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A New Steroid Derivative Stabilizes G-Quadruplexes and Induces Telomere Uncapping in Human Tumor Cells

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ABSTRACT

Human telomeric DNA consists of tandem repeats of the sequence d(TTAGGG) with a 3′ single-stranded extension (the G-overhang). The stabilization of G-quadruplexes in the human telomeric sequence by small-molecule ligands inhibits the activity of telomerase and results in telomere uncapping, leading to senescence or apoptosis of tumor cells. Therefore, the search for new and selective G-quadruplex ligands is of considerable interest because a selective ligand might provide a telomere-targeted therapeutic approach to treatment of cancer. We have screened a bank of derivatives from natural and synthetic origin using a temperature fluorescence assay and have identified two related compounds that induce G-quadruplex stabilization: malouetine and steroid FG. These steroid derivatives have nonplanar and nonaromatic structures, different from currently known G-quadruplex ligands. Malouetine is a natural product isolated from the leaves of *Malouetia bequaaertiana* E. Woodson and is known for its curarizing and DNA-binding properties. Steroid FG, a funtumine derivative substituted with a guanylhydrazone moiety, interacted selectively with the telomeric G-quadruplex in vitro. This derivative induced senescence and telomere shortening of HT1080 tumor cells at submicromolar concentrations, corresponding to the phenotypic inactivation of telomerase activity. In addition, steroid FG induced a rapid degradation of the telomeric G-overhang and the formation of anaphase bridges, characteristics of telomere uncapping. Finally, the expression of protection of telomere 1 (POT1) induced resistance to the growth effect of steroid FG. These results indicate that these steroid ligands represent a new class of telomere-targeted agents with potential as antitumor drugs.

Telomeres are nucleoprotein structures that cap the ends of eukaryotic chromosomes; these regions protect chromosome ends from fusion and from illegitimate recombination and repair (Blackburn, 2001). Telomere replication is sustained in proliferative somatic cells and in most cancer cells by telomerase, a ribonucleoprotein complex that elongates the chromosome ends to compensate losses occurring at each cell division, because of the inability of polymerase to fully replicate telomeric extremities (McEachern et al., 2000). In somatic cells, the absence of telomerase provokes a progressive shortening of the telomeric DNA at each round of division that ultimately leads to replicative senescence once a critical telomere length has been reached (Shay and Wright, 2002). Numerous observations, notably that inhibition of telomerase activity limits tumor cell growth (Hahn et al., 1999), have led to the proposal that telomere and telomerase are potential targets for cancer chemotherapy (Lavelle et al., 2000; Neidle and Parkinson, 2002; Shay and Wright, 2002).

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ABBREVIATIONS: hTERT, human telomerase reverse transcriptase; PCR, polymerase chain reaction; POT1, protection of telomere 1; TBE, Tris-borate-EDTA; DAPI, 4,6-diamidino-2-phenylindole; TRF, terminal restriction fragment; steroid FG, a funtumine derivative substituted with a guanylhydrazone moiety; EtBr, ethidium bromide; TRF, telomere repeat factor.

In humans, the telomere is composed of tandem repeats of the G-rich duplex sequence 5′-TTAGGG-3′, with the G-rich 3′ strand extending beyond the duplex to form a 130- to 210-base overhang, called the G-overhang (Makarov et al., 1997; Wright et al., 1997). Telomeres are believed to exist in different conformations together with several telomere-associated proteins, such as telomere repeat factors (TRF1, TRF2) and POT1 (Smogorzewska and de Lange, 2004). The G-overhang is accessible for telomerase extension in the open state or inaccessible in a capped (or closed) conformation that involves the formation of a telomeric loop motif (Smogorzewska and de Lange, 2004). Although the telomeric loop structure has not been defined in detail, it may be created by the invasion of the G-overhang into the duplex region of the telomere (Griffith et al., 1999). Uncapping of the telomere ends leads to telomeric dysfunction characterized by end-to-end fusion, inappropriate recombination, anaphase bridges, and G-overhang degradation that may lead to either apoptosis or senescence (Blackburn et al., 2000; Duan et al., 2001; Karlseder et al., 2002; Li et al., 2003).

Because of the repetition of guanines, the G-overhang is prone to formation of a four-stranded G-quadruplex structure that has been shown to inhibit telomerase activity in vitro (Mergny et al., 2002; Davies, 2004). Small molecules that stabilize G-quadruplexes are effective as telomerase inhibitors, and several series of compounds have been identified using techniques such as temperature melting fluorescence assays on oligonucleotides (Mergny et al., 2001), electrophoresis analysis of quadruplex formation (Koeppel et al., 2001), electrospray ionization mass spectrometry (Rosu et al., 2003a), and the telomeric repeat amplification protocol that measures telomerase activity in cell extracts (Gomez et al., 2002) (for review, see Guittat et al., 2004). The ligands that stabilize G-quadruplex structures include cationic porphyrins (Han et al., 1999, 2001; Dixon et al., 2007), perylenes (Fedoroff et al., 2000), amidoanthracene-9,10-diones (Perry et al., 1998), 2,7-disubstituted amidofluorenones (Perry et al., 1999), acridines (Read et al., 1999; Harrison et al., 2003), ethidium derivatives (Koeppel et al., 2001; Rosu et al., 2003a), disubstituted triazines (Riou et al., 2002), fluoroquinoanthroxazines (Kim et al., 2003a), indoloquinolines (Caprio et al., 2000), dibenzophenanthrolines (Mergny et al., 2001), bisquinacridines (Teulade-Fichou et al., 2003), pentacyclic acridinium (Gowan et al., 2001), telomestatin (Shin-ya et al., 2001), and the recently discovered bisquinolinium derivatives (Lemarteleur et al., 2004; Pennarun et al., 2005; De Cian et al., 2007) (for review, see Kerwin, 2000; Cuesta et al., 2003; Guittat et al., 2004; Pendino et al., 2006). Because of the peculiar features of the quadruplex structure, compared with classic double-stranded B-DNA, a selective recognition of telomeric G-quadruplex by small-molecule ligands should be possible (Neidle and Parkinson, 2002; Parkinson et al., 2002; Clark et al., 2003; Ambrus et al., 2006). Some partial selectivity for G-quadruplex relative to duplex DNA was obtained with triazine (Riou et al., 2002) and with ethidium derivatives (Rosu et al., 2003a), and selectivity was significantly enhanced with the natural product telomestatin (Kim et al., 2002, 2003b; Rosu et al., 2003b), with a new series of 2,6-pyridin-dicarboxamide derivatives (Pennarun et al., 2005), and with a porphyrin derivative (Dixon et al., 2007).

To test the paradigm of the lifespan control by telomerase activity and telomere length, G-quadruplex ligands were evaluated in cells. This paradigm is at least partially true because a functional telomerase inhibition was observed in cell lines treated for several weeks with a subtoxic dosage of a compound that provokes a telomere shortening, and this shortening was correlated with the induction of senescence (large morphology of cells and SA-β-galactosidase activity) (Riou et al., 2002). It was also observed that G-quadruplex ligands induce more rapid effects on cell growth than initially expected based on telomerase inhibition. Apoptosis and short-term responses were observed with triazine derivatives (12459, 115405), telomestatin, and more recently with the pyridine dicarboxamide derivatives (307A, 360A) (Riou et al., 2002; Tauchi et al., 2003; Pennarun et al., 2005).

The observation that BRACO-19 causes chromosome end-to-end fusions associated with the appearance of p16-associated senescence led to the proposal that G-quadruplex ligands mostly act to disrupt the telomere structure (Incles et al., 2004). Such telomeric dysfunction was also observed in cell lines treated with RHPS4 or with 307A and in cell lines resistant to 12459, with typical images of telophase bridges (Gomez et al., 2003a; Leonetti et al., 2004; Pennarun et al., 2005). Further indirect evidence that G-quadruplex ligands target telomere replication arose from experiments in mutant cell lines resistant to these ligands: These cells present telomere capping alterations, overexpression of human telomerase reverse transcriptase (hTERT), and telomere shortening cross-resistance for different classes of ligands (Gomez et al., 2003a,b; Leonetti et al., 2004; Pennarun et al., 2005).

We have demonstrated that G-quadruplex ligands interfere with the conformation and the length of the telomeric G-overhang, an effect that is thought to be more relevant than the double-stranded telomere erosion as a marker for telomestatin cellular activity (Gomez et al., 2004). G-overhang degradation was found to be associated with the onset of senescence (Gomez et al., 2004) or with the onset of apoptosis (Douarre et al., 2005). Recent publications also indicate that G-quadruplex ligands may act by dissociation of telomere binding proteins POT1 and TRF2, uncapping telomeres to make them available for extension (Gomez et al., 2006a,b; Tahara et al., 2006).

To search for novel and more potent G-quadruplex ligands, we have screened a bank of derivatives from natural and synthetic origin using a temperature melting fluorescence assay. Two steroids derived from the natural products malouetine and funtumine were identified and characterized for telomeric G-quadruplex stabilization and telomere elongation inhibition in vitro. Funtumine substituted by a guanylhydrazone moiety (steroid FG) induced antiproliferative activities in HT1080 cells associated with telomere shortening, G-overhang degradation, and anaphase bridge formation suggesting that this steroid ligand is a high-affinity G-quadruplex ligand that binds to telomeres in human cells. These steroid ligands represent a new class of telomere-targeted agents that have potential as antitumor drugs.

Materials and Methods

Compounds and Cells. All oligonucleotides were synthesized and purified by Eurogentec (Seraing, Belgium). Malouetine dichloride, funtumine, and funtumine guanylhydrazone were components of the Institut de Chimie des Substances Naturelles chemical library. Malouetine dichloride and funtumine were isolated from *Malouetia*

bequertiana (Janot et al., 1960) and *Funtumia latifolia* (Janot et al., 1958; Quevauviller and Blanpin, 1958), respectively. Funtumine guanylhydrazone was prepared from funtumine by reaction with aminoguanidine bicarbonate as described previously (Meyer et al. (1967). The structure and purity of the compounds were verified by liquid chromatography/mass spectrometry and NMR spectroscopy (data not shown). Compounds were solubilized at 10 mM in dimethyl sulfoxide. Additional dilutions were made in water. HT1080 human lung carcinoma, H460 lung carcinoma, and HeLa cervix carcinoma were from the American Type Culture Collection (Manassas, VA). BJhTERT human foreskin fibroblast immortalized with human telomerase reverse transcriptase (hTERT) has been described previously (Bodnar et al., 1998). The cells were grown in Dulbecco's modified Eagle's medium with GlutaMAX (Invitrogen, Carlsbad, CA), supplemented with 10% fetal calf serum and antibiotics. The HT1080GFP-POT1 cell line was described previously (Gomez et al., 2006b).

Fluorescence Experiments. Initial screening experiments were performed on a LightCycler real-time PCR instrument (Roche, Basel, Switzerland) as described previously (Darby et al., 2002), using a fluorescent oligonucleotide F21D (5′-FAM-GGGTTAGGGTTAGGGTTAGGG-DabCyl-3′), alone or in the presence of 10 μM compound. Assays were performed in a buffer containing 0.5 μM F21D, 10 mM cacodylate, pH 8.0, 0.1 M LiCl, and 5 mM KCl. Excitation wavelength was 470 nm, and emission of fluorescein was recorded at 530 nm.

Quantitative experiments and dose-response results were obtained by real-time PCR (MX3000P; Stratagene, La Jolla, CA) using F21D or F21T (analogous to F21D but with the 3′ quencher tetramethylrhodamine) fluorescent oligonucleotides (0.2 μM). Melting profiles were recorded in a 10 mM Li cacodylate buffer, pH 7.2, with 50 to 100 mM NaCl or 5 to 10 mM KCl. Ionic strength was kept constant by the addition of LiCl. Excitation wavelength was 496 nm, and emission was recorded at 516 nm. For competition experiments, various concentrations of the double-stranded ds26 competitor were added before the melting experiment (Kaiser et al., 2006).

PCR Stop Assay. The stabilization of G-quadruplex structures was investigated by a PCR-stop assay (Lemarteleur et al., 2004) using a test oligonucleotide and a complementary oligonucleotide that partially hybridizes to the last G-repeat of the test oligonucleotide. Sequences of the test oligonucleotides (21G) and the corresponding complementary sequence (anti21G) used here are presented in Fig. 3.

Assay reactions were performed in a final volume of 25 μl in a 10 mM Tris buffer, pH 8.3, with 50 mM KCl, 1.5 mM $Mg(OAc)_2$, 7.5 pmol of each oligonucleotide, 1.5 units of *Taq* polymerase, and the amount of the ligand indicated in Fig. 3. Reaction mixtures were incubated in a thermocycler with the following cycling conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Amplified products were resolved on a 12% nondenaturing polyacrylamide gel in 1× TBE and stained with SYBR Green I (Roche). Fluorescence was analyzed with a Typhoon 9210 PhosphorImager (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Cell Growth Assays. For long-term cell growth studies, cells were seeded at 1×10^4 cells/ml (5 ml) into a 25-cm^2 tissue-culture flask in the presence or the absence of steroid derivatives, cultured for 4 days, then trypsinized and counted. At each passage, 1×10^4 cells/ml were replated into a new culture flask with fresh medium containing drug solution. Results were expressed as the cumulated population doublings as a function of the time of culture as described previously (Riou et al., 2002). MTT survival assays (4 days or 2 days) were performed in quadruplicate in 96-well plates, as recommended by the manufacturer (Sigma, St. Louis, MO).

Anaphase Bridge Analysis. To determine the presence of anaphase bridges, cells were seeded on glass coverslips in complete culture medium and treated with steroid ligands for 24, 48, and 96 h, then stained with DAPI (Sigma) and mounted. Images of anaphases were recorded with an Axiovert 200 M inverted microscope (Carl Zeiss, Oberkochen, Germany) coupled with a Coolsnap HQ camera controlled by MetaMorph software (Molecular Devices, Sunnyvale, CA). At least 50 metaphases were examined.

Detection of SA-β-Galactosidase Activity. Endogenous senescence-associated β-galactosidase activity was assessed by a staining using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as described previously (Dimri et al., 1995) on HT 1080 cultures treated with steroid FG (0.7 μM) or steroid B1 (10 μM) at days 4, 8, and 12.

Solution Hybridization Experiments. The nondenaturing hybridization assay to detect the 3′ telomere G-overhang was performed as described previously (Gomez et al., 2004). Aliquots (2.5 μg) of undigested genomic DNA were hybridized at 50°C overnight with 0.5 pmol of [γ-^{32}P]ATP-labeled (5′-$(CCCTAA)_3$CCC-3′)oligonucleotide (21C) in sodium hybridization buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, and 1 mM EDTA) in a volume of 20 μl. For competition with Pu22myc (5′-GAGGGTGGGGAGGGTGGGGAAG-3′), the reactions were performed in the presence of 10 μM Pu22myc. Reactions were stopped by the addition of 6 μl of loading buffer (20% glycerol, 1 mM EDTA, and 0.2% bromphenol blue). Hybridized samples were size-fractionated on 0.8% agarose gels in 1× TBE buffer containing ethidium bromide (EtBr). Gels were dried on Whatman filter paper. Ethidium fluorescence and radioactivity were determined using a Typhoon 9210 PhosphorImager (GE Healthcare). The procedure allows detection of the amount of single-strand overhang available for hybridization. Results were expressed as the relative hybridization signal normalized to the fluorescent signal of EtBr and represented the mean of three independent experiments.

TRF Analysis. Aliquots of 5 μg of undigested genomic DNA were hybridized at 37°C overnight with 0.5 pmol [γ-^{32}P]ATP-labeled (5′-$(CCCTAA)_3$CCC-3′) oligonucleotide in sodium hybridization buffer in the presence of RsaI and HinfI restriction enzymes in a volume of 20 μl. Reaction was stopped with 2 μl of proteinase K solution (1% SDS and 1 mg/ml proteinase K) and incubated for 30 min at 50°C. Hybridized samples were size-fractionated on 0.8% agarose gels in 1× TBE buffer. The gels were stained with EtBr, washed, and dried on filter paper (Whatman, Maidstone, UK). Ethidium fluorescence and radioactivity were determined and telomeric smears were revealed by using a Typhoon 9210 PhosphorImager (GE Healthcare). The mean length of the TRF corresponds to the peak of the integration curve from three separate experiments.

Results

Stabilization of Telomeric G-Quadruplex Structures by Steroid Derivatives. We initially screened 1000 small molecules, with a broad diversity of structures, using a fluorescence melting assay with a G-quadruplex forming oligonucleotide F21D that mimics 3.5 repeats of the human telomeric motif (Mergny et al., 2001; Gomez et al., 2004). Each compound was first evaluated at 10 μM in a buffer containing 100 mM LiCl and 5 mM KCl. Under these conditions, the F21D probe has an apparent melting temperature ($T_{1/2}$) of 48°C. Ligands that specifically interact with a G-quadruplex increase the melting temperature of the F21D, as evidenced by plotting fluorescence emission versus temperature. Two compounds, malouetine dichloride and funtumine guanylhydrazone (Fig. 1), gave significant variations of $\Delta T_{1/2}$ (>5°C) (Table 1). These two compounds are steroids from the pregnane series substituted at positions 3 and 17. Malouetine is a steroidal alkaloid isolated from the leaves of *Malouetia bequaertiana* E. Woodson (genera Apocynaceae) (Janot et al., 1960), known for its curarizing properties (Huu Laine and Pinto Scognamiglio, 1964; McKenzie, 2000) and its ability to bind double-stranded DNA (Gourevitch et al., 1981). Funtumine guanylhydrazone is a funtumine derivative substi-

tuted with a guanylhydrazone moiety. Funtumine, a steroid isolated from the leaves of *Funtuma latifolia stapf*, was inactive in the fluorescence melting assay ($T_{1/2} < 2°C$ at 10 μM). An epimer of the funtumine guanylhydrazone derivative was reported to present cardiotonic properties (Meyer et al., 1967).

The effect of the two steroids on the stabilities of G-quadruplex structures formed by F21D and F21T (these two probes differ by the nature of the 3′ quencher, dabcyl or tetramethylrhodamine, respectively) in different cation conditions and ligand concentrations were studied. As summarized in Table 1, results obtained with the two oligonucleotides were in qualitative agreement. The steroid FG far more efficiently stabilized quadruplexes than malouetine. In K^+ conditions (i.e., cation conditions; see Table 1) steroid FG induced a 2- to 3-fold higher $\Delta T_{1/2}$ than malouetine (Table 1). It is noteworthy that malouetine had nearly no effect on G-quadruplexes in Na^+ conditions, whereas the steroid FG had only a slightly lower $\Delta T_{1/2}$ in Na^+ than in K^+ ($\Delta\Delta T$ = 2–4°C).

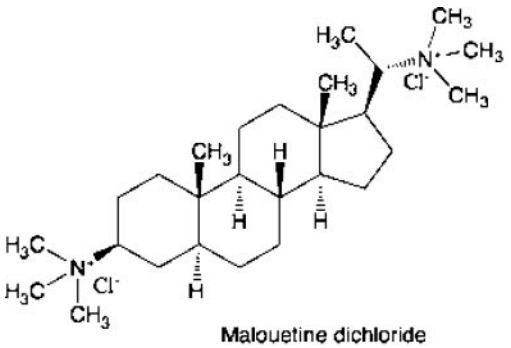
The steroid FG was also compared with 360A, a pyridine dicarboxamide derivative (Pennarun et al., 2005), for the F21D fluorescence melting assay in K^+ conditions. The steroid FG concentration necessary to achieve the same melting curve as that with 2 μM 360A was 20 μM (result not shown). Thus, we estimated that this ligand is approximately 10-fold less potent than 360A.

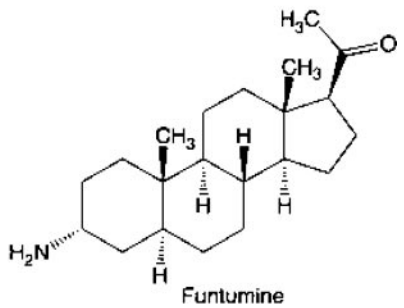
To determine the selectivity of the interaction for the telomeric G-quadruplex relative to duplex DNA, the melting temperature of F21T (0.2 μM) in the presence of steroid FG (5 μM) was monitored in the presence of a 26-nucleotide duplex oligonucleotide (ds26) competitor (at 3, 10, and 30 μM) (Fig. 2). In the presence of 3 μM ds26 oligonucleotide (i.e., a 15-fold molar excess), the stabilization induced by steroid FG was not significantly lowered. Higher competitor concentrations led to a partial loss of stabilization (Fig. 2); at 30 μM ds26 (i.e., a 150-fold molar excess), $\Delta T_{1/2}$ was only + 2°C. Thus, the selectivity is at least 15-fold for G-quadruplex DNA relative to duplex DNA.

G-quadruplex stabilization was also evaluated by the PCR stop assay using the 21G and anti-21G oligonucleotides (Lemarteleur et al., 2004). In this assay, 5′ to 3′ extension by *Taq* polymerase to produce a final double-stranded DNA product is inhibited when the target 21G oligonucleotide folds in a G-quadruplex structure. As shown in Fig. 3, the steroid FG induced a dose-dependent inhibition of PCR product formation. Slight inhibition was evident at 3 μM and was complete at 30 μM. Together, these results indicate that steroid FG is a potent and selective G-quadruplex ligand able to impair telomere repeat elongation and/or replication.

Steroid Ligands Induced Senescence in HT1080 Cells. To examine the effects of malouetine and steroid FG on HT1080 cells, we first determined the drug concentrations that inhibited cell viability after 4 days of culture (Fig. 4A). Results indicated that malouetine had very limited antiproliferative properties with an IC_{50} value higher than 30 μM, whereas steroid FG had a potent inhibitory effect with an IC_{50} equal to 1.8 ($\pm$ 0.3) μM. Steroid FG also had potent antiproliferative effects on H460 lung carcinoma cells (IC_{50} = 2 μM) and to a lesser extent against HeLa cells (IC_{50} = 5.5 μM) and BJhTERT immortalized foreskin fibroblast cells (IC_{50} = 6 μM).

To examine the long-term effects of these ligands on HT1080 cells, we treated cells with concentrations of steroid lower than the IC_{20} and measured growth and cell morphologies. Malouetine (at 3 and 10 μM) and steroid FG (at 0.3 and 0.7 μM) were evaluated. Treated cells were replated every 4 days (with fresh compound added at each replating), and the cumulated population doubling was measured (Fig. 4B). Treatment of HT1080 cells with 10 μM malouetine or 0.7 μM steroid FG induced a population-doubling plateau at day 8, followed by cell growth arrest at days 12 and 16, respectively. The morphologic examination of the cells at the plateau phase showed an increased proportion of flat and giant cells





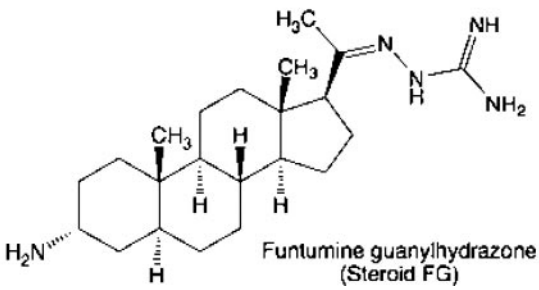


Fig. 1. Chemical structures of steroid derivatives.

with the phenotypic characteristics of senescence (Fig. 5). Noticeable increases in the size of the nuclei and in the number of binucleated cells were observed after steroid treatment (Fig. 5A). These giant cells also stained positively for the senescence associated β-galactosidase activity (SA-β-gal) at day 12 of treatment (Fig. 5B). Cells harvested earlier (i.e., after 4 or 8 days of steroid treatment), did not express SA-β-gal activity (result not shown), suggesting that the senescence occurs at the terminal phase of the culture. At lower ligand concentrations (3 μM malouetine or 0.3 μM steroid FG), HT1080 cells were able to grow continuously up to 36 days with only a slight decrease in doubling time compared with control untreated cells (Fig. 4B).

Steroid Ligands Induced TRF Length Shortening. Treatment of tumor cells with G-quadruplex ligands has been previously reported to induce telomere shortening (Riou et al., 2002). To determine whether steroid ligands inhibited telomere replication, a TRF analysis was performed on DNA samples from steroid-treated HT1080 cells (Fig. 6). For steroid FG (0.7 μM), a TRF shortening was clearly detectable at day 8 of the treatment (Fig. 6A). Telomestatin (2 μM) induced a detectable TRF shortening after 4 days of treatment (Fig. 6A); this concentration provoked a plateau arrest at day 12 (result not shown). In contrast, malouetine (10 μM) treatment did not result in significant telomere shortening at days 4 or 8. The quantification of these experiments indicated a mean TRF length shortening of 300 and 600 base pairs for steroid FG and telomestatin, respectively (Fig. 6B). These results suggest that steroid FG induced a rapid double-stranded telomere shortening associated with its cell growth arrest properties.

At ligand concentrations that do not impair the HT1080 cell growth (for steroid FG, 0.3 μM), a more significant telomere shortening was observed after 20 days of treatment (Fig. 6A). The TRF size decrease corresponded to 600 bases (Fig. 6B). For malouetine (3 μM), a slight decrease of the telomere length was detectable in some experiments (Fig. 6A), but this variation was not significant (Fig. 6B), suggesting that malouetine has at best modest effects on telomere length.

Steroid FG Induces a Decrease in Length of the Telomeric G-Overhang. Recent studies have indicated that the telomeric G-overhang represents one of the direct targets of quadruplex ligands (Gomez et al., 2004; Douarre et al., 2005). We analyzed the effect of steroid FG on the telomeric G-overhangs from HT1080 cells. As shown before, hybridization of a telomeric C-rich probe (21C) under nondenaturing conditions allowed the measurement of the relative single-stranded G-overhang signal in undigested genomic DNA samples (Cimino-Reale et al., 2001; Gomez et al., 2004). Treatment of HT1080 cells with 0.7 μM steroid FG had no effect after 4 days but a strong decrease in the G-overhang signal (55 ± 8.5%) was observed after 8 days (Fig. 7, A and B). In contrast, treatment of HT1080 cells with malouetine (10 μM) only induced a modest decrease in the G-overhang signal (16 ± 11%) after 8 days of treatment (Fig. 7, A and B).

Previous results with telomestatin (Gomez et al., 2004)

TABLE 1

$\Delta T_{1/2}$ of G-quadruplex structures in the presence of steroid ligands and in different cationic conditions

Unless noted, values are provided with 1°C or better accuracy.

Oligonucleotide	$\Delta T_{1/2}$ (°C)			
	F21D (0.2 μM)		F21T (0.2 μM)	
Cation conditions	Na^+ 50 mM Li^+ 50 mM	K^+ 5 mM Li^+ 95 mM	Na^+ 100 mM	K^+ 10 mM Li^+ 90 mM
Steroid FG				
2 μM	3.6	5.1	4.3	8.9
5 μM	4.2	9.5	9.4 ± 1.9	14.1
10 μM	11 ± 2.2	13.8	13.7	18.4
Malouetine				
2 μM	0.9	3	N.S.	3.5
5 μM	1.3	4.6	N.S.	3.8
10 μM	1.4	6.5	1.1	7

N.S., no stabilization.

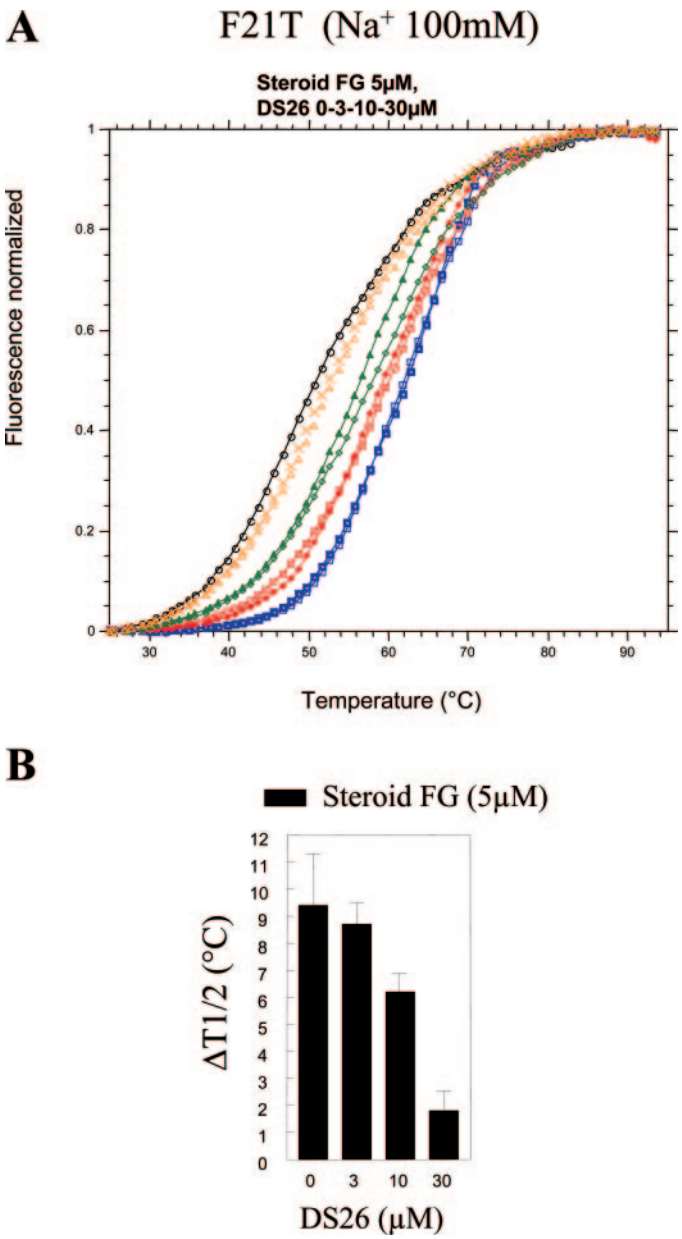


Fig. 2. Effect of steroid FG on melting of G-quadruplex structure as analyzed by fluorescence. A, fluorescence-melting behavior of F21T oligonucleotide (0.2 μM) in 10 mM lithium cacodylate buffer, pH 7.0, and 100 mM NaCl in the absence (black curve) or presence (blue curve) of steroid FG (5 μM) and in the presence of 3 μM (red curve), 10 μM (green curve), and 30 μM (yellow curve) of ds26 double-stranded oligonucleotide competitor. B, $\Delta T_{1/2}$ for steroid FG (5 μM) in the presence of ds26 competitor.

indicate that the apparent decrease in G-overhang signal may result from the stabilization of the quadruplex, making it less prone to hybridization to its complementary C-rich probe. To exclude this possibility, we performed the following experiment: Steroid FG (3–100 μM) was added to purified DNA just before the hybridization reaction. This resulted in a detectable inhibition of the G-overhang signal, only at a concentration equal to 30 μM, but in a cation-dependent manner (Fig. 8B). The inhibition was almost completely reversed in presence of an oligonucleotide that adopts a G-quadruplex structure (Pu22myc, 10 μM) (Fig. 8, A and B) (Gomez et al., 2004). Because the in vitro inhibition of the G-overhang hybridization assay is only observed at a 50-fold higher concentration of steroid FG than that used to treat cells (0.7 μM), and the cellular effect was detected after a lag time of 8 days, we reasonably concluded that the ligand induced an effective degradation of the telomeric G-overhang in vivo rather than a modification of the G-overhang conformation that interferes with the hybridization reaction.

Steroid FG Induced Anaphase Bridge Formation in HT1080 Cells. Alterations of telomere capping have been reported during the treatment with G-quadruplex ligands (Izbicka et al., 1999a; Leonetti et al., 2004; Burger et al., 2005; Douarre et al., 2005; Pennarun et al., 2005). These alterations may be evidenced by the formation of anaphase bridges. We examined the anaphase bridge formation in steroid-treated HT1080 cells. Typical images of anaphase bridges were obtained in HT1080 cells treated for 24 h with 0.7 μM steroid FG, which represented 59 $\pm$ 6% of the anaphases examined (n = 50), compared with 0% for controls (Fig. 8C). It is noteworthy that anaphase bridge formation is an early event, in that it is observed within 24 h, before any evidence for antiproliferative or cytotoxic activity. Treatment for 4 days strongly reduced the amount of mitotic cells in the preparation and therefore the number of anaphase bridges (result not shown). Thus, anaphase bridge formation seems to precede the antiproliferative effects of the ligand. These data suggest that steroid FG is able to induce the degradation of the telomeric G-overhang and to increase the formation of anaphase bridges, indicating that this ligand induces an alteration of the telomere capping in HT1080 cells.

Expression of GFP-POT1 Induced Resistance to Steroid FG. Expression of a green fluorescent protein-POT1 fusion (GFP-POT1) in HT1080 cells increases telomere length and G-overhang signal (Colgin et al., 2003; Gomez et al., 2006b). This cell line model is resistant to the cellular effect of telomestatin (Gomez et al., 2006b). We have examined whether the overexpression of POT1 modulates the cytotoxic activity of steroid FG for short-term treatment (48 h). HT1080GFP-POT1 cells had a noticeable resistance to the effects of steroid FG, compared with parental HT1080 cells (Fig. 9). These results suggest that the cellular effect of the compound is due to a direct effect on telomeres.

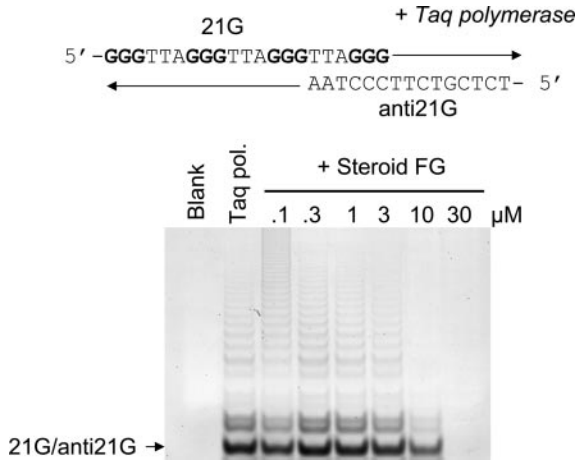


Fig. 3. Effect of steroid FG on the formation of telomeric 21G quadruplex by a PCR-stop assay. Upper part of the figure illustrates the principle of the assay. The 21G oligonucleotide was amplified with a complementary oligonucleotide (anti-21G) overlapping the last G-repeat by *Taq* polymerase. Lower part of the figure shows the effect of increasing concentrations of the steroid FG on the PCR-stop assay. The double-stranded 21G/anti21G DNA formed by PCR amplification is indicated by an arrow.

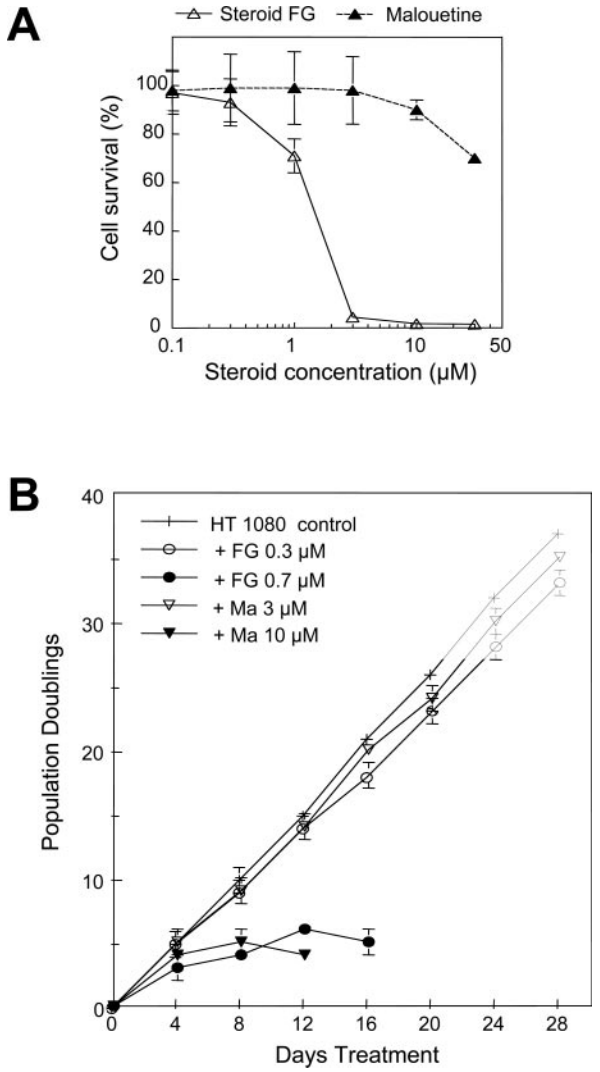


Fig. 4. Short-term and long-term effects of steroid ligands on HT1080 cell line. A, survival curves of HT1080 cells treated for 4 days in the presence of various steroid ligand concentrations. B, proliferation curves for long-term cultures of HT1080 cells in the absence or presence of steroid FG (FG, 0.3 and 0.7 μM) or malouetine (Ma, 3 and 10 μM). The proliferation is expressed in population doublings as a function of days of culture. A growth arrest of the culture appears at day 8 for malouetine (10 μM) and day 12 for steroid FG (0.7 μM). No arrest was observed at lower ligand concentrations (0.3 and 3 μM) even after 28 days of culture.

Discussion

It has been demonstrated previously that G-quadruplex stabilizing compounds derived from polycyclic structures from natural origin or from synthetic chemistry efforts are potent telomere-interacting agents in vitro and produce senescence or apoptosis in several cancer cell lines (Kerwin, 2000; Mergny et al., 2002; Neidle and Parkinson, 2002; Pendino et al., 2006). We show here that steroid diamine derivatives are also potent G-quadruplex ligands that interact with the human telomeric sequences and inhibit telomere elongation. It is noteworthy that these derivatives differ greatly from previously characterized G-quadruplex ligands because these compounds are nonplanar and nonaromatic. Structural studies will be required to determine their precise mode of interaction with telomeric G-quadruplexes.

Steroidal diamines exert a variety of effects on cells (Mahler and Baylor, 1967) and include substances from natural origin, such as the plant alkaloids irehdiamine A and malouetine (Janot et al., 1960; Goutarel et al., 1967). Their chemical similarity to hormonal steroids suggests that these compounds are able to interact with DNA, as directly shown for malouetine and dipyrandium (Gourevitch et al., 1981; Hui et al., 1989). Biophysical studies led to the conclusion that these derivatives partially insert between base pairs and induce a kink in AT-rich DNA structures (Hui et al., 1989). DNA interacting properties have been the basis for the discovery of several classes of G-quadruplex ligands, including porphyrin, acridine, and ethidium derivatives (Harrison et al., 1999; Izbicka et al., 1999b; Koeppel et al., 2001; Kerwin et al., 2002; Guittat et al., 2003), and our results seems to confirm this rule. The chemical modification of these DNA-interacting agents (i.e., trisubstituted acridines for BRACO-19), led to an important improvement in the selectivity for G-quadruplexes over duplex DNA (Burger et al., 2005).

Competition with a double-stranded oligonucleotide (ds26) in the G-quadruplex fluorescence melting assay indicated that malouetine had a poor selectivity but that the steroid FG

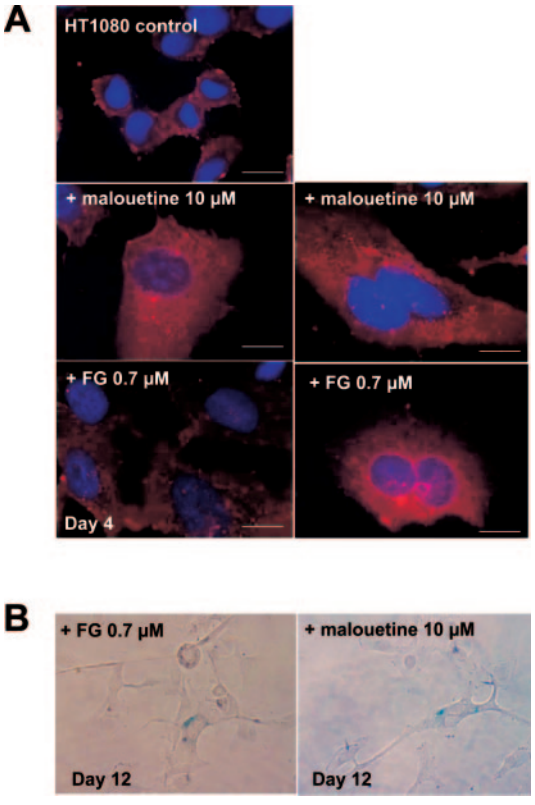


Fig. 5. Treatment with steroid ligands induce morphological cell alterations and β-galactosidase expression in HT1080 cells. A, HT1080 cells treated with malouetine (10 μM) or steroid FG (0.7 μM) for 4 days were examined for morphological modifications. Fluorescence for β-actin (red) and for DAPI (blue) were determined on fixed cells and merged. Ligand treatment induced the formation of giant cells with increased cytoplasm and nuclei (left) and the formation of cells with two nuclei (right). B, SA-β-galactosidase activity in HT1080 cells treated with steroid FG (0.7 μM) or malouetine (10 μM) for 12 days. Cells observed by phase contrast microscopy show the appearance of a blue coloration and morphologic modifications characteristics of senescent cells.

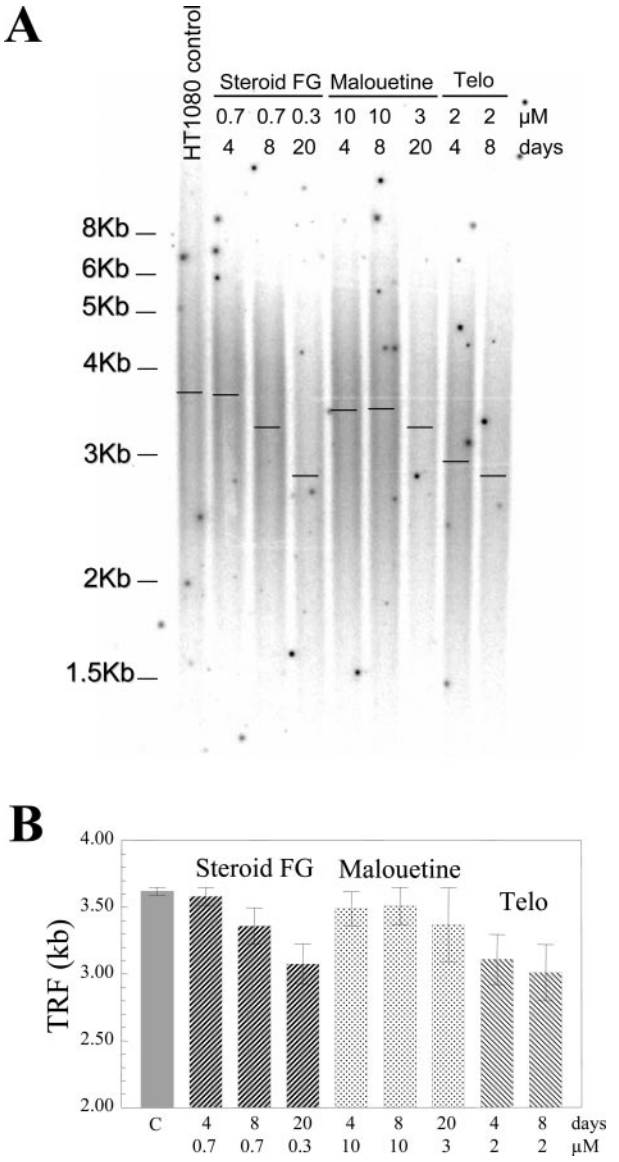


Fig. 6. Effects of steroid ligands on double-stranded telomere length in HT1080 cells. A, TRF analysis of DNA samples from HT1080 cells untreated (control) or treated with steroid FG, malouetine, or telomestatin (Telo) for 4, 8, and 20 days at indicated concentrations. The mean TRF length is indicated by a horizontal line and corresponds to the peak of the integration curve measured relative to DNA molecular weight markers. B, mean TRF values (kb) from three independent experiments after steroid FG, malouetine, and telomestatin (Telo) treatment for 4, 8, and 20 days, at their respective indicated concentrations. Control untreated HT1080 cells ("C") are shown on the left.

selectively bound to G-quadruplex rather than duplex DNA. Although the selectivity was less than that obtained for telomestatin or pyridine-dicarboxamide derivatives (Rosu et al., 2003b; Lemarteleur et al., 2004; Pennarun et al., 2005), these results indicate that it is possible to improve the quadruplex/B DNA selectivity for steroid ligands (Goutarel et al., 1967).

Preliminary experiments using a Pu22myc oligonucleotide, corresponding to the G-quadruplex-forming sequence from the c-myc promoter, indicated that steroid FG does not discriminate between these two types of G-quadruplexes (B. Brassart, unpublished results), similar to many previously reported G-quadruplex ligands (Lemarteleur et al., 2004). However, because the stabilization was very dependent on the nature of the cation (Table 1), one might propose that these molecules have a strong preference for the potassium over the sodium form of the telomeric quadruplex. This observation makes the recent determination of the potassium form of the telomeric quadruplex (Ambrus et al., 2006; Xu et al., 2006) very important, as rational drug design approaches may now be initiated on this physiologically relevant quadruplex.

Biochemical assays indicated that the steroid ligands are less potent than the pyridine dicarboxamide derivative 360A or telomestatin (Lemarteleur et al., 2004; Pennarun et al., 2005). For example, malouetine induced the senescence of HT1080 cells at a 20-fold higher concentration than did telomestatin. However, steroid FG induced senescence on HT1080 cells at submicromolar concentrations. Steroid FG exhibited all the characteristics of a telomere interacting agent (double-stranded telomere erosion, G-overhang degradation, anaphase bridge induction), previously reported for other potent G-quadruplex ligands (Izbicka et al., 1999b; Riou et al., 2002; Gomez et al., 2004; Leonetti et al., 2004;

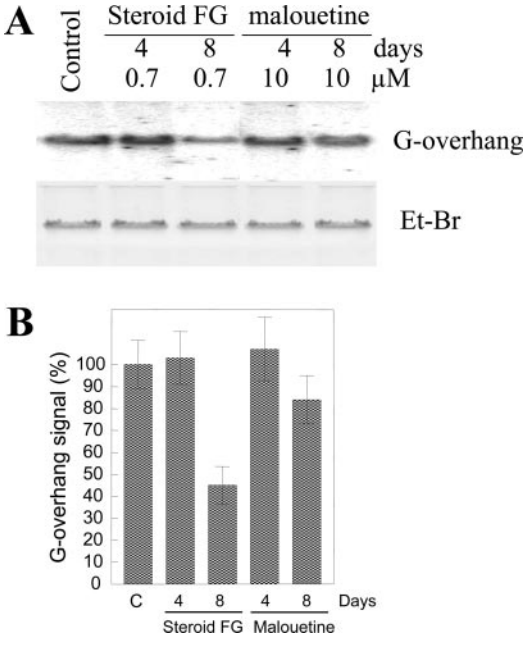


Fig. 7. Effect of steroid ligands on the telomeric G-overhang in HT1080 cells, evaluated by nondenaturing solution hybridization with 21C telomeric probe (5′-CCC(TAACCC)$_3$-3′). A, HT1080 cells treated with steroid FG (0.7 μM) or malouetine for 4 and 8 days, as indicated. Steroid FG induced a strong decrease of the 3′ G-overhang signal in HT1080 cells treated with the ligand for 8 days, compared with control untreated cells (*G-overhang*, signal of the gel with the CCC(TAACCC)$_3$ probe; *Et-Br*, ethidium bromide staining of the gel). B, quantification of the steroid effect in HT1080-treated cells. The G-overhang hybridization signal was normalized relative to the Et-Br signal. The results are expressed relative to untreated DNA (defined as 100%) and correspond to the mean (± S.D.) of three independent experiments, including the one presented in A.

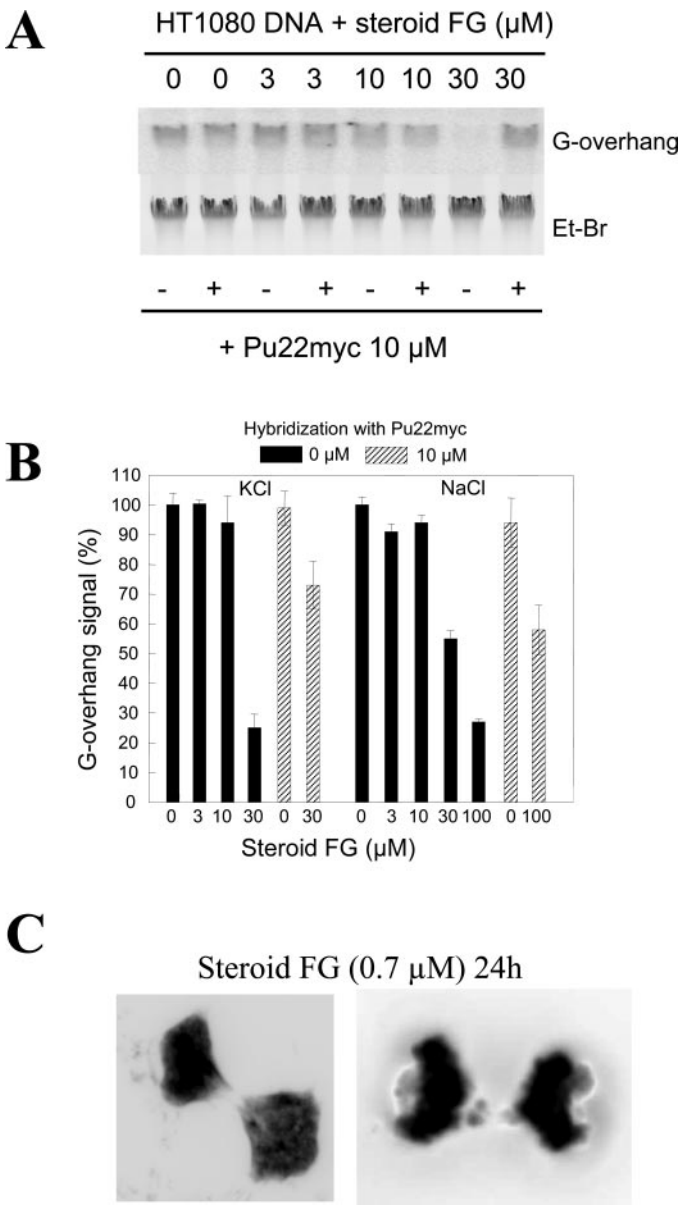


Fig. 8. Effect of steroid FG on the telomeric G-overhang on purified genomic DNA, evaluated by nondenaturing solution hybridization with the telomeric probe 21C. A, steroid FG (3–30 μM) inhibits the in vitro hybridization reaction to the G-overhang from purified HT1080 DNA. The inhibition is detectable at 30 μM. A competition with Pu22myc (10 μM) reverses the hybridization inhibition (*G-overhang*, signal of the gel with the CCC(TAACCC)$_3$ probe; *Et-Br*, ethidium bromide staining of the gel). B, quantification of the steroid FG effect against purified HT1080 DNA with or without competition with Pu22myc in the presence of KCl (50 mM) or NaCl (50 mM). The inhibition was more efficient in KCl and was reversed by Pu22myc competition in both hybridization conditions. G-overhang hybridization signal is normalized relative to the Et-Br signal. The results are expressed relative to untreated DNA (defined as 100%) and correspond to the mean (± S.D.) of three independent experiments. C, steroid FG induces anaphase bridge formation. Representative images of anaphase bridges in HT1080 cells treated for 24 h with steroid FG (0.7 μM). Cells were stained DAPI and images recorded. Steroid FG induces a 59% (± 6%) increase in anaphase bridge formation, compared with control untreated cells (0%).

Burger et al., 2005; Douarre et al., 2005; Pennarun et al., 2005; Tahara et al., 2006). We observed differences between in vitro and cellular effects (Figs. 7 and 8) suggesting that steroid FG might have better cellular penetration or intracellular distribution than telomestatin or 360A. It is noteworthy that bis-guanidinium cholesterol derivatives have been developed as transfection agents, because of their membrane solubility and the ability of the two guanidinium functions to bind the phosphate group of DNA (Vigneron et al., 1996).

Steroid FG also had short-term antiproliferative effects. Preliminary observations indicated that the steroid FG (3 μM) induced apoptosis and DNA damage in HT1080 cells after 24-h treatment (B. Brassart, unpublished results). This feature was previously reported for other G-quadruplex ligands with mild selectivity for quadruplex relative to duplex DNA, such as triazine derivatives (Riou et al., 2002; Douarre et al., 2005). The guanylhydrazone side chain of steroid FG may lead to polyamine biosynthesis inhibition (Davidson et al., 1998). Therefore, we cannot exclude that this ligand has another mechanism of action related to a target in addition to G-quadruplexes. It is noteworthy that the overexpression of POT1 in HT1080 cells induced a significant resistance to the short-term effects of steroid FG emphasizing that telomere targeting contributes, at least in part, to the cytotoxic effect of the compound. On the other hand, because of the presence of many G-quadruplex-forming sequences in other parts of the genome (Huppert and Balasubramanian, 2005; Todd et al., 2005), it is possible that the ligand impairs gene transcription or DNA replication triggering the apoptotic response. Further experiments are undertaken to answer this point.

In conclusion, we reported here a new class of steroid telomere-interacting agents that bind G-quadruplexes and induce telomere uncapping. The steroid FG may have potential for antitumor treatment. The ease with which this steroid can be obtained, together with known chemistry for accessing modifications to this molecule, will allow improvements to the selectivity and the potency of these derivatives.

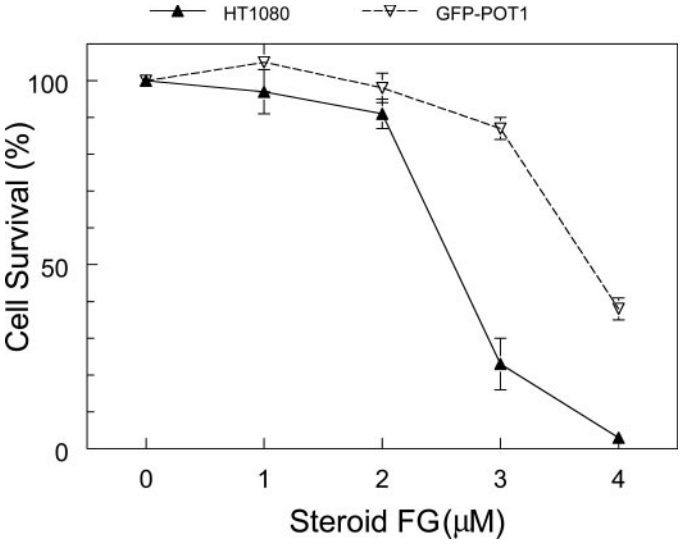


Fig. 9. Expression of GFP-POT1 in HT1080 cells protected cells from the short-term growth inhibitory effect of steroid FG. Survival curves (MTT assay) of HT1080 and HT1080GFP-POT1 cells treated for 48 h in the presence of various steroid FG concentrations, each point in quadruplicate. The IC_{50} values for two independent experiments were 1.8 and 2.5 μM for HT1080 cells and 3.5 and 3.7 μM for HT1080GFP-POT1 cells.

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