerase, a specific reverse transcriptase that elongates the telomeres, is thought to enable cancer cells to proliferate in an unlimited manner, thereby correcting the normal telomere erosion that occurs during cell division. The level of interest in G-quadruplex has increased due to their ability to inhibit telomerase activity. We have investigated chromosomal binding and the cellular effects induced by pyridine derivative G-quadruplex ligand in human normal and tumour cells. We show from our analysis that this G-quadruplex ligand preferentially binds the terminal regions of chromosomes in both normal and tumour cells. This compound also induces DNA damage signals in a strictly ATM-dependent manner, inhibits cell proliferation and induces apoptosis. We further observed by telo-FISH and Chromosome Orientation-FISH that this compound induces specific telomere aberrations either during or after replication and mainly consisting of sister telomere fusions and recombination events principally involving the lagging strand telomeres. We have also observed that ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia Related) reduce telomere instability independently of apoptosis suggesting its direct role in preventing inappropriate DNA repair at the telomeres. We have further demonstrated that, even at elevated concentrations, G-quadruplex ligand has limited effects on proliferation of normal cells, and does not induce apoptosis or telomere aberrations. Interestingly, we observed induction of reversible premature senescence in primary fibroblasts. Taken together, our results suggest that the protein composition and/or organization of the telomeres differ markedly between normal and cancer cells, and provide higher telomere stability to normal cells.

1.1 Structure of telomeres

Telomeres are nucleoprotein structures located at the ends of chromosomes (Figure 1A). Human telomeric DNA contains double-stranded repeats of the motif TTAGGG (5 – 20 Kb) followed by a G-rich 3’-overhang (Makarov et al., 1997) (Figure 1B). In human chromosomes, the long terminal protrusions of single-stranded G-rich sequence have been reported to vary from 50 to more than 500 nucleotides (Makarov et al., 1997; Stewart et al., 2003). Telomeres have also been considered to be transcriptionally silent, but mammalian
Telomeres are transcribed into telomeric repeat-containing RNA (Azzalin et al., 2007; Schoeftner & Blasco, 2008; Ho et al., 2008) (Figure 1B). Telomeric DNA is capped by shelterin, a telomere-specific multiprotein complex (de Lange, 2005). Three shelterin subunits, TRF1 (Telomeric Repeat Factor 1) (Zhong et al., 1992; Broccoli et al., 1997), TRF2 (Telomeric Repeat Factor 2) (Broccoli et al., 1997; Bilaud et al., 1997) and POT1 (Protection Of Telomere 1) (Baumann & Cech, 2001; Loyza et al., 2004; Hockemeyer et al., 2005) directly recognize TTAGGG repeats and are interconnected by three additional shelterin proteins, TIN2 (TRF1 Interacting Nuclear Protein 1) (Kim et al., 1999; O’Connor et al., 2006), TPP1 (TINT1-PIP1-PTOP1) (Hockmeyer et al., 2007; Ye et al., 2004; O’Connor et al., 2006; Chen et al., 2007; Xin et al., 2007) and Rap1 (Repressor activator protein 1) (Li et al., 2000; Li & de Lange, 2003; O’Connor et al., 2006), forming a complex that allows cells to distinguish telomeres from sites of DNA damage.

Fig. 1. Human telomeres evidenced by hybridization with a PNA-Cy3 (Peptide Nucleic Acid – Cyanine 3) probe on chromosome counterstained with DAPI (A) and schematic representation of shelterin on telomeric DNA in the presence of telomeric repeat-containing RNA (B).

Telomeres can adopt a protective conformation, the T-loop structure, in which the telomeric 3’-overhang is incorporated into the proximal double stranded telomeres (Griffith et al., 1999) (Figure 2A). Junction-specific binding would also allow TRF2 to stabilize a strand invasion structure that is thought to exist at the strand invasion site of the T-loop (Stansel et al., 2001; Fouche et al., 2006). The T-loop has been proposed to prevent telomeres from being recognized as a DNA double strand breaks (DSBs) and thus from activating cell cycle checkpoints, inappropriate DNA repair and cell death (Smogorzewska & de Lange, 2004). Nevertheless, the DNA damage machinery recognizes functional telomeres during replication (Verdun et al., 2005). At the telomeres, a localized DNA damage response (DDR) seems to be required for processing and the formation of protective structures such as the T-loop after replication.

The guanine-rich sequences of telomeric DNA are susceptible to form in vitro G-quadruplex as a consequence of the propensity of guanine to associate with each other in a stable hydrogen-bonded arrangement, the G-quartet (Neidle & Parkinson, 2003) (Figure 2B and 2C). G-quartet is stabilized by a monovalent cation (Na+ or K+) localized in the centre of the structure (Figure 2B) (Williamson et al., 1989; Sen & Gilbert, 1990). A three-dimensional arrangement of three G-quartets (Figure 2D) can result in a variety of G-quadruplex structures. The four-stranded quadruplex structural types depend on the number and the orientation of the DNA strands. Indeed, intramolecular G-quadruplexes comprise one DNA strand whereas dimeric and tetrameric intermolecular quadruplex involve 2 and 4 DNA strands.
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strands respectively. G-quadruplex heterogeneity also depends on the orientation of the DNA strands (parallel or anti-parallel) and the guanine conformation (syn or anti).

Fig. 2. Structure of the telomere. Schematic representation of T-loop structure (A). Chemical structure of guanine (B), of a G-quartet (C) and depiction of a G-quadruplex (D).

1.2 Telomerase
The maintenance of telomeric repeats in most eukaryotic organisms requires telomerase, which consists of a reverse transcriptase and an RNA template that dictates the synthesis of the G-rich strand of telomere terminal repeats (Greider & Blackburn, 1987; Autexier & Lue, 2006). The catalytic function of this enzyme depends minimally on two components: TERT (Telomerase Reverse Transcriptase) protein (Kilian et al., 1997) and telomerase RNA (TR: Telomerase RNA template) (Feng et al., 1995; Blasco et al., 1995). However, other proteins (dyskerin, Ku70, Ku80, nucleolin, hsp23, hsp90) have also been proposed to associate with human telomerase (Mitchell et al., 1999; Chai et al., 2002; Ting et al., 2005; Khurts et al., 2004; Forsythe et al., 2001; Cohen et al., 2007). By copying a short template sequences within its intrinsic RNA moiety, telomerase synthesizes the telomeric DNA strand running 5’ to 3’ toward the distal end of the chromosome, thereby extending it. Regulated extension of the chromosomal DNA termini occurs to compensate for the shortening that results from nuclease action and incomplete terminal DNA replication (Makaroy et al., 1997; Blackburn, 2005; de Lange, 2005). Telomerase gene expression is active only in germ cells and stem cells and is repressed in most somatic cells, although limited expression is found in normal cycling cells (Masutomi et al., 2003). The expression of telomerase is reactivated in most tumours and is thought to enable cancer cells to proliferate in an unlimited manner by maintaining and protecting telomeres. Telomerase has therefore become a target for the development of new cancer drugs. In addition to telomerase however, other mechanisms to maintain telomere length have been identified in human tumours. Indeed, telomere lengthening is achieved in some cancer cells by recombination events between telomeres, known as alternative lengthening of telomeres (ALT) (Muntoni & Reddel, 2005).

1.3 Functions of telomeres
1.3.1 Telomeres protect chromosome ends
Telomeres are nucleoprotein structures essential for chromosome replication and genome integrity because they protect against instability-promoting events (degradation of the terminal regions of chromosomes, fusion of a telomere either with another telomere or with a broken DNA end, or inappropriate recombination) (Zakian, 1995). Telomeres allow cells to distinguish natural chromosome ends from damaged DNA by inhibiting the canonical DNA
damage response (NHEJ and HR). Telomerase, the T-loop and the shelterin complex serve to prevent telomeres from being recognized as DNA damage. Indeed, TRF2 can inhibit the ATM-dependent DNA damage response (Celli & de Lange, 2006; Karlseder et al., 2004; Denchi & de Lange, 2007, Bae & Baumann, 2007) whereas POT1 can inhibit inappropriate recombination at the telomeres (He 2006) and NHEJ (Denchi & de Lange, 2007). However, functional human telomeres are recognized by proteins involved in the DNA damage response, highlighting that a localized DNA damage response at the telomeres after replication is essential for recruiting the processing machinery that promotes formation of a chromosome end protection complex (Verdun et al., 2005). Without the protective activity of shelterin or when they shorten, telomeres are no longer hidden from the DNA damage surveillance and DNA repair pathways that may inappropriately process chromosome ends (Palm & de Lange, 2008).

1.3.2 Telomere shortening and replicative senescence

The replication of telomeres by conventional mechanisms is inevitably incomplete, leading to telomere shortening at each round of cell division (the end-replication problem) (Olovnikov, 1973). On the other hand, the processing reactions required to re-create a 3’ end also lead to telomere shortening after replication (Dionne & Wellinger, 1998). Telomerase, the unique enzyme in the cell that can add telomeric repeats de novo to the 3’ end, counteracts these losses. In humans, however, the expression of the telomerase is highly regulated and most somatic cells do not possess any telomerase activity, and as a result telomere shortening is prevalent in proliferating cells. Because end-replication results in telomere shortening with each round of replication, the telomeres of human somatic cells act as a mitotic clock, shortening with age both in vitro and in vivo in a replication dependent manner (Olovnikov, 1973; Makarov et al., 1997). When telomeres reach a critical length (Hayflick & Moorhead, 1961), dysfunctional telomeres trigger a damage response leading to growth arrest (cellular senescence) or cell death (apoptosis) (Figure 3). Indeed, uncapping of one telomere, because of damage or loss of telomere sequences or because of destabilization of the protein complex, triggers a DNA damage response and an attempt by the cell to repair the unprotected extremity (Takai et al., 2003; d’Adda di Fagagna et al., 2003). Dysfunctional telomeres are sensed as double strand DNA breaks, activating the DNA damage response checkpoints, including ATM and ATR (Karlseder et al., 1999; d’Adda di Fagagna et al., 2003). Hence, dysfunctional telomeres became associated with DNA damage response factors such as γ-H2AX, 53BP1, Rad17 and Mre11 leading to the formation of TIFs (Telomere dysfunction-Induced Foci) in an ATM- and ATR-dependent manner (Takai et al., 2003; Konishi & de Lange, 2008) and to the induction of replicative senescence. Cellular senescence is a cell-cycle arrest event in which cells show characteristic morphological changes and stain positively for senescence-associated β-galactosidase (SA-β-gal) activity (Hayflick & Moorhead, 1961; Dimri et al., 1995; Cosme-Blanco et al., 2007), and proceed through central signalling pathways leading to the activation of the p53 and retinoblastoma tumour suppressor proteins (pRb). Therefore, telomere shortening is considered to be the main causal mechanism underlying replicative senescence, but telomere deprotection, DNA damage, numerous stresses and/or signalling imbalance can also induce senescence (d’Adda di Fagagna et al., 2003; Cosme-Blanco et al., 2007). Other sources of telomere damage (exogenous events, such as UV irradiation, or endogenous factors, such as reactive oxygen species) may lead to sudden telomere shortening and
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uncapping presumably with very similar consequences (von Zglinicki, 2000; Oikawa et al., 2001). Owing to its anti-proliferative effects, cellular senescence triggered by telomere dysfunction and/or erosion is considered to be a strong protective mechanism against unlimited proliferation (Harley et al., 1991) and an efficient tumour suppressor mechanism (Sedivy, 2007; Feldser & Greider, 2007).

Fig. 3. Induction of replicative senescence and cellular immortalization.

1.4 Replication of telomeres

Bidirectional replication initiates at defined origins but telomeric chromatin that has the ability to form unusual structures might be a source of difficulties for the passage of the replication fork (Fouche et al., 2006). Leading strand replication occurs continuously whereas lagging strand replication is discontinuous and the DNA is synthesized via Okazaki fragments. Telomere replication occurs in two phases, S-phase and G2/M phase (Verdun & Karlseder, 2006 and 2007) which implicate specific mechanisms to initiate, control and coordinate the synthesis of leading and lagging strands at the telomeres and to allow G-tail formation and recapping of the telomeres after their replication.

The telomere structure, T-loop, G-quadruplex and D-loop, cause fork progression problems (Fouche et al., 2006; Verdun & Karlseder, 2006). During the G1 phase, the T-loop structure prevents telomeres from being recognized as DSBs. During S-phase however, T-loop opening and/or difficulties during the passage of the replication fork induce a localized DNA damage response (Verdun et al., 2005) but do not engage the downstream elements of the cascade that would lead to cell cycle checkpoint enforcement. Naturally, stalling of the replication fork at the telomeres induces an ATM/ATR-dependent DNA damage response. Proteins involved in the DNA damage response such as MRE11/RAD50/NBS, RPA,
trimeric complex of Rad9/Rad1/Hus1 and β DNA polymerase are detected at telomeres at the end of S phase (Verdun et al., 2005; Verdun & Karlseder, 2006 and 2007; Jazayeri et al., 2006; Gilson & Geli, 2007). RPA, POT1 and helicases from the RecQ family (BLM and WRN) regulated by TRF2 and POT1 could be implicated in the resolution of unusual DNA structure and G-quadruplexes on the G-rich strand (Crabbe et al., 2004; Shen & Loeb, 2001; Opresko et al., 2004 and 2005) to facilitate progression of the replication fork. ATM and MRE11 located at telomeres during G2/M phase suggest that ATM-dependent damage is required for generation of the 3’ overhang at the leading telomere (Verdun & Karlseder, 2006) implicating Apollo through its 5’ resection activity (Lenain et al., 2006; van Overbeek & de Lange, 2006 ; Dimitrova & de Lange, 2009). Apollo, a member of the SNM1/PSO2 family of nucleases, contributes to the repair of interstrand crosslink and the cleavage of hairpins during V(D)J recombination and is recruited to chromosome ends via interaction with TRF2 (Ye et al., 2010 ; Wu et al., 2010). Steir et al. have reported that 80% of the C-rich strands terminate in CCCAATC-5’ (Sfeir et al., 2005), suggesting the actions of a specific nuclease that is POT1-dependent. Indeed POT1 is implicated in the generation of the correct sequence at chromosome ends (Hockmeyer et al., 2005). ATM-dependent signalling at telomeres in G2 is required for T-loop re-formation (Verdun et al., 2005; Verdun & Karlseder, 2006). Proteins implicated in homologous recombination (RAD51, RAD52 and XRCC3) and telomeric proteins (TRF2, TRF1 and TIN2) are recruited at telomeres to facilitate T-loop formation after replication (Stansel et al., 2001; de Lange, 2005; Verdun et al., 2005; Verdun & Karlseder, 2006; Amiard et al., 2007, Fouche et al., 2006).

1.5 Telomeres, telomerase and cancer
1.5.1 Telomere stabilization

When human cells bypass replicative senescence via the inactivation of p53 or Rb, they continue to divide in spite of very short telomeres leading to the deprotection of chromosome ends (Counter et al., 1994; Stewart & Weinberg, 2000; Kim et al., 2002; Londono-Vallejo, 2008). As the substrates of repair mechanisms, critically shortened telomeres are highly recombinogenic leading to the formation of dicentric chromosomes by NHIEJ, anaphase bridges, engagement of the breakage-fusion-breakage (BFB) cycle (Murname, 2006; Bailey & Murname, 2006), and the generation of novel chromosomal variants that could lead to the emergence of a pro-cancer genome. Indeed, genomic instability driven by dysfunctional telomeres is associated with the transition from benign lesions to malignant cancer. Eventually, the number of unstable chromosomes being too high, cells cannot divide further without losing vital genetic material and thus they initiate mitotic catastrophe and die (crisis) (Figure 3). Consequently, crisis represents another powerful barrier to uncontrolled cell proliferation. To escape from death, cells that enter crisis must acquire a mechanism of telomere maintenance that is most often achieved by the re-expression of telomerase (Counter et al., 1992 and 1994) (Figure 3). In stabilizing telomere length, telomerase reactivation facilitates an indefinite replication potential, which is a hallmark of tumour cells. The reactivation of telomerase thus allows cells to stabilize their genome and divide indefinitely. Amplification of the hTERT locus and duplication/translocation of this locus has been linked to the reactivation of telomerase. In addition, the promoter of the hTERT gene is a target for numerous oncogenes (such as c-myc) or tumour suppressors (e.g. p53). The telomerase ribonucleoprotein recognizes telomeric DNA during S phase (Jady et al., 2006; Tomlinson et al., 2006) and sequentially
adds telomeric repeats to the 3’ end. Telomerase then undergoes a translocation reaction to enable another round of nucleotide addition (Morin, 1989; Autreix & Lue, 2006; Hug & Lingner, 2006). Transformed cells may also acquire alternative mechanisms of telomere maintenance (ALT) based on homologous recombination between telomeres (Figure 3) (Bryan et al., 1997; Muntoni & Reddel, 2005; Stewart, 2005). ALT cells are characterized by high level of telomeric exchange (Londono-Vallejo et al., 2004; Bailey et al., 2004; Dunham et al., 2000), highly heterogeneous telomeres and ALT-associated PML bodies colocalized with telomeres (Muntoni & Reddel, 2005; Dunham et al., 2000).

1.5.2 Telomere recombination and chromosomal instability

Dysfunctional telomeres are sensed as double strand DNA breaks which activates the DNA damage response checkpoints, including the ATM and ATR pathways (Karlseder et al., 1999; d’Adda di Fagagna et al., 2003). In human cells, the activation of ATM and ATR, members of the phosphatidylinositol 3-kinase-like kinase family, leads to the phosphorylation and activation of the central cell cycle regulator p53, CHK1 (checkpoint kinase 1) and CHK2 (checkpoint kinase 2), which in turn facilitates G1 and G2 arrest (Shiloh, 2003; Khanna et al., 2001). ATM responds to DSBs (Shiloh, 2003) whereas ATR responds to lesions after they have been processed to single-stranded DNA intermediates (Zhou et al., 2003). Two main pathways, HR (homologous recombination) or NHEJ (non-homologous end joining), can repair DNA damage. The HR pathway is a very accurate repair mechanism because sister chromatid serves as a template to guide repair of the broken strand during the S and G2 phases of the cell cycle. NHEJ, which is potentially less accurate because two termini of broken DNA are ligated, is the prevailing repair pathway during the G1 and M phases.

The loss of telomere function can result in telomere fusion events in an NHEJ-dependent manner (Smogorzewska et al., 2002; Celli & de Lange, 2005) containing telomeric repeat DNA. Prior to replication, telomere fusion results from the linkage of the G-strand of one chromosome end to the C-strand of another chromosome (van Steensel et al., 1998; Bailey et al., 2001). These telomere fusions induce the formation of dicentric chromosome with telomere sequences at the fusion point and anaphase bridge. In contrast, after replication, telomere fusion can result from the linkage of two chromatids of different chromosomes resulting from C-strand synthesis with blunt ends or two sister chromatids leading to the propagation of the breakage/fusion/breakage (B/F/B) cycle (Fouladi et al., 2000). The B/F/B cycle is a well-established mechanism that causes genome instability leading to complex chromosomal rearrangements and cancer genome amplification. Indeed, deficiency of several proteins such as ATM, TRF2, POT1 and DNA-PKcs result in telomere fusion events that are dependent on factors involved in NHEJ (van Steensel et al., 1998; Veldman et al., 2004; Yang et al., 2005; Bailey et al., 2001; Bailey & Murnane, 2006; Gilley et al., 2001; Metcalfe et al., 1996). In addition, CO-FISH (Chromosome Orientation-Fluorescence In Situ Hybridization) analysis has shown the existence of telomere-DSB fusion (Crabbe et al., 2004; Bailey & Murnane, 2006).

Homologous recombination has been observed at dysfunctional telomeres. Indeed, recombination can occur within the T-loop structure (T-loop HR or telomere rapid deletion) (Wang et al., 2004; de Lange & Petrini, 2000), between sister telomeres (Telomere Sister Chromatid Exchange or T-SCE) (Rudd et al., 2007; Baird, 2008; Bailey et al., 2004) and...
between telomere and chromosome-internal telomeric sequences leading to the formation of TDM (Telomeric DNA-containing Double Minute Chromosomes) (Zhu et al., 2003, Palm & de Lange, 2008).

Another source of telomere-driven instability is the modification of the telomere nucleoprotein complex. The best characterized of these modifications is the inactivation of TRF2 in cells, which leads to rampant uncapping and chromosome fusions in the presence of telomere repeats (van Steensel et al., 1998).

1.6 Telomeric G-quadruplexes are new targets for cancer therapy

Because telomerase is reactivated in most human tumours but not in normal human cells, it is regarded as a potential drug target. Several classes of telomerase inhibitors have now been developed and inhibit this enzyme through the targeting of its RNA (Asia et al., 2003; Herbert et al., 2005; Jackson et al., 2007; Gomez-Millan et al., 2007; Gryanov et al., 2007) or catalytic components (Ward & Autexier, 2005; El-Daly et al., 2005). However compounds that stabilize the telomeric G-quadruplex have been shown to inhibit the activity of telomerase and disrupt telomere capping and maintenance, making the human telomeric DNA G-quadruplex also an attractive target for cancer therapeutic intervention (Mergny & Helene, 1998; Riou et al., 2002; Gowen et al., 2001; de Cian et al., 2008).

Indeed, the intramolecular telomeric G-quadruplex (Neidle & Parkinson, 2003; Dai et al., 2008) has been considered to be an attractive target for anticancer drug design since quadruplex ligands were found to inhibit telomerase (Sun et al., 1997; Zahler et al., 1991; Zaug et al., 2005). However genomic analyses using several algorithms have revealed that more than 370 000 sequences have the potential to form G-quadruplex structure in the human genome (Huppert & Balasubramanian, 2005; Huppert, 2008). Indeed, many G-rich sequences in the human genome are susceptible to the formation of a G-quadruplex (e.g. ribosomal RNA, repetitive G-rich microsatellites and the promoters of several proto-oncogenes including c-MYC and c-KIT) (Maizels, 2006; Eddy & Maizels, 2008; Huppert & Balasubramanian, 2005 and 2007; Todd et al., 2005 and 2007; Qin & Hurley, 2008; Siddiqui-Jain et al., 2002; Phan et al., 2004; Yang & Hurley, 2006; Rankin et al., 2005; Fernando et al., 2006; Shirude et al., 2006). Several proteins have been reported to interact and/or resolve such unusual DNA conformations (Hurley, 2002; Bates et al., 2007; Oganesian & Bryan, 2007; Fry, 2007) supporting the existence of a G-quadruplex. Thus, G-quadruplex ligands have to selectively bind to and stabilize telomeric G-quadruplexes to inhibit telomerase activity. Many quadruplex ligands have now been identified such as telomestatin, BRACO19, TMPyP4 and RHSP4 (Monchaud et al., 2008; Gowen et al., 2002; Han et al., 2001; Gavathiotis et al., 2003; Shin Ya et al., 2001; de Cian et al., 2008), all selected in vitro for their ability to interact with telomeric G-quadruplex. However, many new compounds are likely to be discovered in the future (Bates et al., 2007). In this context, we have investigated chromosomal binding and the cellular effects induced by new pyridine-derived G-quadruplex ligands of the 2,6 pyridine-dicarboxamide series in human normal and tumour cells, and also in ATM- and ATR- (Ataxia Telangiectasia-mutated and Rad3-related) deficient cells. Pyridine derivatives that function as G-quadruplex ligands were selected in vitro on the basis of their ability to interact with a telomeric G-quadruplex (dTm values of 21 – 26 °C) and to inhibit telomerase (IC50 values of 0.22 - 0.45 µM) (Pennarun et al., 2005). Among these new compounds, 307A and 360A (Figure 4) displayed the best compromise between activity and selectivity in vitro. Finally, we chose 360A as it is the most toxic G-quadruplex ligand in human normal cells (Pennarun et al., 2005).
2. In vivo binding of G-quadruplex ligand 360A

The existence of G-quadruplexes was shown in vitro many years ago (Wang & Patel, 1993; Parkinson et al., 2002; Neidle & Parkinson, 2003), but it has not yet been definitively demonstrated that G-quadruplexes exist in vivo, apart from Escherichia coli (Duquette et al., 2004) by electron microscopy and ciliates (Schauffitzel et al., 2001 and 2010) using an antibodies specific for telomeric guanine-quadruplex DNA. The replication band, which is the region where replication and telomere elongation take place, is not stained suggesting that G-quadruplexes are resolved during replication. In human cells, G-quadruplex-interacting proteins (Oganesian & Bryan, 2007), the physiological relevance of G-quadruplexes at the telomere (Phatak & Burger, 2007; Qin & Hurley, 2008), and in vitro studies (Su et al., 2010; Yang et al., 2009) strongly support the in vivo existence of such structures at the telomere. BMVC (3,6-bis(&-methyl-4-vinylpyridinium)carbazole diiodide) has a high sensitivity and binding preference for quadruplex d(TTAGGG)₄ over duplex DNA (Chang et al., 2004). By analyzing BMVC fluorescence at the ends of metaphase chromosomes and other regions of chromosomes, Chang et al. have shown the presence of G-quadruplexes in the human genome and in telomere-proximal regions (Chang et al., 2004). G-quadruplex ligands were selected in vitro on the basis of their highly selective interactions with telomeric G-quadruplexes and for their potent inhibitory effects on telomerase.

Because the in vivo existence of G-quadruplex is still unresolved, we investigated the targeting in human normal and cancer cells by the G-quadruplex ligand using tritiated-360A (3H-360A) (Granotier et al., 2005). The selectivity of 3H-360A for G-quadruplex was first checked in vitro using competitive equilibrium dialysis (Granotier et al., 2005). The binding of 3H-360A to purified genomic DNA was then determined by competition experiments with various oligonucleotides and the results highlighted that 3H-360A has a preference for G-quadruplex. Interestingly, the addition of oligonucleotide that hybridized to the telomeric G-overhang decreased the binding of 3H-360A, suggesting that at least 35% of 3H-360A bound to genomic DNA at the telomeric G-overhang (Granotier et al., 2005). G-quadruplex formation would be more likely on the 3’ telomeric overhang because the Watson-Crick double helix is the predominant DNA form under physiological conditions. Interaction of G-quadruplex ligand and the telomeric single-strand overhang have been reported for telomestatin (Gomez et al., 2004). In agreement with this, DMS footprinting and exonuclease hydrolysis has revealed that G-quadruplex preferentially forms at the very 3’ end of the telomeric DNA (Tang et al., 2008). Using this method, it will be interesting in the future to analyze the position of the G-quadruplex stabilized by 360A. Indeed, G-quadruplex formation at the very 3’ end of the chromosome may be a regulatory mechanism at the
telomeric overhang that mediates T-loop formation, DNA repair and telomere maintenance by telomerase and/or ALT.

Autoradiography has been used previously to investigate the distribution of tritium using a liquid emulsion, NTB2. Charged particles from tritium ($\beta$-rays) interact with a silver halide crystal of the autoradiography emulsion. This interaction induces the formation of a latent image within the crystal, initiating the formation of silver grains during subsequent photographic development of the emulsion (Boren et al., 1975). Thus, by autoradiography of cancer cells cultured with $^3$H-360A, we were able to show previously in our laboratory that the G-quadruplex ligand enters the cells within six hours of the start of treatment and progressively accumulate in the nuclei of living cells (Figure 5A and Granotier et al., 2005).

Autoradiography of metaphase spreads from cancer cells treated with $^3$H-360A (T98G cells with short telomeres and CEM1301 cells with long telomeres) demonstrated that the silver grain density was significantly higher at the ends of the chromosomes than at the interstitial regions, thus highlighting that the G-quadruplex ligand preferentially bound to the terminal regions of the chromosomes (Figure 5B and 5C and Granotier et al., 2005). Interestingly, autoradiographs of the CEM1301 cells that have very long telomeres did not show a greater silver grain density at the ends of chromosomes compared with T98G cells (Figure 5C) revealing that the frequency of binding of $^3$H-360A to chromosome ends does not appear to depend on telomere length and supporting the idea that $^3$H-360A might be bound to telomeric overhangs (Granotier et al., 2005). The G-quadruplex ligand specifically interacts with the terminal regions of chromosomes but we cannot rule out the possibility of specific binding sites located in interstitial sequences. Indeed G-quadruplexes have been shown to exist in other physiologically important G-rich regions (Siddiquin-Jain et al., 2002; Xu et al., 2006; Sun et al., 2005; de Armond et al., 2005; Todd et al., 2007, Huppert, 2008).

RNase treatment of metaphase spreads decreased the number of silver grains (data not shown) suggesting that 360A also binds RNA, in accordance with the existence of an RNA G-quadruplex (Phan & Patel, 2003; Kumari et al., 2007; Huppert, 2008). Nevertheless, autoradiography analysis of metaphase spreads treated with RNase provided evidence that $^3$H-360A binds to telomeric DNA quadruplex at the ends of chromosomes, but not to telomeric repeat-containing RNA (TERRA) (Azzalin et al., 2007; Azzalin & Lingner, 2008; Ho et al., 2008). Indeed, Azzalin et al. have shown previously that UUAGGG-containing RNA exists in cells at higher levels than complementary CCCUAA-containing RNA. High levels of UUUAGGG-containing RNA might result from the strong RNA resistance induced by G-quadruplex formation (Xu et al., 2008). Using a light-switching pyrene probe, Xu et al. have now demonstrated that human TERRA RNA forms a G-quadruplex structure in living cells (Xu et al., 2010) and hence we cannot exclude the possibility that $^3$H-360A binds to the RNA G-quadruplexes located at the telomeres.

Autoradiography has revealed G-quadruplexes in interphase nuclei and in metaphase spreads, suggesting that their existence is not restricted to only one phase of the cell cycle. Telomere replication and T-loop resolution also do not seem to interfere with the formation and/or the stabilization of G-quadruplex but it is presently unclear whether a T-loop and G-quadruplex can form on the same telomere.

Silver grain densities were found again to be significantly higher at the ends of chromosomes in PHA-activated PBL cells from a healthy donor (Figure 5C), indicating the preferential binding of $^3$H-360A to terminal regions of chromosomes from normal as well as cancer cells (Granotier et al., 2005).
Fig. 5. Detection of $^3$H-360A by autoradiography in human cancer and normal cells. A: Autoradiographs of T98G glioblastoma cells cultured without (Control) or with $^3$H-360A for 24h and showing accumulation of the radioactive compound in the nuclei stained with Mayer’s hemalun solution. B: Autoradiography of metaphase chromosomes from T98G cells cultured with $^3$H-360A for 48 h. Black arrows indicate silver grains on the terminal regions and red arrows indicate silver grains on the interstitial regions. C: Densities of silver grains on terminal (T) and interstitial (I) regions of chromosomes from T98G (short telomeres) and CEM1301 (long telomeres) and normal cells (PBL, peripheral blood lymphocytes PHA-p stimulated). Silver grains were counted in 25 metaphases/group for the untreated control and in 50 metaphases/group for cultures incubated with $^3$H-360A. I values were normalized to areas of terminal regions by dividing the total numbers of grains on the interstitial regions in each metaphase by the mean ratio of the interstitial and terminal areas estimated by Metamorph software (T98G: $2.93 \pm 0.44$ at 24 h, $n = 20$; $3.52 \pm 0.48$ at 48 h, $n = 20$ and $3.34 \pm 0.29$ at 72 h, $n = 20$; CEM1301: $3.03 \pm 0.44$, $n = 12$; PBL: $3.64 \pm 0.87$, $n = 20$). Boxes include 50% of the values centred on the median (the horizontal line through the box). The vertical lines begin at the 10th percentile and end at the 90th percentile. T values were significantly greater than I values: *t-test $P < 0.0001$.

Overall, autoradiography analysis of metaphase spreads has revealed that $^3$H-360A binds to telomeres, indicating G-quadruplex formation. However, this result might be an under-estimation of this binding because not all the decays can be detected by this method. In vitro studies have indicated the polymorphic nature of the telomeric G-quadruplex (Dai et al., 2008), and the $^3$H-360A ligand may therefore be unable to bind all the G-quadruplex structural conformations. In addition, $^3$H-360A is naturally unstable due to progressive radiolysis, so we cannot totally exclude a partial degradation of this G-quadruplex ligand. We have shown elsewhere that tritium induces DNA damage and/or cell cycle alterations so that the metaphase spreads analyzed could be from the cells less affected by the G-quadruplex ligand. G-quadruplex elimination by the DNA repair machinery might also explain that not all telomeres are detected with silver grains. Previous findings from our laboratory revealed that a specific G-quadruplex ligand interacts with the terminal ends of human chromosomes and support the hypothesis that G-quadruplex ligands induce and/or stabilize G-quadruplex structures at the telomeres in
both human normal and cancer cells. Fluorescence titration experiments with oligonucleotides have demonstrated that 360A might actively induce the formation of a tetramolecular quadruplex, acting as a chaperone for the association of the four strands (de Cian & Mergny, 2007), therefore highlighting that 360A triggers G-quadruplex formation and locks it into a preformed structure. We have also shown by autoradiography that the G-quadruplex ligand (360A) binds preferentially to the terminal regions of chromosomes in tumour and normal cells (Granotier et al., 2005). This led us to investigate the cellular effects induced by 360A in cancer cells.

3. Cellular effects induced by the G-quadruplex ligand 360A in human cancer cells

3.1 G-quadruplex ligands reduce viability and induce apoptosis in both telomerase-positive and ALT cancer cells

We have reported previously that pyridine derivatives displaying strong selectivity for G-quadruplex structures inhibit cell proliferation in telomerase-positive tumour cells and induce apoptosis after more than three population doublings (Pennarun et al., 2005 and 2008). This is similar to the effects of other G-quadruplex ligands (telomestatin, TMPyP4, RHSP4 and BRACO19) (Phatak & Burger, 2007; Tahara et al., 2006; Gomez et al., 2004; Incles et al., 2004; Gowan et al., 2002). In these earlier studies, cancer cells were treated with several doses of G-quadruplex ligands (ranging from 1 to 5 µM for telomestatin, RHSP4 and BRACO19 and from 50 to 100 µM for TMPyP4). Comparisons of the effects of these ligands is problematic however as they were evaluated in different cancer cell lines. It will be important therefore to compare the inhibition of cell proliferation by various G-quadruplex ligands in the same cancer cell types.

3.1.1 The action of the G-quadruplex ligands is independent of telomerase inhibition

We have shown that 360A blocks cell proliferation and induces apoptosis in ALT tumour cells, suggesting that this G-quadruplex ligand does not only inhibit telomerase activity (Pennarun et al., 2005). Consistently, other G-quadruplex ligands (TMPyP4, RHSP4, BRACO19) have also been shown to inhibit cell proliferation in ALT cells (Kim et al., 2003; Gowan et al., 2001; Incles et al., 2004; de Cian et al., 2008), although telomestatin does not do so (Kim et al., 2003). Moreover, the overexpression of hTERT or a dominant-negative of hTERT in telomerase-positive cell lines does not modify the anti-proliferative effects of the triazine derivative, 12459, (de Cian et al., 2008) indicating that the suppression of cell growth by G-quadruplex ligands is independent of its function in telomerase inhibition. Consistent with this, we have further shown that active pyridine derivatives require several rounds of replication to induce cell growth arrest.

Flow cytometry analysis have shown that the treatment of cancer cells with a G-quadruplex ligand induces a marked decrease in the percentage of cells in G0/G1 phase, dramatically increases the percentage of cells in S phase and also in sub-G1, indicating that 360A induces cell death in cancer cells. The detection of TUNEL-positive cells (Pennarun et al. 2005) in 360A-treated telomerase positive- and ALT cells confirmed that this G-quadruplex ligand induces apoptosis in human cancer cells regardless of the status of their telomere maintenance mechanism.

The effectiveness of the G-quadruplex ligand depends more on its specificity for the G-quadruplex structure. In vitro studies have found that 360A more actively induces the G-
quadruplex structure compared with other G-quadruplex ligands tested (telomestatin, TMPyP4 and BRACO19) (de Cian & Mergny, 2007). G-quadruplex ligands lock into the G-quadruplex structure but this situation is complicated by the existence of a number of different G-quadruplex structures (dimeric, tetrameric, intra- or inter-molecular). TMPyP4, that facilitates the formation of intermolecular G-quadruplex structures, suppresses the proliferation of ALT cells and telomerase-positive cells whereas telomestatin, which promotes the formation of intramolecular G-quadruplex, only suppresses the proliferation of telomerase-positive cells (Kim et al., 2003). TMPyP4 induces anaphase bridges in sea urchin embryos whereas telomestatin does not have this effect (Kim 2003) suggesting that the selectivity of G-quadruplex ligands for intra- or inter-molecular structures is important for their biological effects. It will be interesting in the future to analyze the selectivity of 360A by electrophoretic mobility shift assay (Kim et al., 2003) or by fluorescent screening method (Paramasivan & Bolton, 2008) using multiple reporter molecules that bind to different features of quadruplex DNA.

3.1.2 360A does not induce telomere shortening but provokes telomeric overhang degradation

360A was found not to induce telomere shortening in our previous analysis (Pennarun et al., 2005). Indeed, Southern blot analysis has revealed no significant change in the mean TRF (Telomere Restriction Fragment) lengths in cells treated with this G-quadruplex ligand, although the possibility that this treatment may have caused slight telomere erosion could not be excluded. In contrast, BRACO19 and TMPyP4 treatments shorten telomeres whereas telomestatin and RHPS4 do not do so. In highlighting that G-quadruplex ligands do not act as telomerase inhibitors these data suggest that they induce telomere deprotection. The inhibition of cell proliferation induced by G-quadruplex ligands in ALT cells and in CEM1301 cells with long telomeres (data not shown) confirms that G-quadruplex ligand targets telomeres independently of any effects upon telomerase activity. In any case, the inhibition of cancer cell proliferation is not due to telomere shortening induced by G-quadruplex stabilization but could be triggered by telomeric overhang degradation. Indeed, non-denaturing hybridization assays have revealed that a pyridine derivative induces an alteration of the length of telomeric G-overhang in cancer cells (Pennarun et al., 2005) in a manner similar to G-quadruplex ligands. Taken together, these data suggest that G-quadruplex ligands destabilize telomere structures in human cancer cells.

3.2 G-quadruplex ligands induce telomere deprotection and telomere aberrations in cancer cells involving lagging strand telomeres

Giemsa staining of metaphase spreads from 360A-treated cancer cells has shown that G-quadruplex ligands induce the formation of dicentric or ring chromosomes and anaphase bridges in telomerase-positive cancer cell lines (Pennarun et al., 2005). This confirms that pyridine derivatives acts at the telomeres and induce telomeric instability that is comparable to deprotected telomeres. Detection of chromosome dicentrics in 360A-treated CEM1301 cells with long telomeres has further indicated that genetic instability is not provoked by critically short telomeres providing new evidence that G-quadruplex ligands modify telomere structures.

Indeed, we have shown by Telomere-Fluorescence in situ hybridization (Telo-FISH) and Chromosome Orientation-FISH (Bailey et al., 2004; Crabbe et al., 2004), that the G-quadruplex ligands induce specific telomere aberrations (sister telomere fusions, sister
telomere losses and telomere doublets) either during or after replication (Figure 6) (Pennarun et al., 2008 and 2010). Telomere aberrations induced by a G-quadruplex ligand mainly consist of sister telomere fusions and also recombination preferentially involving the lagging strand telomeres (Pennarun et al., 2008).

Fig. 6. Telomere aberrations revealed by CO-FISH in human cancer cells. Lagging strand telomeres are labelled in red and leading strand telomeres in green. Representative examples of a control chromosome (A), a sister telomere fusion (B), a sister telomere loss involving the lagging strand (C) and a telomere doublet involving the lagging strand (D) are shown.

Delocalization of telomeric proteins (for example POT1 and TRF2) from the telomere induced by 360A (Figure 7A and 7B) and other G-quadruplex ligands (Tahara et al., 2006; Gomez et al., 2006; Salvati et al., 2007; Rizzo et al., 2009) and telomere aberrations induced by 360A in cancer cells confirmed that those compounds provoke telomere deprotection events and not just the inhibition of telomerase. Rizzo et al. have provided evidence that RHSP4 damages telomeric chromatin during replication (Rizzo et al., 2009), confirming the results from our laboratory.

Fig. 7. TRF2 delocalization from the telomere revealed by immunostaining with anti-TRF2 antibody (green) in CEM1301 cancer cells treated for 8 days with 5 µM 360A or 0.05% DMSO. Nuclei and metaphase chromosomes were counterstained with DAPI (blue). Histograms show the mean number of TRF2 foci per nucleus (A) and the mean number of telomeres on metaphase chromosomes with TRF2 foci ± SE (B).
360A has been shown to stabilize the G4 structure within the TRF2 mRNA thereby repressing the expression of this telomeric protein (Gomez et al., 2010). By interacting with supercoiled DNA and participating to the formation and to the dissolution of the T-loop, TRF2 is essential for telomere replication. Therefore the decrease of TRF2 may reduce the presence of this telomeric protein at telomeres leading to dysfunctional telomeres. Taken together, these data reveal that G-quadruplex ligands inhibit cell proliferation and stimulate apoptosis by inducing a broad spectrum of telomere aberrations and telomere deprotection in human cancer cells.

3.3 Increase in telomere aberrations in 360A-treated ATM- or ATR-deficient cells
Deprotection of the telomere is known to induce DNA damage response pathways leading to cell cycle arrest, genomic instability, apoptosis or senescence. In addition, several DNA damage response factors (e.g. ATM) are involved in telomere replication and in the formation of a T-loop in the S and G2 phases. ATR, another key protein involved in the DNA damage response, is essential for maintenance of genomic stability. ATR belongs to a family of phosphatidyl-inositol 3-kinase-like kinases, which includes ATM (Cimprich & Cortez, 2008). ATM is primarily activated by DNA double-stranded breaks, whereas ATR responds to a broad spectrum of DNA damage events, in particular those interfering with DNA replication. In that context, we investigated the cellular effects of 360A in ATM- and ATR-deficient cells. In cancer cells, immunofluorescence staining showed that 360A induces DNA damage signals (γ-H2AX foci) in a strictly ATM-dependent manner (Pennarun et al., 2008) similar to telomestatin (Tauchi et al., 2003), thus showing that G-quadruplex stabilization induces telomere deprotection. However, in cells treated with 360A, nuclear foci of 53BP1 only partly co-localize with PNA telomeric signals, indicating that G-quadruplex ligands, such as telomestatin (Bates et al., 2007), induce other DNA damage events at interstitial sites.

We have further characterized the biological effects of 360A in stable cells expressing siRNA molecules that target ATM or ATR. We have found that ATM, which is involved in the cellular response to DNA damage, strongly suppresses 360A-induced telomere instability (Pennarun et al., 2008). The increase in telomere aberrations in 360A-treated ATM deficient cells were not to be related to defects in cell cycle checkpoints or to apoptosis induction, suggesting a direct role of ATM in preventing inappropriate DNA repair at the telomeres. We have further reported that an ATR deficiency causes telomere instability both in primary human fibroblasts from Seckel syndrome patients and in HeLa cells (Pennarun et al., 2010). Telomere aberrations resulting from an ATR deficiency are mainly generated during and/or after telomere replication and involve both leading and lagging strand telomeres. Moreover, we have demonstrated that an ATR deficiency strongly sensitizes cells to the effects of the G-quadruplex ligand 360A, leading to enhanced sister fusions and chromatid-type telomere aberrations involving specifically the lagging strand telomere. RHSP4 has also been found to induce an ATR dependent ATM signalling (Rizzo et al., 2009) consistent with our results. These data emphasize that ATR also plays a critical role in telomere maintenance during and/or after telomere replication in human cells.

The increase in telomere instability that we observed in ATM- and in ATR-deficient cells treated with G-quadruplex ligand indicates the importance of ATM and ATR and suggests that key proteins implicated in the DNA damage response also contribute to telomere stability during and/or after replication, thus playing an important role in human telomere maintenance.
4. Effects of the G-quadruplex ligand 360A in human normal cells

G-quadruplex ligands inhibit cellular proliferation, and provoke cell cycle arrest and apoptosis by inducing telomere aberrations and telomere deprotection, i.e. they do not only function as a telomerase inhibitor. We previously observed that after seven days of treatment, no effects of G-quadruplex ligands could be found in primary astrocyte cultures (Pennarun et al., 2005), suggesting a differential effect of these factors in cancer and normal cells. In most somatic cells, telomerase activity is not detectable whereas telomerase is expressed in highly proliferative cells such as germ cells and stem cells. In this context, we investigated the cellular effects induced by 360A in human telomerase-positive (PBL, peripheral blood lymphocytes PHA-p stimulated) and telomerase-negative normal cells (NHF27 fibroblasts). It is noteworthy however that in normal fibroblasts, a transient expression of hTERT and very low telomerase activity can be detected during S phase (Masutomi et al., 2003). The telomerase activity detected in primary fibroblasts during S phase is considerably lower than that in cancer cells and other normal cells (such as activated lymphocytes). In normal cells, telomere maintenance by telomerase activity impedes replicative senescence but telomerase activity decreases with age, thereby inducing a telomere shortening.

4.1 The G-quadruplex ligand 360A marginally represses the proliferation of normal cells and can induce premature senescence

We previously observed that after seven days of treatment, no effect of G-quadruplex ligands was evident in primary astrocyte cultures (Pennarun et al., 2005). This result was confirmed by assessing the effects of this treatment on the population doublings in long-term cultures. Peripheral blood lymphocytes isolated from 15 healthy volunteers, and stimulated with PHA-p and IL2 (Interleukine 2) were treated with 360A. For long-term exposure studies, these cells were grown in flasks and exposed to 360A at various concentrations (1, 5 and 10 µM). Control cells were treated with the corresponding concentrations of DMSO (0.01, 0.05 and 0.1%). Every 3-4 days, the cells were counted using trypan blue, and then reseeded with fresh medium containing a new dose of compound. Indeed, the 1 µM dose of 360A was found not to decrease the rate of PBL proliferation (Figure 8), whereas this concentration dramatically reduced proliferation and induced cell death in cancer cells (Pennarun et al., 2005; 2008 and 2010). Higher concentrations of 360A (5 and 10 µM) slightly decreased PBL proliferation in a donor-dependent manner (Figure 8).

Fig. 8. Cell growth curves for PBLs isolated from the fresh blood samples of normal volunteers (one colour per donor) and cultured with 1, 5 and 10 µM 360A (▲) or the corresponding concentrations of DMSO (■).
In telomerase-positive cancer cells, 360A induces apoptosis after 12-14 days, (2-5 population doublings) (Pennarun et al., 2005; 2008 and 2010). After 14 days of treatment, DMSO had no effect on the mean population doubling time of PBLs (4.94 ± 0.55 for 0.01% – 4.96 ± 0.46 for 0.05% and 4.77 ± 0.33 for 0.1%), whereas 360A slightly decreased this rate (4.52 ± 0.36 for 1 µM, 3.31 ± 0.31 for 5 µM and 2.90 ± 0.59 for 10 µM). This result emphasizes that the lack of apoptosis in 360A-treated normal cells is not linked to their reduced cell proliferation compared with cancer cells. Autoradiography analysis have previously demonstrated the preferential binding of the G-quadruplex ligand to the chromosome ends of PBLs (Granotier et al., 2005), thus providing evidence that the differential effects of the G-quadruplex ligand in human normal cells is not linked to the lack of binding of 360A.

We found that 360A slightly decreases the rate of PBL proliferation without inducing cell cycle arrest (analyzed by propidium iodide staining and FACS, Figure 9). Determination of the cellular DNA content by flow cytometry shows that the G-quadruplex ligand induces a gradual increase in the percentage of cells in G1 phase over time in treated populations of PBLs (Figure 4). By TUNEL staining and sub-G1 quantification by FACS we have further found that 360A does not induce cell death in PBLs. Indeed, our TUNEL assay results reveal less than 5% of PBLs treated with 1, 5 and 10 µM 360A or the corresponding concentrations of DMSO for 13 days are apoptotic (data not shown).

In telomerase-negative normal cells, a WST assay revealed a reduced effect of 360A in primary NHF27 fibroblasts (IC\textsubscript{50} > 16 µM) so we further characterized the biological effects of 360A by assessing population doublings in long-term cultures. Primary fibroblasts were treated with 1 and 5 µM 360A or the corresponding concentrations of DMSO (Figure 10A). A 20 day treatment with 1 and 5 µM of 360A slightly reduced the proliferation of primary fibroblasts in a dose-dependent manner without inducing cell death. After 50 days of treatment, 360A reduced the mean doubling population of primary fibroblasts (6 and 18 population doublings for 360A- and DMSO-treated NHF27 respectively). Moreover, telomerase-negative primary fibroblasts treated with 1 µM of 360A can be maintained in culture for 50 days without cell death.
death, highlighting the fact that 360A decreases cell viability and/or cell proliferation in telomerase-positive cancer cell lines, whereas telomerase-positive and telomerase-negative human normal cells are more resistant to G-quadruplex ligand treatment.

Fig. 10. Proliferation and morphology of primary fibroblasts treated with G-quadruplex ligand 360A or DMSO. A: Representative cell growth curves for primary fibroblasts cultured with various concentrations of 360A (1 and 5 µM) or the corresponding concentrations of DMSO (0.01% and 0.05%). Mean population doublings from duplicate cultures ± standard deviation, are representative of four independent experiments. B: Phase-contrast micrographs showing the cellular morphology of primary fibroblasts treated with 0.05% DMSO (left, *10 objective) or 5 µM 360A (middle, *10 objective and right, *20 objective).

Light microscopy revealed a decrease in the cell density and morphological changes that became detectable after about 20 days of treatment. Indeed, treated primary fibroblasts exhibited a typical senescent phenotype with a flattened and enlarged cell shape (Figure 10B). By propidium iodide staining and FACS analysis, we analysed the progression of primary fibroblasts treated with 1 and 5 µM of 360A or the corresponding concentrations of DMSO for 10, 14, 22 and 29 days. The G-quadruplex ligand was found not to increase the percentage of cells in S-phase as in cancer cell populations, or the percentage of cells in G1-phase such as in PBLs but did increase the percentage of cells in G2/M phase (Figure 11) without increasing the percentage of cells in sub-G1 phase. This confirmed the lack of a cell death in 360A-treated primary fibroblasts.

The induction of senescence was confirmed by the detection of senescent associated β-galactosidase-positive cells at pH6.0, an established senescence marker (Dimri et al., 1995) (Figure 12A).

At day 19, we detected 35.4 ± 15.2 and 61.7 ± 2.3% of senescent cells in primary fibroblast cultures treated with 1 and 5 µM 360A, respectively, and a 1.8 ± 0.6% of senescent cells in DMSO-treated primary fibroblasts (Figure 12B), thus showing that G-quadruplex ligand does not induce apoptosis but premature senescence in normal cells in a dose-dependent manner. Some G-quadruplex ligands (e.g. telomestatin, BRACO19, RHPS4) but not all (TMPyP4) show selective toxicity towards cancer cells (Gomez et al., 2006; Cheng et al., 2007; Salvati et al., 2007; Rha et al., 2000). Indeed TMPyP4 inhibits the cell proliferation of normal cells (fibroblasts, adult keratinocytes and epithelial cells) and cancer cells (neuroblastoma, breast cancer, cervical cancer, pancreatic cancer, colon cancer and a prostate cancer cell line) (Rha et al., 2000). The slow growth of normal cells versus the rapid proliferation of tumour cells may be involved in the selective toxicity of G-quadruplex ligand. But we have found that 360A does not induce cell death in normal cells and Salvati...
et al, have further reported that highly proliferating human peripheral blood lymphocytes are resistant to RHPS4 suggesting that growth rate is not the main cause of G-quadruplex ligand tumour selectivity (Salvati et al., 2007).

Fig. 11. Cell cycle analysis of primary fibroblasts cultured with 1 and 5 µM 360A or 0.05% DMSO for 10, 14, 22 and 29 days. The percentages of cells in different phases of the cell cycle are expressed with the respect to the total number of viable cells (corresponding to an analysis of 10^6 cells). Histograms show the mean of duplicate cultures and are representative of three independent experiments.

Fig. 12. Premature senescence induced by G-quadruplex ligand in primary fibroblasts cultured with 1 or 5 µM 360A or the corresponding concentration of DMSO for 19 days. A: Senescent associated-β galactosidase staining (blue) of senescent cells. Nuclei were stained with propidium iodide (red). B: Histograms showing the percentage of senescent cells (150-550 nuclei analyzed per condition).
G-quadruplex ligands induced premature senescence in primary fibroblasts but no cell death. Masutomi et al. have detected low levels of telomerase activity in primary fibroblasts during S phase. The disruption of telomerase activity in human primary fibroblasts slows down proliferation, alters the maintenance of the 3’ single-stranded telomeric overhangs and induces senescence without cell death (Masutomi et al., 2003) suggesting that the periodic expression of hTERT benefits cell proliferation and delays the onset of replicative senescence. Because 360A induces a premature senescence, this G-quadruplex ligand may inhibit transient telomerase activity in normal cells. It was therefore of interest to evaluate the transient telomerase activity levels in 360A-treated primary fibroblasts during S phase. To this end, we analyzed BJ-TERT cells, primary human fibroblasts with long telomeres immortalized through the stable expression of the catalytic component of human telomerase (Jiang et al., 1999). Our results showed that 360A-treated BJ-TERT cells are more resistant to this ligand than primary fibroblasts. Indeed, BJ-TERT cells treated with 5 µM of 360A can be maintained in culture for 34 days without any evidence of premature senescence (<5% of senescent cells) confirming the link between telomerase activity and G-quadruplex resistance in normal cells.

We have previously shown by autoradiography that 360A binds preferentially to the terminal regions of chromosomes in human cancer and normal cells (Granotier et al., 2005), supporting the hypothesis for the in vivo formation of G-quadruplexes at the telomeres of these cells. The differential effects of 360A in normal and cancer cells are not linked to the lack of a G-quadruplex and/or the lack of 360A binding in normal cells. Nevertheless, we cannot at this stage exclude G-quadruplex structure variation and/or the differential regulation of G-quadruplexes during cell cycle progression between normal and cancer cells. In vitro studies using oligonucleotides have given some insights into the structural polymorphisms of the G-quadruplex. (Hurley, 2002; Bates et al., 2007; Oganesian & Bryan, 2007; Dai et al., 2008). These polymorphisms and the dynamic equilibrium of telomeric G-quadruplexes, between human normal and cancer cells, may be important for considerations for the design of future therapeutic interventions.

Finally, we analyzed the proliferation of cancer cells (human glioblastoma, T98G) and normal cells (primary fibroblasts, NH27) at the end of a 360A treatment. T98G cells and primary fibroblasts were cultured with 1 and 5 µM 360A or the corresponding concentration of DMSO for several days (4 and 7 days for T98G, 15 and 22 days for NH27). After the treatment period had ended, T98G and NH27 cells were cultured in medium without G-quadruplex ligand until day 20 for T98G and day 50 for NH27 (Figure 13).

After treatment with 1 µM 360A, we observed a resumption of proliferation in T98G cells treated for 4 and 7 days (Figure 13A) and in NH27 cells treated for 15 and 22 days (Figure 13B). After a treatment with 5 µM 360A, elimination of the G-quadruplex ligand at day 4 provoked a resumption of proliferation in cancer cells, but elimination of 360A at day 7 did not do so (Figure 13C). However, elimination of this G-quadruplex ligand at the 5µM dose did induce the resumption of proliferation, even after 15 days of treatment (Figure 13D). In primary fibroblasts, the percentage of senescent cells decreased after the end of treatment, when we detected the resumption of proliferation (data not shown). We found that 360A also induced reversible premature senescence in primary fibroblasts but provoked apoptosis in cancer cells. Replicative senescence is thought to be an essentially irreversible growth arrest phenomenon but a p53-dependent reversible senescence pathway has been identified in normal cells as a response to dysfunctional telomeres (Beausejour et al., 2003; Dirac & Bernards, 2003). It will be of interest to study the implications of p53 function in normal cells treated with G-quadruplex ligand.
Fig. 13. Cell growth curves for T98G glioblastoma cells (A and C) and NHF27 primary fibroblasts (B and D) cultured with 1µM 360A (top), 5µM 360A (bottom) or the corresponding concentrations of DMSO. Cells were analyzed after the end of treatment at day 4 (stop D4) or day 7 (stop D7) for T98G cells and day 15 (stop D15) or day 22 (stop D22) for NHF27 cells. Mean population doublings from duplicate populations ± standard deviation are shown.

4.2 The G-quadruplex ligand 360A does not induce telomere instability in normal human cells

To gain further insight into the cellular effects of 360A in primary human normal cells, we analyzed genetic stability as an endpoint. We have previously shown that G-quadruplex ligands induce dicentric and ring chromosomes in telomerase-positive cancer cell lines (Pennarun et al., 2005). However, Giemsa staining of PBL chromosomes did not reveal chromatid breaks or dicentric chromosomes, suggesting that 360A does not induce chromosome instability in PBLs at a 1, 5 or 10 µM dose for 7, 10 and 15 days. Giemsa staining of primary fibroblasts chromosomes treated with 5 µM 360A for 7 days confirmed that this G-quadruplex ligand does not induce chromosome instability in normal cells.
We further characterized telomere stability in 360A-treated PBL and primary fibroblasts by telo-FISH after a 7 day treatment. In PBLs, we detected telomere aberrations (sister telomere fusions, sister telomere losses and telomere doublets) but their frequency remained stable in G-quadruplex ligand-treated normal cells (Figure 14A). We detected 9.1% ± 1.3 chromosomes with telomere aberrations in 360A-treated PBLs (n=1387 chromosomes) and 9.9% ± 1.1 chromosomes with abnormal telomeres in DMSO-treated PBL (n=1385 chromosomes), indicating that 360A does not induce telomere instability in normal cells but does so in cancer cells (Pennarun et al., 2008 and 2010). It is noteworthy that the frequency of telomere aberrations in normal cells was found substantially lower than that in cancer cells. Indeed, Telo-FISH analysis revealed 2.98 ± 0.36 damaged telomere in 360A-treated PBLs and 2.98 ± 0.34 in DMSO-treated PBLs (Figure 14B), highlighting that this G-quadruplex ligand does not induce telomere aberrations in human primary peripheral cells. Autoradiography analysis of metaphase spreads of PBLs treated with 3H-360A further demonstrated the preferential binding of this G-quadruplex ligand to the terminal regions of chromosomes (Granotier et al., 2005) suggesting that the selective toxicity of 360A is not due to its lack of binding to telomeres in normal cells. Telo-FISH of primary fibroblast cultures treated with 360A for seven days (data not shown) further confirmed that this G-quadruplex ligand does not induce telomere instability in human normal cells, in contrast with the cancer cells. RHPS4, is another G-quadruplex ligand that does not induce telomere aberration in normal fibroblasts (Salvati et al., 2007) further suggesting that the telomere organization and the cellular response to damaged telomeres is substantially different between normal and cancer cells.

Fig. 14. The G-quadruplex ligand 360A does not induce telomere aberrations in primary blood lymphocytes at a 5 µM 360A dose in a 7 day culture. A: Example of telomere evidenced by Telo-FISH in normal cells (a-d): control chromosome (a), sister telomere fusion (b), telomere doublet (c) and sister telomere loss (d). B: Histograms showing the percentage of chromosomes with telomere aberrations (sister telomere fusions, sister telomere losses, and telomere doublets) ± standard error (n=32-34 metaphases analyzed per condition). C: Box graphs showing the percentage distribution of damaged telomeres in DMSO- and in 360A-treated PBLs for seven days. Boxes include 50% of the values centred on the median (the horizontal line through the box). The vertical lines begin at the 10th percentile and end at the 90th percentile.
Differential Effects of the G-Quadruplex Ligand 360A in Human Normal and Cancer Cells

5. Discussion

Taken together, our results demonstrate that even at elevated concentrations, the G-quadruplex ligand 360A has limited effects on the proliferation of normal cells, in which they do not induce apoptosis. Interestingly, we found that 360A induces reversible premature senescence in primary human fibroblasts. We mainly observed that this G-quadruplex ligand does not induce telomere aberrations in normal human telomerase-positive and telomerase-negative cells, highlighting the differential effects of this ligand in human normal and cancer cells (Table 1).

The cellular effects induced by this G-quadruplex ligand suggest that the organization and/or cellular responses activated by damaged telomeres differ significantly between human normal and cancer cells.

5.1 Telomere organization in the nucleus

Telomere organization is cell cycle dependent, with the assembly of telomeres into a telomeric disk in the centre of the nucleus occurring in G2 phase in normal cells (primary human lymphocytes, primary human fibroblasts and normal human epithelial tissue) (Chuang et al., 2004). Telomeres are widely distributed throughout the nucleus in the G0/G1 and S phases. Moreover, the three dimensional telomere organization is distorted in tumour cells and telomeric aggregates are thus formed. Transient telomeric aggregations that potentially cause irreversible chromosomal rearrangements are suggestive of genomic instability. The spatial organization and distribution of telomeres in the nucleus, and during the cell cycle, are important for genomic stability. On the other hand the telomere organization in the nucleus of cancer cells could promote the chromosome instability induced by G-quadruplex ligands. Hence, it will be very interesting in the future to investigate telomere organization in normal and cancer cells treated with G-quadruplex ligands to evaluate whether G-quadruplex stabilization disrupts the three dimensional arrangement of the telomere organization in the nucleus.

5.2 Telomeric 3' overhang length

At the very ends of the chromosomes, the telomeric 3' overhang is critical for telomere elongation by telomerase, for forming the T-loop structure, and therefore for normal telomere function and genome stability (Rahman et al., 2008). Most cancer cell lines possess shorter overhangs whereas most normal cells have longer 3' overhangs (Lee 2008). The size difference of the telomeric 3' overhang between normal and cancer cells could explain the differential effects of the G-quadruplex ligand. Stabilization of G-quadruplexes by specific ligands along a long 3' overhang in normal cells would not prevent the formation of the T-loop. We can also speculate that G-quadruplex resolution by endonucleases and/or helicases would leave a sufficiently long overhang for normal telomere function. In this regard we have previously shown that 360A reduces the telomeric overhang in cancer cells (Pernarun et al., 2005). It will be now interesting to measure the 3' overhang in human normal cells treated with G-quadruplex ligand using the previously described oligonucleotide ligation assay (Cimino-Reale et al., 2001). The telomerase inhibitor telomestatin, which is known to stabilize G-quadruplexes, preferentially inhibits the growth of cancer cells compared to normal cells (fibroblasts and epithelial cells). However, telomestatin does not inhibit cell growth, does not reduce the telomeric overhang and does not induce the dissociation of TRF2 from telomeres in normal and hTERT-positive
<table>
<thead>
<tr>
<th></th>
<th>Human cancer cells</th>
<th>Human normal cells</th>
<th>Pyridine derivative G-quadruplex ligand (360A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Telomerase positive</td>
<td>Telomerase positive (Peripheral blood lymphocytes)</td>
<td>Telomerase negative (Primordial blood)</td>
</tr>
<tr>
<td>Preferential binding to telomeres</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>significant decrease</td>
<td>slight decrease</td>
<td>slight decrease</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>accumulation in S</td>
<td>no cell cycle arrest</td>
<td>no cell cycle arrest</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Senescence</td>
<td>no</td>
<td>n.d.</td>
<td>yes</td>
</tr>
<tr>
<td>DNA damage signaling</td>
<td>yes</td>
<td>no</td>
<td>n.d.</td>
</tr>
<tr>
<td>Telomere shortening</td>
<td>no</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Shortening of the 3’ overhang</td>
<td>yes</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Delocalization of telomeric proteins</td>
<td>yes</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Telomere aberrations</td>
<td>yes</td>
<td>no</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* Human glioma (T98G, CB193 and U118-MG), SV40-transformed human normal fibroblast (AS3WT2), SV-40 transformed ataxia-telangiectasia fibroblast from normal (GM03657) and ataxia-telangiectasia donors (GM03189), cervical carcinoma (HeLa)

n.d.: not determined
fibroblasts (Tahara et al., 2006). We can thus hypothesize that there is a link between the lack of reduction of the telomeric 3’ overhang length, the TRF2 stability at telomeres and resistance to the effects of G-quadruplex ligands.

5.3 Telomeric proteins

Variations in the number of telomeric proteins fixed to double- or single-stranded telomeric DNA can modulate the sensitivity of cells to G-quadruplex ligands. In cancer cell lines, we have previously shown that 360A induces TRF2 delocalization from the telomeres but we don’t yet know if 360A also induces TRF2 delocalisation in normal cells. In any event, the G-quadruplex ligands telomestatin and RHSP4 have been shown to induce TRF2 and POT1 delocalization from the telomeres only in cancer cells (Tahara et al., 2006; Zaug et al., 2005; Salvati et al., 2007; Bates et al., 2007) without inducing TRF1 delocalization. The differential effects of telomestatin on TRF1 and TRF2 could be explained by their DNA-binding activities. Indeed, TRF1 binds to the telomere more strongly than TRF2 (Hanaoka et al., 2005). The differential effects of telomestatin on TRF2 localization in cancer and normal cells could therefore be linked to different DNA-binding activities between these cell types.

The telomere-disrupting agent telomestatin also induces a DNA damage response and apoptosis in newly generated neurons which have low levels of TRF2, whereas neural progenitor cells which have high levels of telomerase and mature neurons with high levels of TRF2 are resistant to telomere damage (Cheng et al., 2007). Finally, TRF2 or POT1 overexpression correlates with the resistance to telomestatin and RHSP4 (Salvati et al., 2007; Cheng et al., 2007; Gomez et al., 2006). TRF2 might therefore counteract the effects of G-quadruplex ligands through the formation of a T-loop that would mask the 3’ overhang and inhibit the recruitment of helicases which are expected to resolve G-quadruplexes and favour the binding of POT1 (Salvati et al., 2007; Opresko et al., 2002; O’Connor et al., 2006). Recently, Wang et al. have reported that the G-quadruplex formation at the 3’ end of telomeric DNA inhibits extension by telomerase and that the BLM helicase requires few nucleotides beyond the G-quadruplex for efficient unwinding (Wang et al., 2011). These data suggest that G-quadruplexes at the extreme 3’ end of a chromosome are resistant to the unwinding activity of helicases. The resistance of normal cells to 360A could therefore be due to the strong binding of TRF2 at the telomeres and/or the lack of TRF2 delocalization induced by G-quadruplex ligand.

5.4 Cellular responses to damaged telomeres

The cellular responses induced by damaged telomeres may significantly differ between normal and cancer cells. At the telomeres, the DNA damage response is activated by TRF2 delocalization (Karlseder et al., 1999; van Steensel et al., 1998; Takai et al., 2003; Celli & de Lange, 2005). In cancer cells, immunofluorescence staining results have shown that 360A-induced DNA damage signals occurs in a strictly ATM-dependent manner, but not in normal cells. Indeed, our own results indicate that 360A does not increase the number of γ-H2AX foci in human primary lymphocytes, suggesting that the cellular response activated by damaged telomeres is significantly different between human normal and cancer cells. However, we don’t yet know if 360A induces less DNA damage in normal cells than in cancer cells and/or if the DNA damage response pathway is more efficient in normal cells than in cancer cells. In agreement with our preliminary results, RHPS4, which induces telomere damage in immortalized fibroblasts, does not induce H2AX phosphorylation in
normal fibroblasts (Salvati et al., 2007). This lack of H2AX phosphorylation is not due to impairment of DNA damage response pathways because bleomycin and the dominant-negative form of TRF2 phosphorylate H2AX in normal fibroblasts.

6. Conclusions

We showed herein that a specific G-quadruplex ligand, 360A, interacts with the terminal ends of human chromosomes in both tumour and normal cells. We find that 360A binds preferentially to human chromosome ends in normal and cancer cells but that the G-quadruplex ligand-induced cell death by apoptosis occurs only in cancer cells. We provide evidence that the G-quadruplex ligand provokes telomere deprotection during replication and induces telomere aberrations only in cancer cells. Taken together, our results indicate that 360A induces telomere instability in cancer cells but not in normal cells. Our results thus suggest that the cellular response activated by damaged telomeres and/or mechanisms implicated in telomere maintenance are significantly different between human normal and cancer cells. We cannot yet exclude the possibility that the protein composition at the telomeres and/or organization of the telomeres differ markedly between normal and cancer cells, and thereby provide normal cells with a higher degree of telomere stability.

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Over the past decades, great advances have been made in understanding the cellular DNA repair pathways. At the same time, a wealth of descriptive knowledge of human diseases has been accumulated. Now, the basic research of the mechanisms of DNA repair is merging with clinical research, placing the action of the DNA repair pathways in the context of the whole organism. Such integrative approach enables understanding of the disease mechanisms and is invaluable in improving diagnostics and prevention, as well as designing better therapies. This book highlights the central role of DNA repair in human health and well-being. The reviews presented here, contain detailed descriptions of DNA repair pathways, as well as analysis of a large body of evidence addressing links between DNA damage repair and human health. They will be of interest to a broad audience, from molecular biologists working on DNA repair in any model system, to medical researchers.

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