The G-Quadruplex-Interactive Molecule BRACO-19 Inhibits Tumor Growth, Consistent with Telomere Targeting and Interference with Telomerase Function

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Abstract

Interference with telomerase and telomere maintenance is emerging as an attractive target for anticancer therapies. Ligand-induced stabilization of G-quadruplex formation by the telomeric DNA single-stranded 3’ overhang inhibits telomerase from catalyzing telomeric DNA synthesis and from capping telomeric ends. We report here the effects of a 3,6,9-trisubstituted acridine compound, BRACO-19, on telomerase function in vitro and in vivo. The biological activity of BRACO-19 was evaluated in the human uterus carcinoma cell line UXF1138L, which has very short telomeres (2.7 kb). In vitro, nuclear human telomerase reverse transcriptase (hTERT) expression was drastically decreased after 24 hours, induction of cellular senescence and complete cessation of growth was seen after 15 days, paralleled by telomere shortening of ca. 0.4 kb. In vivo, BRACO-19 was highly active as a single agent against early-stage (68 mm^3) tumors in a s.c. growing xenograft model established from UXF1138L cells, if given chronically at 2 mg per kg per day i.p. BRACO-19 produced growth inhibition of 96% compared with controls accompanied by partial regressions (P < 0.018). Immunostaining of xenograft tissues showed that this response was paralleled by loss of nuclear hTERT protein expression and an increase in atypical mitoses indicative of telomere dysfunction. Cytoplasmic hTERT expression and its colocalization with ubiquitin was observed suggesting that hTERT is bound to ubiquitin and targeted for enhanced degradation upon BRACO-19 treatment. This is in accord with a model of induced displacement of telomerase from the telomere. The in vitro and in vivo data presented here is consistent with the G-quadruplex binding ligand BRACO-19 producing an anticancer effect by inhibiting the capping and catalytic functions of telomerase. (Cancer Res 2005; 65(4): 1489-96)

Introduction

The integrity of human telomeric DNA is maintained in the overwhelming majority of tumor cells by the telomerase enzyme complex (1), in contrast to the progressive attrition of telomere length in most normal somatic cells that occurs as a consequence of the end replication effect (2). Telomerase performs several key roles, notably maintenance of telomere length by catalyzing the synthesis of telomeric DNA repeats (3) and protection of the 3’ end of the ssDNA overhang (4). This latter capping function is critical for cellular integrity since if the 3’ end becomes exposed (5, 6), then DNA damage response pathways are activated (7, 8) and cells rapidly succumb to senescence and apoptosis. These events are also seen when telomerase is inhibited or suppressed, which results in telomeres becoming critically shortened following successive rounds of replication (9–11).

Telomerase plays a key role in tumorigenesis (12). This, together with the presence of telomerase in >85% of all tumors and across all tumor types, suggest that inhibition of telomerase would be an effective anticancer therapeutic strategy. A number of distinct approaches to inhibition of the enzyme complex and its unique mechanism of telomeric DNA catalysis have been suggested (13–16). None have reached the clinic as yet, although phase I clinical trials with anti-telomerase peptide epitopes have provided promising indications that down-regulation of telomerase leads to therapeutic advantage (17).

A key problem of direct telomerase inhibition is the extended time-lag required before telomeres reach the critically short length required for senescence and apoptosis to be triggered, although this may be offset by the existence of sensitive subpopulations of cells with very short telomeres (18). A small-molecule approach has been devised that targets telomeres rather than telomerase per se (19). It exploits the single-stranded G-rich overhang at telomere 3’ ends (20), and induces them to fold into four-stranded quadruplex complexes (21). Formation of a quadruplex DNA structure is incompatible with telomerase attachment to the 3’ end (22), suggesting that quadruplex-binding and quadruplex-promoting small molecules can in principle effectively inhibit both the catalytic and capping functions of telomerase. The more potent in vitro quadruplex-binding ligands produce telomere shortening and senescence in cancer cells (e.g., refs. 23–26). We have previously described the rational design of trisubstituted acridine compounds, with potent and selective activity against telomerase, yet with up to 20-fold less short-term cytotoxicity (27, 28). A member of this family (BRACO-19, Fig. 1A) produces growth inhibition and senescence (29) in human tumor cell lines at subcytotoxic concentrations, after days rather than weeks. In vivo activity against human uterine xenograft was also found, where the established cytotoxic agent taxol was used to produce tumor regression and BRACO-19, given after debulking, was able to suppress tumor regrowth (29).

We report here a study of the effects of BRACO-19 on hallmarks of telomerase/telomere modulation in vitro and in vivo. We show
telomere shortening in cells and describe significant single agent
*in vivo* antitumor activity in an early-stage human tumor xenograft
model with short telomeres. This activity is associated with major
changes in expression levels of the catalytic human telomerase
reverse transcriptase (hTERT) subunit of telomerase and in
chromosomal integrity, indicating that BRACO-19 is targeting
telomeres and telomerase *in vivo*.

**Materials and Methods**

The trisubstituted acridine compound BRACO-19 was synthesized using
the routes previously established by us (27, 28).

**Sulforhodamine B Proliferation Assay**

Cells (2,000/well) were seeded into 96-well plates in 0.1 mL RPMI 1640
supplemented with 10% FCS (30). Cells were grown overnight at 37
°C/5%
CO2 and BRACO-19 was added in 0.1 mL of medium to obtain final drug
dilutions between 0.1 and 10 mol/L. Cell proliferation was
determined 5 days after continuous exposure to drug by sulforhodamine
B staining. The plates were read at 515 nm with a Millipore Cytofluor
2350-
microplate reader.

**Telomere Length**

Mean telomere restriction fragment length was determined using the
Telo-TAGGG-telomere length kit from Roche (Mannheim, Germany),
following the manufacturer's instructions. Genomic DNA was isolated from
pellets of cells grown in culture with 10 mmol/L bromodeoxyuridine.

**Senescence-Associated β-Galactosidase Staining**

Senescence was assayed with the Senescence-Associated
β-Galactosidase Staining
procedure (31). Cells (10,000/well) were seeded in 6-well plates in either 5 mL of
vehicle control (containing PBS in a concentration equal to the highest drug
dose) or BRACO-19 in concentrations of 0.1 and 10 mmol/L for 15 days. Drug
and medium were replaced every 4 days. At day 15, cells were washed with
PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde, and stained.

**In vivo Studies**

**Tumor Model.** The UXF1138L uterus carcinoma cell line was originally
established from a patient tumor by Prof. Heiner Fiebig (32). All animal
experiments were conducted in compliance with the guidelines of the
German Animal Welfare Act (Tierschutzgesetz) and the UKCCCR
guidelines on experimental neoplasia (33). Thymus aplastic nude mice of
NMRI genetic background were used for establishment and serial
propagation of the human tumor xenograft UXF1138LX from the cell line.

Tumor fragments (size, ~30 mm³) were implanted s.c. into nude mice and
treatment was initiated when tumors reached a median volume of ~68 mm³
(~6 days after transplantation, early stage) or between 130 and
200 mm³ (advanced stage; Table 1). This s.c. xenograft staging system has
been defined by the U.S. National Cancer Institute’s Drug Development
Program. Tumors with a median volume of 190 mm³ (100-400 mm³)
are termed advanced stage. A model is considered early stage if treatment is
initiated when tumor sizes range from 63 to 200 mm³ (34). UXF1138LX is
a fast growing tumor (average doubling time in log-growth = 4 days) and
has a >95% take rate, hence fulfilling National Cancer Institute criteria for a
suitable early-stage tumor xenograft model (32, 34). A group contained 6 to
8 mice each with 12 to 16 tumors.

**Treatment and Data Evaluation.** BRACO-19 was dissolved in PBS
(vehicle) and given i.p. or p.o. at nontoxic doses of 2 and 5 mg per kg per
day, qdx5 or q3dx2 for 3 weeks or as indicated in Table 1. Less than five
doses were given in the first week, if randomization could not start at the
beginning of a week (see advanced stage model, Table 1). Dose and schedule
were determined as being well tolerated before start of experiments. Tumor
growth was followed twice weekly by serial caliper measurement, body
weights recorded, and tumor volumes were calculated using the standard
formula, it has to be noted that 1 mm³ equals 1 mg of tumor weight. Data
were evaluated using the National Cancer Institute guidelines for
assessment of anticancer drug effects in s.c. growing human tumor
xenografts (33, 34). Using specifically designed software, the median relative
tumor volume in mm³ is the appropriate variable deduced from this
formula, where length is the largest dimension and
width the smallest dimension perpendicular to the length (34, 35). Whereas
tumor volume in mm³ is the appropriate variable deduced from this
formula, it has to be noted that 1 mm³ equals 1 mg of tumor weight. Data
were evaluated using the National Cancer Institute guidelines for
assessment of anticancer drug effects in s.c. growing human tumor
xenografts (33, 34). Using specifically designed software, the median relative
tumor volume was plotted against time. Relative tumor volumes were
calculated for each single tumor by dividing the tumor volume on day X by
that on day 0 (time of randomization). Tumor doubling time of test and
control groups was defined as the period required to double the initial
tumor volume (100%) to 200%. Growth curves were analyzed in terms of
maximal tumor inhibition/optional % treated versus control (T/C)
where changes in tumor volume (ΔTV) for each treated (T) and control (C) group

**Figure 1.** A, structure of BRACO-19.

**B, acute cytotoxicity of BRACO-19 in
UXF1138L cells in vitro in a 5-day
proliferation assay. Three independent
experiments were done. Points, mean ±
SD. C, telomere length of UXF1138L cells
in vitro ± 1 μmol/L BRCAO-19, 15-day
exposure. PD time UXF1138L = 30.5 ±
1.9 hours. White horizontal bars, mean
telomere restriction fragment length and
a 2.7- to 2.3-kb length decrease. Lanes 1
and 2, molecular weight marker; lane 2,
low molecular weight control probe
supplied with the Telo-TAGGG kit; lane 5,
high molecular weight control probe; lane
3, telomere restriction fragment length of
UXF1138L control cells; lane 4, telomere
restriction fragment length of UXF1138L
cells treated with 1 μmol/L BRCAO-19.
were calculated for each day. Tumors were measured by subtracting the median tumor volume on day of first treatment (staging day) from the median tumor weight on the specified observation day (34). These values were used to calculate a % T/C as follows:

$$\% T/C = (\Delta T / \Delta C) \times 100, \text{ where } \Delta T > 0 \text{ or}$$

$$\% T/C = (\Delta T / T_i) \times 100, \text{ where } \Delta T < 0$$

and $T_i$ = median tumor volume at start of the treatment (34). The optimum (minimum) value obtained is used to quantitate antitumor activity and the day at which this effect occurs is indicated. Partial tumor regressions are defined as tumor volume decreases to 50% of less of the tumor volume at the start of treatment, complete regressions are the instances in which the tumor burden decreases below 63 mm$^3$ at any time during the experimental period (34, 35).

Statistical data analysis was done using nonparametrical Wilcoxon Mann-Whitney statistics. Median relative tumor volumes of each treatment group were compared with those of the vehicle control groups. $P < 0.05$ were considered statistically significant. The degree of variability/range of values for the median was assessed in terms of 95% confidence intervals. SPSS 2000, SYSTAT version 10 software was used.

**Immunohistochemistry and Immunofluorescence**

**Cultured Cells.** UXF1138L cells were grown on 8-well chamber slides (Corning Inc., Acton, MA) and treated with vehicle (PBS) or BRACO-19 at concentrations of 1 and 10 μmol/L for 24 hours. Cells were then washed, fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.25% Triton X-100 for 5 minutes, and blocked in 10% goat serum for 1 hour. PBS was used as washing buffer. For triple staining, cells were incubated with 4% paraformaldehyde for 30 minutes. Sections were then blocked with 10% normal goat serum in PBS and simultaneously with monoclonal ubiquitin antibodies (Cell Signaling Technologies, Beverly, MA) diluted 1:200 in PBS. For immunofluorescence detection of hTERT and ubiquitin, TRITC-conjugated anti-rabbit and FITC-conjugated anti-mouse secondary antibodies (Sigma, St. Louis, MO; 1:400; 1:100 diluted in 10% goat serum) were added for 2 hours at room temperature followed by three washes in PBS and a 5-minute incubation with 4',6-diamidino-2-phenylindole (Sigma, 2 mg/mL, diluted 1:1,000 in PBS). The slides were mounted and immediately analyzed using a Leica DM 4000 microscope with a cooled camera (Quorum Technologies, Guelph, Ontario, Canada) and the Openlab software to generate overlays.

hTERT-specific immunoperoxidase staining was developed using the DAKO Envision+ system (K-4010 horseradish peroxidase, Rabbit Envision 3,3'-diaminobenzidine Plus kit, DAKO Cytomation) with 3,3'-diaminobenzidine as chromogen. 3,3'-Diaminobenzidine-stained slides were counterstained with hematoxylin, dehydrated, and cover-slipped.

**Tissues.** UXF1138LX tumor tissues were collected from the *in vivo* experiments upon termination of the study and immediately fixed in 10% PBS buffered formalin for 24 hours followed by routine paraffin embedding procedures (36). Five-micrometer sections were cut, dewaxed, and antigen retrieval done in citrate buffer (pH 6.0)/microwave for 30 minutes. Sections were then blocked with 10% normal goat serum in PBS and stained as described above. Rabbit immunoglobulin G (DAKO, Glostrup, Denmark) was used as a negative control. To enhance contrast, tissues were counterstained with hematoxylin. In addition, for each treatment a H&E-stained section was prepared. hTERT-specific staining intensity was documented using a digital Leica DM 6000 microscope and camera system (Leica, Wetzlar, Germany). Both immunoperoxidase and H&E-stained sections were viewed and evaluated by independent pathologists (Drs. T. Treger and J. Wong, SWCHSC, Toronto, Canada). Atypical mitoses were counted in control and BRACO-19 treated tissues from three different mice and 100 fields (100 fields, average 225,000 cells) of each section, and a mean of atypical mitoses per field ± SD was generated.

**Results**

**BRACO-19 Induces Hallmarks of Telomerase Inhibition**

**In vitro.** The uterine carcinoma cell line UXF1138L, with very

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose/route/schedule</th>
<th>Median tumor volume at randomization (mm$^3$)</th>
<th>Optimal % T/C [d]</th>
<th>Tumor doubling time (d)</th>
<th>Mann-Whitney $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>10 ml/kg i.p. 1-3, 6-10, 13-17</td>
<td>189</td>
<td>100</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>BRACO-19</td>
<td>2 mg/kg i.p. 1-3, 6-10, 13-17</td>
<td>128</td>
<td>83 [13]</td>
<td>4.1</td>
<td>NS</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>10 ml/kg i.p. 1-5, 8-12, 15-19</td>
<td>95</td>
<td>100</td>
<td>21</td>
<td>—</td>
</tr>
<tr>
<td>BRACO-19</td>
<td>2 mg/kg p.o. 1-5, 8-12</td>
<td>110</td>
<td>89 [7]</td>
<td>13.1</td>
<td>NS</td>
</tr>
<tr>
<td>BRACO-19</td>
<td>2 mg/kg i.p. 1-5, 8-12, 15-19</td>
<td>129</td>
<td>91 [3]</td>
<td>14.4</td>
<td>NS</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>10 ml/kg p.o. 1, 4, 9, 12</td>
<td>68</td>
<td>100</td>
<td>19.8</td>
<td>—</td>
</tr>
<tr>
<td>BRACO-19</td>
<td>5 mg/kg p.o. 1, 4, 9, 12</td>
<td>51</td>
<td>73 [28]</td>
<td>19.1</td>
<td>NS</td>
</tr>
<tr>
<td>BRACO-19</td>
<td>2 mg/kg i.p. 1-5, 8-12, 15-19</td>
<td>68</td>
<td>4.1 [28]</td>
<td>PR 0.018</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Days [d] of maximum effects are indicated in brackets. Vehicle control is phosphate buffered saline. Abbreviation: NS, not significant; PR, partial regression.
short telomeres (average telomere restriction fragment length of 2.7 kb, Fig. 1C), was used. The IC\textsubscript{50} for BRACO-19 in UXF1138L cells is 2.5 \textmu M, the IC\textsubscript{100} is 5 \textmu M, and the concentration that would cause zero growth inhibition was found 1 \textmu M, in a 5 day proliferation assay (Fig. 1B). Drug concentrations of 0.1 to 1.0 \textmu M were used for longer-term treatments (15 days) and 1.0 to 10 \textmu M were for examining target effects in short-term (24 hours) studies.

The expression of active telomerase and its inhibition by BRACO-19 in UXF1138L cells was confirmed by the TRAP assay (data not shown) and by immunohistochemical staining for expression of the telomerase catalytic subunit hTERT (Fig. 2A and B). UXF1138L control cells readily expressed hTERT indicated as a strong nuclear (dark brown) immunoperoxidase stain (Fig. 2A), whereas cells treated with 1 \textmu M BRACO-19 for 24 hours showed dramatically reduced nuclear hTERT expression (Fig. 2B). However, residual cytoplasmic hTERT staining was observed accompanied by the occurrence of atypical mitoses (Fig. 2B and C). Nuclei of BRACO-19-treated cells seemed enlarged compared with control UXF1138L (Fig. 2A and B).

To investigate whether BRACO-19 could cause telomere length reduction upon long-term exposure, we treated UXF1138L cells with 1 \textmu M drug for 15 days. Beyond that time period, BRACO-19, but not control treated cells ceased growth, underwent apoptosis and detached or inflated and showed extensive replicative senescence, which was accompanied by typical morphologic changes (Fig. 2D and E). These were observed even at 10-fold lower BRACO-19 concentrations. Genomic DNA was isolated from UXF1138L cells after 15 days and analyzed for telomere length compared with untreated controls by Southern blotting. Telomere erosion of 0.4 kb occurred (Fig. 1C), which corresponds to the loss of ~34 nucleotides per round of cell division in UXF1138L cells (population doubling time of 30.5 hours).

**Effects of BRACO-19 as a Single Agent in Early-Stage and Established UXF1138LX Xenografts In vivo.** UXF1138L cells were initially injected s.c. in nude mice and then propagated as fragments to produce the UXF1138LX xenograft. The response to BRACO-19 of early stage (median relative tumor volume = 68 mm\textsuperscript{3}, confidence intervals 95% min. = 62.5 mm\textsuperscript{3} and 95% max. = 78.7 mm\textsuperscript{3}) and established UXF1138LX tumors (median relative tumor volume = 110-129 mm\textsuperscript{3}, confidence intervals 95% min. = 111.4 mm\textsuperscript{3} and 95% max. = 174.2 mm\textsuperscript{3}) in nude mice was compared (Table 1). BRACO-19 was given i.p. and p.o. in various schedules at nontoxic doses of 2 and 5 mg per kg per day, respectively (Table 1). Tumor growth was determined by twodimensional caliper measurement. The oral dosing regimen was always inactive and the animals had to be sacrificed due to high tumor burden (1.5 cm) before overall termination of the study (Table 1; Fig. 3). Chronic, i.p. BRACO-19 administration, qdx5 for 3 weeks, was only efficient in inhibiting tumor growth in early-stage xenografts but not advanced-stage xenografts (Table 1). Significant (P = 0.018) tumor growth inhibition by BRACO-19 was seen in early-stage xenograft tumors when the compound was given i.p. at 2 mg per kg per day, starting 6 days after transplantation of UXF1138LX fragments. At this time, tumors could be considered established according to National Cancer Institute criteria as outlined in Materials and Methods, but remained static and had not resumed logarithmic growth (Fig. 3). Under these conditions, marked single-agent antitumor activity was observed, with some animals in the group showing complete regressions (5 of 12 tumors). Overall, the data assessed as median relative tumor volumes, showed partial tumor regressions with an optimal T/C on day 28 of 4.1%, equal to 95.9% inhibition of tumor growth compared with control (Fig. 3; Table 1). Partial tumor regressions were most pronounced on day 17 with BRACO-19 treatment, which was stopped on day 19, at which point slow regrowth occurred. A twice weekly oral administration of 5 mg/kg drug did not produce significant tumor growth inhibition in the same experiment (optimal T/C on day 28 = 73%, equal to 27% growth inhibition).

**Pharmacodynamic Monitoring of BRACO-19 Effects in UXF1138LX Xenograft Tissues.** Effects of BRACO-19 in vivo were determined by evaluating hTERT expression levels in both, control
and treated human tumor xenograft tissues (Fig. 4A-D), as well as by analysis of the occurrence of atypical mitoses and anaphase bridges, which indicate telomeric dysfunction (Fig. 4D-F).

Figure 4A shows strong nuclear hTERT staining in UXF1138LX tissue treated with vehicle control (PBS). The specificity of hTERT expression is supported by its presence in the in vitro growing cell line (Fig. 2A) and complete lack of staining of the control group tissue sections when they were probed with rabbit immunoglobulin G instead of anti-hTERT antibodies (Fig. 4B). In marked contrast, a large decrease in nuclear hTERT protein levels was seen, when UXF1138LX tumors were treated with several cycles of BRACO-19 (Fig. 4D). Xenografts that did respond to treatment with tumor regression after 2 mg/kg i.p. dosing not only showed a reduction in nuclear hTERT levels but also a striking increase in atypical mitotic figures (Fig. 4D). The mean number of atypical mitoses rose from 0.4 in control tumors to 5.7 per field (225 cells) in the responsive tissues (Fig. 5). In addition, anaphase bridges were seen (Fig. 4E and F). There was also a significant decrease in hTERT levels in cells derived from the 5 mg/kg p.o. treated tumors despite lack of growth inhibition (Fig. 4C). The extent of atypical mitoses was less pronounced, but considerable (average = 3 per field). Although nuclear hTERT protein levels were strongly diminished in all BRACO-19 treatment groups, slight cytoplasmic hTERT staining had occurred. Effects seen in BRACO-19 treated tumor tissues were consistent with drug effects in UXF1138L cells in vitro (Figs. 2A-C and 4).
BRACO-19 Treatment Leads to Decreased Nuclear hTERT Expression with Colocalization of hTERT and Ubiquitin in the Cytoplasm. Ubiquitination is the predominant mechanism for protein destruction (37). Conjugation of proteins to ubiquitin can also influence their cellular localization (37). We studied whether the loss of nuclear hTERT protein expression and its detection in the cytoplasm is a result of ubiquitin-regulated degradation after stabilization of G-quartet formation by BRACO-19, and thus, hTERT displacement from the 3’ telomeric overhang. UXF1138L cells were treated with 1 and 10 μmol/L BRACO-19 for 24 hours and double stained for expression of hTERT (TRITC-labeled, red) and ubiquitin (FITC-labeled, green). 4′,6-Diamidino-2-phenylindole (blue) was used as a third fluorochrome to contrast the cell nuclei. UXF1138L control cells (PBS treated) expressed nuclear hTERT, whereas ubiquitin expression was localized in the nucleus and to some extent in the cytoplasm (Fig. 6, top). When the expression of hTERT and ubiquitin in control cells was merged, only ubiquitin (green) was seen in the cytoplasm (Fig. 6, top). Treatment of UXF1138L cells with 1 μmol/L BRACO-19 for 24 hours led to an overall reduction of hTERT protein, and in particular in nuclear localization; however, detectable hTERT levels were seen in the cytoplasm (Fig. 6, middle). Ubiquitin also translocated partly from the nucleus into the cytoplasm upon BRACO-19 exposure. Moreover, cytoplasmic ubiquitin colocalized with hTERT, suggesting that they might be bound together and hence, that hTERT has been tagged by ubiquitin for subsequent destruction in the proteasome (Fig. 6, middle; see yellow to orange areas in the merge). This effect was enhanced when UXF1138L cells were treated with 10 μmol/L BRACO-19 (Fig. 6, bottom).

Discussion

Evidence of telomere shortening in UXF1138L cells (2.7 → 2.3 kb) produced by exposure to BRACO-19 at a noncytotoxic concentration, shows that the ligand acts at the telomere. We have also recently shown that BRACO-19 produces a 17% decrease in telomere length in MCF-7 (6 → 5 kb) cells after 39 days of long-term exposure (38). Telomere shortening has also been reported for several other G-quadruplex binding agents (23, 24, 26) in a range of tumor cell lines, with the degree of shortening dependent on mean initial telomere length. There is clear heterogeneity in telomere length in the UFX1138L cell line (Fig. 1C), with a significant population of cells having exceptionally short telomeres. These may be especially sensitive to BRACO-19 exposure and thus to rapid triggering of senescence (18).

BRACO-19 administration to early-stage UFX1138LX xenograft tumors results in a rapid and progressive decrease in tumor volume, up to a maximum median reduction of 96% compared with control. Although an overall partial tumor regression was seen during the course of i.p. treatment with BRACO-19 in these small tumors, some individual tumors completely regressed (5 of 12). The two previous reports of single-agent antitumor activity in xenografts produced by a G-quadruplex binding ligand underline the effectiveness of this class of telomerase inhibitory agents in early-stage tumors and “residual” disease models (29, 39). We have previously reported a synergistic effect of BRACO-19 in taxol-pretreated A431 human vulval carcinoma xenografts (29), which have a longer mean telomere length. The cationic porphyrin TMPyP4 produces increased survival of mice bearing the breast cancer xenograft MX-1 in an adjuvant setting (after debulking of tumor with cyclophosphamide) as well as tumor growth inhibition by TMPyP4 given alone in early-stage PC-3 prostate cancer xenografts (39). Interestingly, PC-3 prostate cancer cells have short (~3 kb) telomeres (40) suggesting that telomere length and tumor size at start of treatment might predict response to G-quadruplex stabilizing agents.

The observation of an almost complete lack of protein expression of the catalytic subunit of telomerase, hTERT, in cultured cells and cells from the treated tumors, but not in controls, is direct evidence that BRACO-19 is affecting this target. We suggest that our data are consistent with two interrelated models, both of which involve BRACO-19 stabilizing quadruplex formation at the 3’ telomeric DNA overhang: (i) in which the quadruplex complex dissociates hTERT from its capping function. The subsequent exposure of the 3’ end leads to cell death and degradation of hTERT by ubiquitin-mediated proteolysis following its release from the telomere complex. (ii) Quadruplex formation may affect the ability of telomerase to maintain telomere length, so that senescence and apoptosis results from effects on a short telomere subpopulation (18). It is plausible that the quadruplex complex itself elicits a DNA damage/cell death response, as has been observed with telomeric-like oligonucleotide sequences (41). The observation of anaphase bridges being produced in vivo is consistent with G-quadruplex ligand stabilization, and has been previously reported for the quadruplex-binding porphyrin TMPyP4 (39, 42).

It is striking that the antitumor effects of BRACO-19 are apparent within a very few days after the start of treatment. This suggests that it is highly unlikely that BRACO-19 is acting as a pure classic enzymatic telomerase inhibitor. The doubling time of the UXF1138L cell line (30.5 ± 1.9 hours) and the mean telomere length of 2.7 kb, even assuming a maximal mean telomere loss per round of replication of 200 nucleotides, is not compatible with such a rapid apoptotic and selective tumor kill response.
The early optimism for telomerase inhibition as a strategy for specific and anticancer therapy has been tempered until recently by the assumption that pure telomerase catalytic inhibition would require an extended time scale before antitumor effects become apparent. This would raise major problems for their clinical applicability. In striking contrast, evidence is now emerging that G-quadruplex ligands produce rapid senescence and selective cell death (43, 44). End-to-end chromosomal fusions leading to apoptosis and senescence after “short-term” treatment, consistent with uncapped telomere states, have been reported by us for BRACO-19, and others for the G-quadruplex stabilizing agent RHP54 (43, 44). Loss of nuclear hTERT expression, occurrence of enlarged nuclei and atypical mitoses seen in BRACO-19-treated UXF1138LX tissues and cell cultures in the present study (Figs. 2 and 4) are additional evidence of the existence of uncapping effects (45). Telomere uncapping mechanisms might have broader applicability and play an important part in the action of several anticancer agents. It could provide an explanation for frequent findings of an “nonspecific” decrease in telomerase activity, hTERT expression, or telomere erosion caused by proapoptotic drugs such as arsenic trioxide, cisplatin, Adriamycin, or paclitaxel (46–50). Cisplatin is known to cross-link G-rich DNA sequences (46) and was shown to inhibit telomerase activity and to poison telomeres after just one round of cell division before induction of apoptosis (48, 49), a scenario resembling telomere uncapping.

The acridine ligand BRACO-19 has high selectivity and affinity for quadruplex DNA (27). We suggest that this target specificity, together with its ability to be rapidly transported into cell nuclei, are important factors in its single-agent antitumor activity and favorable therapeutic window in the uterus carcinoma xenograft UXF1138LX, which is refractory to most conventional antitumor agents (32). We consider that the high polarity of the BRACO-19 molecule may be a major factor in the lack of response with oral administration, although there was still a degree of reduction in hTERT expression and telomere dysfunction as indicated by atypical mitoses. We shall report in due course on BRACO-19 derivatives that have superior pharmacologic profiles.

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References

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Angelika M. Burger, Fangping Dai, Christoph M. Schultes, et al.


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