Reevaluation of telomerase inhibition by quadruplex ligands and their mechanisms of action

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Quadruplex ligands are often considered as telomerase inhibitors. Given the fact that some of these molecules are present in the clinical setting, it is important to establish the validity of this assertion. To analyze the effects of these compounds, we used a direct assay with telomerase-enriched extracts. The comparison of potent ligands from various chemical families revealed important differences in terms of effects on telomerase initiation and processivity. Although most quadruplex ligands may lock a quadruplex-prone sequence into a quadruplex structure that inhibits the initiation of elongation by telomerase, the analysis of telomerase-elongation steps revealed that only a few molecules interfered with the processivity of telomerase (i.e., inhibit elongation once one or more repeats have been incorporated). The demonstration that these molecules are actually more effective inhibitors of telomeric DNA amplification than extension by telomerase contributes to the already growing suspicion that quadruplex ligands are not simple telomerase inhibitors but, rather, constitute a different class of biologically active molecules. We also demonstrate that the popular telomeric repeat amplification protocol is completely inappropriate for the determination of telomerase inhibition by quadruplex ligands, even when PCR controls are included. As a consequence, the inhibitory effect of many quadruplex ligands has been overestimated.

G-quadruplex | telomere | telomeric repeat amplification protocol assay | direct assay

The unlimited proliferative potential of cancer cells depends on telomere maintenance (1). As a consequence, several classes of telomerase inhibitors have been developed for anticancer purposes. Optimal telomerase activity requires an unfolded single-stranded telomeric overhang (2–4). This overhang may adopt an unusual DNA structure called a G-quadruplex, composed of G-quartets and formed in the presence of cations (5). Therefore, ligands that selectively bind to and stabilize telomeric G-quadruplex structures could act as indirect telomerase inhibitors (Fig. 1A) (6). The number of identified G-quadruplex ligands has grown rapidly over the past few years (for a recent review, see ref. 7); some of these molecules exhibit potent *in vivo* antitumor activity, and clinical trials started recently.

Although various methods are available to measure telomerase activity, the telomeric repeat amplification protocol (TRAP) is most widely used (8). With a few notable exceptions (6, 9–12), TRAP [or a variant called TRAP-G4 (13)] has been the standard method to measure telomerase inhibition by G-quadruplex ligands (reviewed in ref. 7). The original method was improved by the use of an internal control (often called ITAS for internal telomerase assay standard) that coamplifies with the telomerase products and allows the detection of nonspecific *Taq* polymerase inhibitors. Notably, the ITAS product does not contain any telomeric sequence and therefore is not able to form a G-quadruplex (5, 14). We demonstrate here that TRAP is inappropriate for the determina-

tion of telomerase inhibition by quadruplex ligands, because these molecules interfere with the PCR of a sequence able to form a G-quadruplex but leave the internal control unaffected. As a consequence, the use of a direct, also called a primer extension, assay is required to measure telomerase inhibition. In contrast to TRAP, this approach not only provided information on the total activity, but also allowed us to analyze specific steps of the telomerase reaction, such as elongation and initiation.

Results

Telomerase Inhibition by G-Quadruplex Ligands Cannot Be Determined by TRAP. TRAP is a two-step assay. In the first step, telomerase is allowed to elongate an oligonucleotide substrate. In the second step, PCR is used to amplify the elongation product. We hypothesized that G-quadruplex ligands might inhibit the PCR step rather than the telomerase-elongation step. We compared inhibition profiles when compound was added before the elongation reaction or just before PCR [full protocols are provided in [supporting information \(SI\) Text](#)]. Fig. 2 shows the result of this experiment carried out with known G-quadruplex ligands (structures shown in Fig. 1B). The IC₅₀s for compound addition before step 1 or before step 2 are reported in Table 1. Strikingly, for all of the compounds tested, inhibition occurred whether the ligand was added before or after telomerase extension. This observation was independent of the nature of the cellular extracts used; data using A431 CHAPS extracts are given in [SI Fig. 7](#). These experiments unambiguously demonstrate that inhibition determined by TRAP in the presence of G-quadruplex ligands was not due to telomerase inhibition. For ligands such as telomestatin (15), Phen-DC3 (16), and 360A (17), the amplification of telomeric products was inhibited but amplification of the internal control that does not form a quadruplex was not.

Telomerase Inhibition by G-Quadruplex Ligands by Using the Telomeric Substrate (TTAGGG)₃. We then directly evaluated telomerase activity using highly active CHAPS extracts from HEK293T cells

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Abbreviations: ITAS, internal telomerase assay standard; TRAP, telomere repeat amplification protocol.

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Fig. 1. Telomerase inhibition by G-quadruplex ligands. (A) Schematic drawing of a G-quartet (*Left*) and illustration of indirect telomerase inhibition by G-quadruplex stabilizing agents at telomeres (*Right*). (B) Structure of G-quadruplex ligands used in this study.

that transiently overexpress hTR and hTERT (18). Telomerase activity was directly visualized upon extension of an oligonucleotide, Telo3R, containing three telomeric repeats 5'-

(TTAGGG)_{3-3'} in the presence of [α -³²P]dGTP. Data for various G-quadruplex ligands are shown in Fig. 3, and IC₅₀ values are reported in Table 1. For all ligands studied, the IC₅₀ values ranged

Fig. 2. Inhibition of TRAP by G-quadruplex ligands. Telomerase activity was evaluated by TRAP in 6, 2, 0.6, and 0 ng of CHAPS extracts (gray slope) from HEK 293T cells that transiently express hTR and hTERT by using incorporation of [α - 32 P]dCTP. TRAP was then carried out with 2 ng of extract and various concentrations of compound: the black slope corresponds to serial 5-fold dilutions of compound starting from 800 nM to 0.2 nM for telomestatin and ligand Phen-DC3 and from 20 μ M to 32 nM for 360A, BRACO-19, and TMPyP4. C, the final concentration of the solvent used for the dilutions. Ligand was either added during or after (indicated by thick gray bars) the telomerase elongation step (for 15 min at 30°C) to investigate the ligand effect on the PCR amplification of telomeric G-quadruplex-prone sequences. Products were resolved on a 7.5% denaturing PAGE. The control (ITAS) was PCR amplification of the 36-nt TSNT oligonucleotide. TMPyP4 likely inhibited PCR amplification because amplification of ITAS was inhibited, even at concentrations lower than required for the total disappearance of telomeric products.

see ref. 7). These molecules preferentially localize and act at telomeres. They inhibit the activity of canonical DNA polymerases (24) and helicases (25, 26) on telomeric substrates and delocalize telomeric proteins from telomeres (27), which, together with their effects on telomerase activity, participate to their selective cytotoxicity toward cancer cells and their efficiency as anticancer drugs. Telomerase is one of the enzymes that must be evaluated in the design and development of new G-quadruplex ligands, and we provide here an approach to validate carefully such candidates. Other assays, such as *in vitro* inhibition of D-loop or T-loop formation (28) in crude nuclear extract, remain to be developed to improve the validation of G-quadruplex ligands.

Materials and Methods

Full protocols are presented in *SI Text*. Compounds 360A, 307A (counterion I^-), and BRACO-19 (29) were kind gifts from P. Mailliet (Sanofi-Aventis, Vitry/Seine, France). The synthesis of Phen-DC3 (counterion $CF_3SO_3^-$) was described (16). The isolation and purification of telomestatin was described (30).

Telomeric Repeat Amplification Protocol. The TRAP reaction was carried out in two steps (elongation and amplification) as previously described. Telomerase extension products were then analyzed by denaturing PAGE.

Direct Assay. Reactions were carried out at 30°C for 45 min in final volume of 20 μ L, containing 2 μ g of CHAPS extract from HEK 293T cells transiently expressing hTR and hTERT (18). Products were analyzed by denaturing PAGE. Extension products were quantified by using ImageQuant 5.2 software and normalized to the signal of the extension products in control solvent.

Quantification of Telomerase Ability to Elongate. By analogy to processivity of other polymerases, telomerase processivity can be measured by the number of telomeric repeats synthesized before half of the enzyme dissociates (31, 32). This definition suggests that elongation is independent on the number of repeats previously added. The signal A_n of each elongation product n ($1 \leq n \leq N$) was quantified and normalized to the total number of guanines incorporated, thus giving the quantity of product having n repeats. The quantification of a direct assay is more robust and is not subject to the distortions observed in the TRAP assay, which may result from (i) the possible misalignment of the reverse primer to the G-rich strand at every PCR cycle during the hybridization step and (ii)

possible unequal amplification of products with different size, especially because the amount of TS may be limiting). Summing these amounts for each added repeat n gives the total amount of generated product. One can then calculate the proportion R_n of product containing n repeats compared with the total amount of generated products (the $3(n-1) + 1$ denominator corresponds to the number of guanines incorporated in each product):

$$R_n = \frac{A_n}{3(n-1) + 1} \cdot \sum_{n=1}^N \frac{A_n}{3(n-1) + 1} \quad [1]$$

These ratios are plotted on a $\log_{10}$ scale and the average telomerase processivity X is deduced by fitting the curves with the geometric progression

$$R_n = R_1 e^{-(n-1)k} = R_1 e^{(n-1) \frac{-\ln 2}{2.303 X}}, \quad [2]$$

which is usually deduced from the slope k of the linear fit in the $\log_{10}$ representation. However, in the presence of some G-quadruplex ligands, it was difficult to fit the curve with this geometric progression, meaning that the ability of telomerase to elongate depended on the number of repeats already synthesized. We therefore chose another representation and determined the probability P_n that telomerase will elongate a product containing n repeats (i.e., the sum of products having $n+1$ repeats divided by the sum of products having n repeats):

$$P_n = \frac{\sum_{n+1}^N R_n}{\sum_n^N R_n} \quad [3]$$

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