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Natural and synthetic G-quadruplex interactive berberine derivatives

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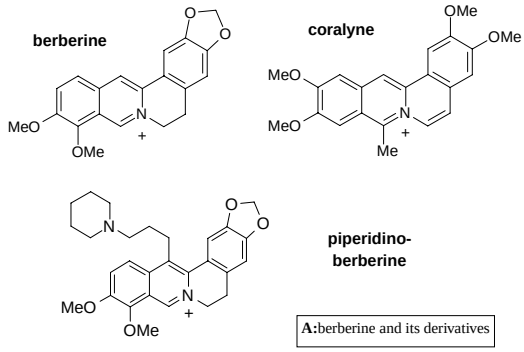
Abstract—The interaction of the natural alkaloid berberine with various G-quadruplex DNA structures and its ability to inhibit telomerase have been examined and compared with those of a synthetic piperidino derivative and the related compound coralyne. The results show that these molecules have selectivity for G-quadruplex compared to duplex DNA, and that their aromatic moieties play a dominant role in quadruplex binding.

G-rich DNA sequences can adopt non-duplex structures based on the association of four guanine bases in a stable hydrogen-bonded arrangement, the G-quartet.[1] Several G-quartets can be stacked on one another and held together by intervening sequences to form G-quadruplex structures. G-quadruplex structures at telomeres may play an important role in telomere maintenance, both from the structural and the functional points of view, where their formation may be in equilibrium with the binding of hPOT1 and other proteins to the 3′-end telomeric DNA single-stranded overhang.[2] Molecules able to induce G-quadruplex structures have been intensively studied for their ability to inhibit telomerase and thereby act as potential anticancer agents. A wide range of such small molecules have been characterized, including acridines, triazines, porphyrins, perylenes and anthraquinones.[3–5]

Berberine is an antibiotic alkaloid originating from Chinese herbal medicine;[6] its anti-bacterial activity has been demonstrated against many species.[7] The drug



Scheme 1.

Keywords: G-quadruplex; Telomerase; Alkaloid; Berberine.
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was subsequently screened for anti-cancer activity following evidence of anti-neoplastic properties.[8] More recently, berberine was shown to inhibit telomere elongation[9] and to bind to G-quadruplex DNA.[10] Coralyne is a synthetic analogue of berberine[11] that also binds to triplex DNA.[12] Competition dialysis experiments[10] have shown that both compounds have selectivity for triplex DNA, and to a minor extent for quadruplex DNA compared to duplex DNA.

Berberine and coralyne include an N^+-containing aromatic moiety (Scheme 1A), which appears suitable for stacking interactions with a G-quartet. In this study, we have examined their ability to induce and stabilize various G-quadruplex structures, including the human G-quadruplex, as well as their efficiency in inhibiting telomerase. We have also examined the effect of adding a side chain analogous to that of the previously studied *N*,*N*′-bis[2-(1-piperidino)ethyl]-3,4,9,10-perylene-tetracarboxylic diimide (PIPER)[13,14] onto the berberine scaffold. Molecular modelling[15] studies of interactions between berberine derivatives and the human parallel G-quadruplex structure[16] indicate that berberine is stacked on the terminal G-tetrad of the quadruplex (Fig. 1), and the side chain of piperidino-berberine can interact with one of the four grooves of the quadruplex.

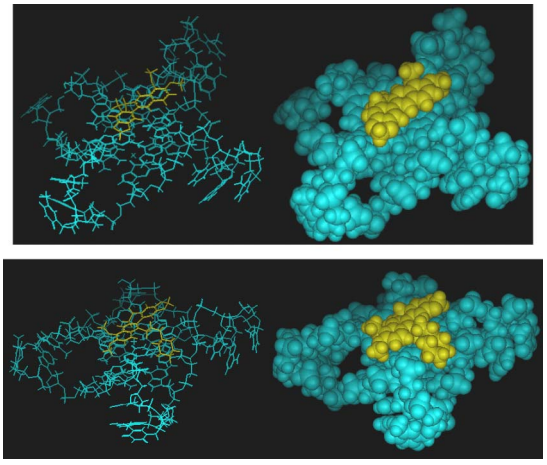
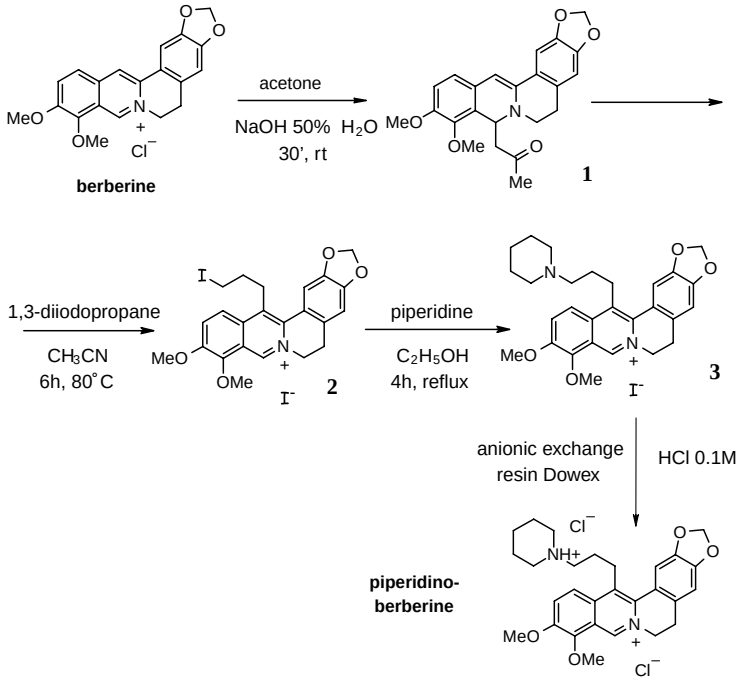


Figure 1. Models for the complexes of berberine (top) and piperidino-berberine (bottom) with a monomeric G-quadruplex (blue) and ligand molecules (yellow).

The synthetic strategy, reported in Scheme 2, uses as a key intermediate the previously described acetonyl-berberine **1**, which can be readily obtained by condensation of berberine with acetone.[17] Compound **1** was transformed to **2** by displacement of the acetonyl group with 1,3-diiodopropane. Substituting the iodine atom on **2** with piperidine gave the berberine derivative **3** with the desired side chain.[18] This was isolated as the chloride salt using an anion exchange resin (Scheme 2).

The ability of the three compounds to form inter- and intramolecular G-quadruplex structures was investigated by polyacrylamide gel electrophoresis (PAGE); Fig. 2),[19] using the DNA oligomers 2HTR and TSG4 (Scheme 1B). 2HTR is able to form only dimeric and/or tetrameric intermolecular G-quadruplex structures;[12–14] TSG4 can also form an intramolecular G-quadruplex structure and can act as a substrate for telomerase elongation in a modified TRAP assay.[14,20] PAGE shows that berberine and its analogues are able to induce G-quadruplex dimeric structures (Fig. 2A), but each to a different extent (Fig. 2B). Coralyne is the most efficient in inducing an intermolecular G-quadruplex. The three compounds are unable to induce a monomeric G-quadruplex structure with TSG4 at



Scheme 2.

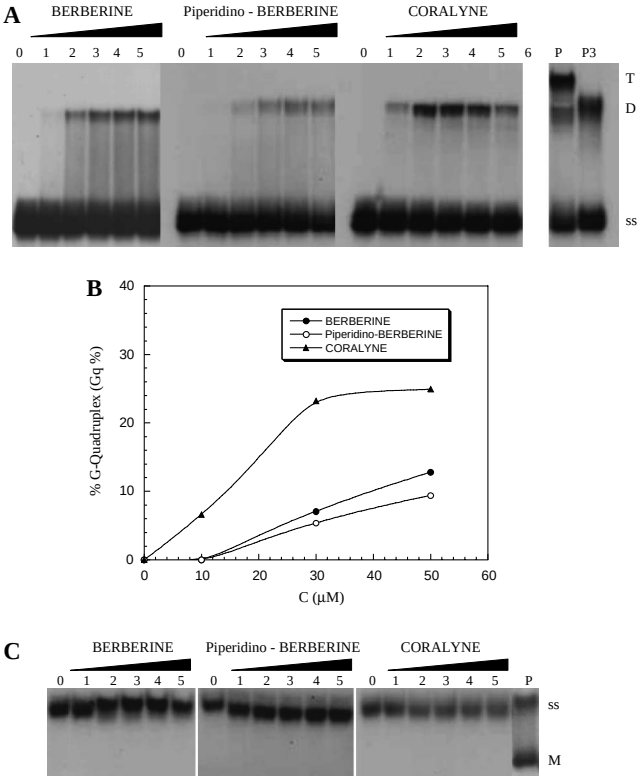


Figure 2. G-quadruplex structure formation induced in 2HTR and TSG4 oligonucleotides by berberine and its derivatives studied by native polyacrylamide gel electrophoresis according to the described experimental procedure. Major bands are identified as single stranded DNA (ss), dimeric (D), tetrameric (T) and monomeric (M) G-quadruplex structures, according to previous studies.[12–14] (A) Experiments were performed at 12 μM oligonucleotide (2HTR) concentration in MES–KCl buffer (pH 6.5) at increasing concentrations of the berberine derivatives and with no drug (lane 0). In lanes P and P3, 2HTR was incubated with 30 μM PIPER and PIPER3, respectively.[13,14] Drug concentrations: 10 μM (lane 1), 30 μM (2), 50 μM (3), 70 μM (4) and 100 μM (5). (B) Percentage of G-quadruplex structures formed (G_q% represents the ratio of the intensity of the relative band on the electrophoresis gel with respect to the total amount of DNA, obtained by Instant Imager[13]) as a function of drug concentration (C (μM)) relative to the band shift assays reported in (A). (C) Same as in (A) but using TSG4 oligonucleotide and a 5 mM KCl–10 mM MES buffer (pH 6.5).

5 mM K^+ concentration (under these experimental conditions no G-quadruplex structure is formed in the absence of drug, while monomeric G-quadruplex would be totally folded in 50 mM KCl:[14] Fig. 2C).

A fluorescence resonance energy transfer (FRET)-based method was used to probe the thermal stability of G-quadruplexes, using fluorescein and tetramethyl-rhodamine conjugates attached to the 5′- and 3′-ends of the oligonucleotide as donor and acceptor moieties,[21] in order to monitor the melting transitions.[22] The oligonucleotide (F21T) used was a 21-mer human telomeric sequence [$GGG(TTAGGG)_3$].[23] The K^+ concentration and other experimental conditions[24] are analogous to those used for the crystallization of the sequence [$AGGG(TTAGGG)_3$].[16] An increase in the G-quadruplex melting temperature (T_m) was observed (Fig. 3)

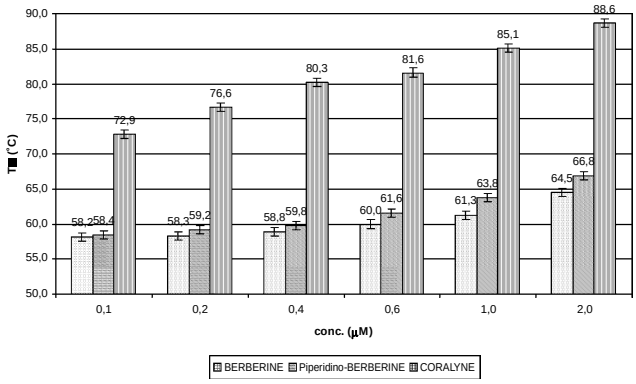


Figure 3. Melting temperatures for F21T at increasing concentrations of berberine, piperidino-berberine and coralyne, as derived from the first derivative of the melting curves obtained by FRET experiments.

with all three compounds. Piperidino-berberine increases G-quadruplex stability compared to berberine. Coralyne, which has a fully aromatic core, is much more active than either. The concentration of coralyne was kept under 2 μM because above this concentration its intrinsic fluorescence becomes significant. We find that the berberine derivatives are able to stabilize a preformed G-quadruplex structure at 50 mM K^+, but are unable to induce an analogous monomeric G-quadruplex structure with 5 mM K^+ (Fig. 2C).

The selectivity of G-quadruplex interacting compounds for G-quadruplex DNA with respect to duplex DNA is relevant to the potential therapeutic applications of these molecules.[25–27] We have analyzed the effect of the three berberine derivatives on the melting temperature of calf thymus DNA,[28] and compared them to the behaviour of ethidium bromide, which also binds to G-quadruplexes, but with low affinity and specificity. Ethidium bromide increases the T_m of CT DNA by ca. 3 °C at a 0.1 drug:DNA molar ratio (R) and by 11 °C at a R of 1 (see Supplementary data). At the same ratios, berberine derivatives do not show any significant effect ($\Delta T_m < 1$ °C), with the exception of coralyne, which produces a slight increase (ΔT_m ca. 3 °C), at R = 1. It is worth noting that at this ratio berberine derivatives show very weak interaction with genomic duplex DNA, while they show significant quadruplex stabilization at lower ratios (in the FRET experiments the DNA concentration was 0.2 μM).

The telomeric repeat amplification protocol (TRAP) assay was used to measure telomerase inhibition.[29] TSG4 oligonucleotide (Scheme 1B) was used as a telomerase substrate:[20] it is able to form an intramolecular G-quadruplex prior to telomerase synthesis, since the K^+ concentration used in the TRAP assay (68 mM) is in the appropriate range for G-quadruplex formation. However in the absence of a G-quadruplex stabilizing ligand, it is efficiently unfolded and extended by telomerase.[14,20] TRAP assays performed at increasing concentrations of the three berberine derivatives are shown in Figure 4: coralyne shows higher telomerase inhibitory activity, with an IC_{50} value of ca. 70 μM, compared to >130 μM for the other two.

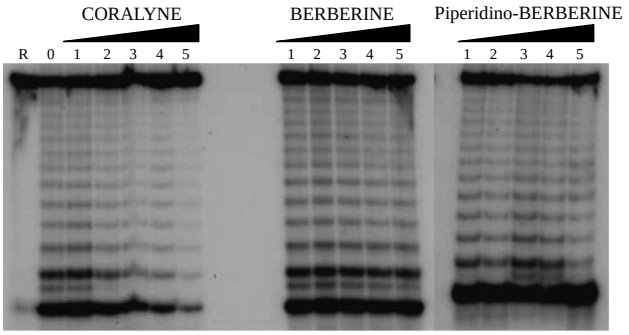


Figure 4. Inhibition of human telomerase by berberine and its derivatives by the telomerase repeat amplification protocol (TRAP) assay, using TSG4 as substrate. In lane 0, no drug was added, in lane R cell extract was not added. The considered drugs concentrations were 30 μM (lane 1), 50 μM (2), 70 μM (3), 100 μM (4) and 130 μM (5).

A comparison between PAGE results using an oligonucleotide (2HTR) with two and one (TSG4) with four G-rich repeats shows that berberine derivatives are selective in inducing intermolecular G-quadruplex with respect to intramolecular G-quadruplex. Analogous selectivity was previously observed for telomestatin and a porphyrin derivative,[30] which was correlated with their different biological effects. The thermal stabilization of a pre-formed intramolecular G-quadruplex shows that these compounds are able to stabilize rather than to induce G-quadruplex intramolecular structure. In both cases, coralyne is the most active compound of this series, probably due to its fully aromatic core. This correlates with the TRAP results, which show that coralyne is the most efficient telomerase inhibitor. It is notable that all the compounds studied show very weak interactions with genomic duplex DNA, suggesting high selectivity for G-quadruplex over duplex DNA (as shown by competition dialysis[31]). This is important if these ligands are to be used as probes for G-quadruplex structures in biological systems, as well as for their development as potential drugs.

Acknowledgments

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Supplementary data

Supplementary data and can be found, in the online version, at doi:10.1016/j.bmcl.2005.12.001.

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15. All modelling used the InsightII package and the X-ray structure of the 22-mer human telomeric DNA sequence $AGGG(TTAGGG)_3$ (PDB code 1KF1).[16] The binding site was defined as the external 3′ G-quartet plane.[32] Initially 200 ligand orientations were randomly centred on the G-quadruplex structure, charges were not considered and the complexes were minimized for 500 steps. The 75 lowest energy structures were then subjected to simulated annealing, then 500 steps of minimization was followed by molecular dynamics over a period of 10 ps. The resulting structures were then minimized.
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18. Berberine and coralyne were purchased from Sigma–Aldrich and used without further purification. Piperidino-berberine: 3 g of berberine chloride was dissolved in 18 ml of water and 4.5 ml of NaOH 50% solution was added. While stirring, 3.0 ml of acetone was added dropwise. After stirring for 30 min at room temperature, the reaction mixture was filtered, washed with 80% methanol to give 2.65 g of **1** (83.5% yield) and dissolved in 75 ml CH_3CN and 7.4 ml of 1,3-diiodopropane was added, then stirred at 80 °C for 6 h. After cooling, the product was filtered, washed repeatedly with acetonitrile and purified by column chromatography on silica gel ($CHCl_3$–MeOH 9:1 and then 8:2) to give 370 mg (8.7% yield) of **2**. This was reacted for 4 h with 0.86 ml of piperidine in 36 ml of refluxing ethanol. After cooling, the reaction mixture was dried under vacuum and the crude product was purified by silica gel column chromatography equilibrated with 10% of water ($CHCl_3$) to give 265 mg (77% yield) of **3**. The hydrochloride was obtained by dissolving it in 0.1 M HCl and passed through a Dowex1 strong basic anion exchange resin. The acid solution was concentrated under vacuum, adding repeatedly an equal volume of chloroform, until a powder was obtained and then dried under vacuum. 8-acetonyldihydroberberine (**1**): ^1H NMR (300 MHz, $CDCl_3$): δ 7.13 (s, 1H, aromatic H), 6.77 (d, J = 8 Hz, 1H, aromatic H), 6.75 (d, J = 8 Hz, 1H, aromatic H), 6.57 (s, 1H, aromatic H), 5.94 (m, 2H, O-CH_2-O), 5.89 (s, 1H, aromatic H), 5.32 (dd, X part of ABX system, 1H, J_{AX} = 7Hz, J_{BX} = 4 Hz, CH-N), 3.89 (s, 3H, CH_3-O), 3.84 (s, 3H, CH_3-O), 3.34 (m, 2H, CH_2-N), 3.07 (dd, A part of ABX system, 1H, J_{AX} = 7 Hz, J_{AB} = 15 Hz, N-CH-CO), 2.79 (m, 2H, CH_2-Ar), 2.41 (dd, B part of ABX system, 1H, J_{BX} = 4 Hz, J_{AB} = 15 Hz, N-CH-CO), 2.05 (s, 3H, CH_3-CO). 13-(3-Iodopropyl)berberine iodide (2): ^1H NMR (300 MHz, $CDCl_3$): δ 10.26 (s, 1H, aromatic H), 8.00 (d, J = 9 Hz, 1H, aromatic H), 7.89 (d,

J = 9 Hz, 1H, aromatic H), 7.09 (s, 1H, aromatic H), 6.89 (s, 1H, aromatic H), 6.10 (s, 2H, O-CH_2-O), 5.08 (br s, 2H, CH_2-N^+), 4.37 (s, 3H, CH_3-O), 4.07 (s, 3H, CH_3-O), 3.51 (t, J = 8Hz, 2H, CH_2-Ar), 3.32 (t, J = 6 Hz, 2H, CH_2-Ar), 3.23 (t, J = 5 Hz, 2H, CH_2-I), 2.30 (m, 2H, $CH_{2(propyl)}$). 13-[3-(1-Piperidino)propyl]berberine iodide (3): ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.69 (s, 1H, aromatic H), 8.20 (d,J = 9 Hz, 1H, aromatic H), 7.93 (d, J = 9 Hz, 1H, aromatic H), 7.10 (s, 1H, aromatic H), 6.88 (s, 1H, aromatic H), 6.15 (s, 2H, O-CH_2-O), 4.78 (br s, 2H, CH_2-N^+), 4.31 (s, 3H, CH_3-O), 4.09 (s, 3H, CH_3-O), 3.63 (t, J = 7Hz, 2H, CH_2-Ar), 3.43 (m, 2H, CH_2-Ar), 3.11 (br s, 4H, CH_2-$N_{piperidine}$), 2.98 (t, J = 8Hz, 2H, CH_2-$N_{piperidine}$), 2.35 (br s, 2H, $CH_{2(propyl)}$), 1.87 (br s, 4H, CH_2-$\beta_{piperidine}$), 1.60 (br s, 2H, CH_2-$\gamma_{piperidine}$). ^{13}C NMR (300 MHz, $CDCl_3$): δ 150.55 (ar.), 150.31 (ar.), 147.68 (ar.), 145.22 (ar.), 144.80 (ar.), 136.51 (ar.), 133.02 (ar.), 132.68 (ar.), 125.62 (ar.), 122.16 (ar.), 121.01 (ar.), 119.86 (ar.), 109.74 (ar.), 108.36 (ar.), 102.32 (ar.), 68.16, 62.43, 57.84, 56.88, 55.65, 52.77, 28.25, 26.88, 25.27, 23.13, 21.75. MS (ESI) m/z: 461.0 [M^+] (calcd for $C_{28}H_{33}N_2O_4$ M = 461.2). 13-[3-(1-Piperidino)propyl]berberine hydrochloride (piperidino-berberine): ^{1}H NMR (300 MHz, D_2O, HDO suppressed): δ 9.57 (s, 1H, aromatic H), 8.03 (d, J = 9 Hz, 1H, aromatic H), 7.97 (d, J = 9 Hz, 1H, aromatic H), 7.13 (s, 1H, aromatic H), 6.94 (s, 1H, aromatic H), 5.99 (s, 2H, O-CH_2-O), 4.75 (m, 2H, CH_2-N^+), 3.98 (s, 6H, CH_3-O), 3.42 (t, J = 7 Hz, 2H, CH_2-Ar), 3.25 (m, 2H, CH_2-Ar), 2.98 (br s, 4H, CH_2-$N_{piperidine}$), 2.72 (t, J = 9 Hz, 2H, CH_2-$N_{piperidine}$), 1.98 (m, 2H, $CH_{2(propyl)}$), 1.74 (m, 4H, CH_2-$\beta_{piperidine}$), 1.55 (m, 2H, CH_2-$\gamma_{piperidine}$). Elemental analysis calculated for $C_{28}H_{34}N_2O_4Cl_2{\cdot}CHCl_3$: 53.3% C, 5.4% H, 4.3% N; found: 54.1% C, 6.1% H, 4.4% N.

19. The DNA oligomers 2HTR and TSG4 (Scheme 1B) were 5′-end labelled by T4 polynucleotide kinase (2.5 U/μg DNA) for 30 min at 37 °C in 50 μl buffer containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 30 μCi [γ-^{32}P]ATP. The reaction was quenched by heating the mixture at 65 °C for 10 min. The reaction mixture was then purified through phenol/chloroform and chloroform extractions. After precipitation with ammonium acetate and ethanol and washing with 80% ethanol, the DNA was dried and then dissolved in water. Radioactivity was quantified using a Beckman LS 5000 TD Scintillation System. Oligonucleotides (8 or 12 μM) were heated at 95 °C for 10 min and quickly cooled in ice to disrupt any preformed structures. They were then incubated for 2 h at 30 °C in the presence of different drug concentrations, in MES–KCl buffer (10 mM MES, pH 6.5, 50 mM KCl). In the case of TSG4 a lower KCl concentration was used ([KCl] = 5 mM), because at higher K^+ concentrations the G-quadruplex monomeric structure is also formed in the absence of drugs. Complexes and structures formed after incubation were studied by native PAGE (15% polyacrylamide gel, TBE 0.5×, KCl 20 mM, run overnight at 4 °C).[12–14]
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24. DNA was dissolved in a stock 20 μM solution in 10 mM TE (Tris, EDTA) buffer (pH 8.0); further dilutions were carried out in 50 mM potassium cacodylate buffer (pH 7.4). The FRET assay was modified for a 96-well format with a DNA Opticon Engine (MJ Research) with excitation at 450–495 nm and detection at 515–545 nm. Fluorescence readings were taken at intervals of 0.5 °C over the range 30–100 °C, with a constant temperature being maintained for 30 s prior to each reading to ensure a stable value.
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28. A 1 mg/ml solution of calf thymus DNA was sonicated to ~500 bp fragments. Sonicated DNA and drug stocks were mixed to obtain a 1 ml solution containing 30 μM DNA, 50 mM potassium cacodylate (pH 7.4) and 3 or 30 μM drugs for [drugs]/[DNA] ratio (R) 0.1 and 1, respectively. DNA melting curves were obtained by 260 nm absorbance measurement on a UV–vis spectrophotometer (JASCO V-530). The temperature range was covered in 70 min, and the absorbance values collected every 10 s.
29. Drugs were added at different concentrations to the reaction mixture (50 μL), containing 50 μM dNTPs, 0.5 μM TSG_4 primer and 1 μL of cell extract (prepared from 10^9 cultured HeLa cells as previously described[33]) in TRAP buffer (20 mM Tris–HCl (pH 7.5), 68 mM KCl, 15 mM $MgCl_2$, 10 mM EDTA and 0.5% Tween 20). Samples were incubated for 2 h at 30 °C, before the addition of the cell extract. After 30 min of incubation at 30 °C, the samples were purified by phenol/chloroform extraction. ^{32}P radiolabelled TSG_4, 0.5 μM CX_{ext} and 2 U *Taq* DNA polymerase (Eppendorf) were added and 27 PCR cycles were performed (94 °C 30″, 50 °C 30″ and 72 °C 1′3O″). Finally, the samples were loaded on non-denaturing 12% polyacrylamide gel. Samples with no drug and with no cell extract were references. A 130 bp 'internal standard' (IS) was used to evaluate PCR amplification efficiency.[34]
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