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Acquired Cellular Resistance to Flavopiridol in a Human Colon Carcinoma Cell Line Involves Up-Regulation of the Telomerase Catalytic Subunit and Telomere Elongation. Sensitivity of Resistant Cells to Combination Treatment with a Telomerase Inhibitor

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ABSTRACT

Flavopiridol is a broad-spectrum inhibitor of cyclin-dependent kinases and of global transcription via the inhibition of positive transcription elongation factor b (P-TEFb). Although flavopiridol is currently undergoing phase II clinical trials, acquired cellular resistance to the compound during treatment is a potential problem, as it is with almost all current anticancer agents. A HCT116 human colon carcinoma cell line with an acquired 8-fold resistance to flavopiridol has been established. We report here that there are changes in these resistant cells in terms of telomere length and telomerase activity, whereas no change in the expression of the P-TEFb subunits CDK9, cyclin T1, cyclin T2a, or cyclin T2b was observed. The level of mRNA expression for the telomerase catalytic subunit hTERT was increased over 2-fold in the resistant cells, and mean telomere length was found to be 2 kb longer than the parental length, although telomerase activity was unchanged. The level of mRNA expression for the telomeric binding protein Pot1 was also increased. We also report that treatment of HCT116 cells with a combination of the G-quadruplex interacting telomerase inhibitor BRACO-19 and flavopiridol results in a 3-fold decrease in population doubling and prevents recovery from treatment with either compound alone. Treatment of flavopiridol-resistant cells with BRACO-19 alone also led to rapid inhibition of cell growth, which is not observed in the parental line. The finding that only the resistant line, with up-regulated telomerase, responds to this G-quadruplex inhibitor is consistent with the hypothesis that the mechanism of BRACO-19 down-regulation of cell growth directly involves the targeting of telomeres and telomerase.

Cyclin-dependent kinases (CDKs), together with their cyclin regulatory subunits and downstream effectors, play a major role in cell-cycle progression. Proteins that inhibit the action of CDKs, such as p53 and p16, are frequently mutated in human cancer, resulting in uncontrolled CDK activity and cellular proliferation (Senderowicz and Sausville, 2000). Flavopiridol (HMR-1275; L-868275; NSC-649890; *N*-methylpiperidinyl chlorophenyl flavone), is an inhibitor of CDKs and is currently undergoing phase II clinical trials (Zhai et al., 2002). The compound has been shown to inhibit several CDKs, including CDK1, CDK2, CDK4, and CDK7, with IC_{50} values ranging from 40 to 400 nM (Losiewicz et al., 1994; Carlson et al., 1996). More recently, flavopiridol has also been shown to inhibit receptor tyrosine kinases, such as epidermal growth factor receptor; receptor associated tyrosine kinases, such as Src; and transducing signal kinases, such as phosphokinase C and Erk-1 (Sedlacek, 2001). The basis of the inhibitory effect of flavopiridol is probably the competitive inhibition of the ATP binding site found in these proteins (De Azevedo et al., 1996).

Several studies have also demonstrated that flavopiridol is a potent inhibitor of transcription in mammalian cells. The compound was found to inhibit transcription globally at concentrations in the 300 nM range (Lam et al., 2001), as a result of the direct inhibition of the elongation phase of transcription [inhibiting positive transcription elongation factor b (P-TEFb)]. P-TEFb is a multisubunit protein that interacts with the C-terminal domain of RNA polymerase II

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ABBREVIATIONS: CDK, cyclin-dependent kinase; P-TEFb, positive transcription elongation factor b; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; FRET, fluorescence resonance energy transfer; TRAP, telomere repeat amplification protocol; WT, wild type; RT, reverse transcriptase; kb, kilobase(s); FAM, 6-carboxylfluorescein hexylamine; TAMRA, 6-carboxyltetramethylrhodamine hexylamine.

to promote transcription (Chao and Price, 2001; Taube et al., 2002). It comprises CDK9 and cyclin T1, T2a, T2b, or K subunits; flavopiridol inhibition of transcription may occur by inhibition of the CDK9 domain in this complex. Flavopiridol has also been demonstrated to bind to duplex DNA although this only occurs at high concentrations (> 500 nM) (Bible et al., 2000a).

Resistance to chemotherapeutic agents is one of the most severe problems in the treatment of human cancers. The mechanisms underlying resistance to flavopiridol have not yet been fully characterized, although several cell lines with resistance to flavopiridol have been generated (see for example, Robey et al., 2001; Smith et al., 2001). It has been shown that flavopiridol resistance in human MCF-7 breast cancer cells is associated with an increase in the expression of the ATP-binding cassette half-transporter ABCG2 (Robey et al., 2001). However, this is not necessarily the case in other cell lines, because it has been found that flavopiridol-resistant OV202 ovarian carcinoma cells do not overexpress this transporter (Bible et al., 2000b). Moreover, a HCT116 human colon carcinoma cell line with acquired resistance to flavopiridol did not overexpress the related multidrug transporters P-glycoprotein MRP1, and no changes in drug accumulation or efflux were noted between resistant and parental cells (Smith et al., 2001). This study also found no change in CDK2 or CDK4 levels or activity and only a small change in the expression of cyclins A, B, D_2, or D_3. Small increases in cyclin E levels were observed in the resistant cells although transfection with this protein did not result in a resistant phenotype.

Telomeres comprise repetitive short DNA sequences together with associated proteins that occur at the ends of all eukaryotic chromosomes, the human repeat being TTAGGG (Moyzis et al., 1988). Telomeric DNA in human cancers typically ranges in length from 2 to 6 kb. The primary role of telomeres is to protect chromosomes from aberrant recombination or end-to-end fusions, although in somatic cells they progressively shorten because of the "end-replication effect" of DNA polymerase being unable to fully replicate the ends. Telomere shortening to a critical length can activate senescence and/or apoptotic pathways (Duan et al., 2001; Karlseder et al., 2002). To prevent these from occurring, telomeres are maintained in length in the overwhelming majority of tumor cells by the action of the enzyme telomerase and by telomere end-capping with various proteins (Morin, 1989). Telomerase activity is detectable in only a subset of normal cells but is present in more than 80% of tumor cells and primary tumors (Kim et al., 1994). A number of observations, notably that inhibition of telomerase limits the growth of tumor cells (Hahn et al., 1999), have led to proposals that telomerase is a potential target for cancer chemotherapy (see, for example, Neidle and Parkinson, 2002; Shay and Wright, 2002). One approach for telomerase inhibition is to promote the formation of secondary structures (G-quadruplexes) within telomeric DNA itself, which cannot be recognized by the RNA template component of telomerase. G-quadruplex stabilization can be achieved by small molecules, such as the trisubstituted acridine compound BRACO-19, which inhibits 50% of telomerase activity at a concentration of 160 nM (Read et al., 2001; Gowan et al., 2002).

Telomerase activity and telomere status are increasingly recognized as important influences on the development of resistance to a number of chemotherapeutic agents. Telomere elongation is associated with resistance to 5-fluorouracil and *cis*-diamminedichloroplatinum, and telomere dysfunction leads to increases in sensitivity to DNA double-strand break–inducing agents such as doxorubicin and actinomycin (Lee et al., 2000; Kuranaga et al., 2001). Interestingly, telomerase inhibition in human malignant glioblastoma cells leads to an increase in susceptibility to cisplatin-induced apoptosis (Kondo et al., 1998). These results suggest that telomere stabilization, possibly involving an up-regulation of telomerase activity, has a role in the maintenance of the resistant phenotype to these compounds. This is consistent with the frequent observations of elevated telomerase levels correlating with disease progression.

In the present study, we have characterized an HCT116 human colon carcinoma cell line with an acquired 8-fold resistance to flavopiridol, in terms of telomere length and telomerase activity. Flavopiridol-resistant cells were found to overexpress mRNA for the telomerase catalytic subunit (hTERT) and showed telomere elongation and elevated expression of mRNA for the telomere binding protein Pot1. Flavopiridol was shown not to interact with telomeric DNA, and no changes in telomerase activity were detectable. In addition, the resistant cells exhibited a decrease in the expression of p53 and p21 proteins, whereas cyclin D1 protein expression was unchanged, as was expression of cyclin T1, cyclin T2a/b, CDK9, and CDK2 proteins. These data suggest that a change in the expression of P-TEFb is probably not involved in the acquisition and maintenance of flavopiridol resistance, whereas telomere stabilization may be required in the generation of the resistant phenotype. This is supported by the observation that treatment with a combination of flavopiridol and the G-quadruplex-interacting telomerase inhibitor BRACO-19 prevents the regrowth of cells during treatment with cytotoxic doses of flavopiridol.

Materials and Methods

Tissue Culture and Compounds. HCT116 human colon carcinoma cells and flavopiridol resistant HCT116 cells were donated by Dr. V. Smith, Institute of Cancer Research (Smith et al., 2001) and were grown as a monolayer maintained in Dulbecco's modified Eagle's media containing 10% (v/v) fetal bovine serum (Invitrogen, Paisley, Scotland, UK), 2 mM L-glutamine, and minimal essential medium nonessential amino acids (Invitrogen) in a 37°C, 5% CO_2 atmosphere. Media were changed weekly. Cells were harvested by washing with phosphate-buffered saline (Dulbecco's phosphate-buffered saline solution A; ICR, London, UK), incubating in trypsin-EDTA (0.05% trypsin in 0.02% EDTA; Invitrogen) at 37°C, neutralizing with media and seeding at appropriate concentrations into tissue culture flasks (Costar; Corning Glassworks, Corning, NY). Flavopiridol was obtained from Dr. V. Smith (ICR, Sutton, UK) and was formulated in DMSO (Sigma), then diluted down with distilled water. The resistant cells had been generated by exposure to increasing concentrations of flavopiridol (starting at 100 nM and increasing to 400 nM) over a period of 3 months. The level of resistance in these cells was checked by a standard growth inhibition assay (see below). They were found to be 8-fold resistant to flavopiridol, in agreement with the original observations.

Sulforhodamine B Growth Inhibition Assay. This was used to screen cells for toxicity to each of the compounds described and was carried out as described in Smith et al. (2001) after 96-h exposure to each compound.

Reverse Transcription and PCR. RNA was extracted from cell pellets using the QIAGEN RNeasy Minikit following the manufac-

turer's instructions. The total RNA concentration was determined by measuring absorbance at 260 nm using a GeneQuant (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The RNA was stored at −80°C until required.

Reverse transcription was carried out using the cDNA cycle kit (Invitrogen) following the manufacturer's instructions, using the primers detailed below. PCR reactions all contained 1× reaction buffer (without $MgCl_2$), 200 μM dNTP mix, 1 μM forward primer, 1 μM reverse primer, and Red Hot DNA Polymerase (0.025 units). $MgCl_2$ was also added as required. All DNA primers were obtained from Cruachem Ltd (Glasgow, Scotland, UK). Reaction products were resolved on a 1% agarose gel (Invitrogen).

Determination of Telomere Length. Telomere length was determined using both Slot and Southern blotting techniques as described previously (Counter et al., 1992; Bryant et al., 1997). Probes were constructed by combining 10 μM concentrations of the oligonucleotides SLOT1 (TTAGGGTTAGGGTTAGGGTTAGGG) or CENT1 (GTTTTGAAACACTCTTTTTGTAGAATCTGC) (Oswell, Southampton, UK), 1× kinase buffer, sterile water, 10 μCi of [γ^{32}-P]ATP and 1.6 units of T4 polynucleotide kinase, then incubating for 1 h at 37°C and were purified using a Bio-spin chromatography column (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). Total genomic DNA was extracted from cell pellets using the QIAamp blood kit (QIAGEN) following the manufacturer's protocol, and 40 μg was resolved on an 8% agarose gel under pulsed-field conditions that was transferred to a nylon membrane (Hybond-XL; Amersham Biosciences). Slot blots were carried out using 100 and 50 ng of genomic DNA. Both membranes were hybridized with probe overnight at 42°C. Bound probe was detected by exposing the membrane to a phosphor screen (Amersham Biosciences) for at least 1 h. The screen was scanned using the Storm 820 PhosphorImager and the bands visualized using ImageQuant software (ver. 3.3). Slot blots were then stripped and reprobed with the control (CENT1/centromere) probe.

Fluorescence Resonance Energy Transfer (FRET). All oligonucleotides and their fluorescent conjugates were purchased from Oswell (Southampton, UK). DNA was initially dissolved as a 50 μM stock solution in purified water; further dilutions were carried out in the relevant buffer. The ability of the compounds to stabilize G-quadruplex DNA was investigated using a fluorescence resonance energy transfer (FRET) assay modified to be used as a high-throughput screen in a 96-well format. The labeled oligonucleotide F21T (5′-*FAM*-dGGG(TTAGGG)$_3$-*TAMRA*-3′) used as the FRET probe was diluted from stock to the correct concentration (400 nM) in a 50 mM potassium cacodylate buffer, pH 7.4, and then annealed by heating to 85°C for 10 min., followed by cooling to room temperature in the heating block. Compounds were stored as 10 mM stock solutions in DMSO; final solutions (at 2× concentration) were prepared using water or 1 M HCl in the initial 1:10 dilution, after which 50 mM potassium cacodylate buffer, pH 7.4, was used in all subsequent steps. Experiments were performed in 96-well plates, which were prepared by aliquoting 50 μl of the annealed DNA to each well, followed by 50 μl of the compound solutions. Measurements were made on an Opticon DNA Engine (MJ Research, Waltham, MA) with excitation at 450 to 495 nm and detection at 515 to 545 nm. Fluorescence readings were taken at intervals of 0.5°C over the range 30 to 100°C, with a constant temperature being maintained for 30 s before each reading to ensure a stable value. Final analysis of the data was carried out using a script written in the program Origin 7.0 (OriginLab Corp., Northampton, MA).

Western Blotting. Western blot analysis of proteins was carried out using asynchronous cells in exponential growth phase as described previously (Sharp et al., 1994). Bands were detected using enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA). Antibodies were obtained from Santa Cruz Biochemicals (p53, cyclin T2a/b, cyclin T1, CDK9, CDK2, β-tubulin) CN Biosciences Ltd, UK (p21), and Stratech Scientific Ltd, UK (cyclin D1). Secondary antibodies were purchased in each case from Amersham Biosciences.

Telomere Repeat Amplification Protocol (TRAP) Assay. The TRAP assay was used to assess telomerase activity in a cell-free assay according to the method described previously (Gowan et al., 2002). In our assays, 5 and 20 ng of protein were used in each reaction.

Growth Curves and Combination Treatment with BRACO-19 and Flavopiridol. Wild-type or flavopiridol resistant HCT116 cells (1×10^5) were seeded and treated twice weekly with 2 μM BRACO-19 (a concentration that greatly exceeds the concentration for 50% telomerase inhibition but is noncytotoxic) and 0.04 μM flavopiridol (IC_{25}). Cells were harvested once weekly and counted using a hemocytometer; 1×10^5 cells were reseeded and retreated. Treatment continued until fewer cells were counted than were initially seeded. For the growth curves, 1×10^5 of each cell type was seeded and counted once weekly, before 1×10^5 cells were reseeded. Media was changed once weekly.

Results

Flavopiridol Resistance Is Accompanied by an Increase in hTERT Expression and Increased Telomere Length but No Increase in Telomerase Activity. Because telomere elongation and increases in telomerase activity have been observed in the resistance to the cancer chemotherapeutic agents 5-fluorouracil and *cis*-diamminedichloroplatinum, we first set out to measure these parameters in the flavopiridol-resistant cells. First, however, both the flavopiridol-resistant HCT116 and the parental wild-type (WT) cells were screened for flavopiridol toxicity using a sulforhodamine B staining assay. In agreement with previous results, the resistant cells displayed an 8-fold resistance to flavopiridol compared with WT cells (IC_{50} values of 0.08 ± 0.03 and 0.61 ± 0.06 μM, respectively). No cross-resistance to BRACO-19 was observed. Southern blotting showed that the mean telomere length in the resistant cells was approximately 2 kb greater than that of the parental line (with a mean telomere length in the 4- to 5-kb range) (Fig. 1a). Slot blotting was also used to measure telomere length and produced identical results (data not shown).

Because of the activity of flavopiridol as a cell cycle inhibitor, one possible basis of resistance may be a selection for slower-growing cells. Hence, such a subpopulation would be intrinsically resistant to compounds that act upon cell-cycle components. To examine whether this was the case, we com-

TABLE 1
Primers

Primer Name	Forward Sequence	Reverse Sequence
GAPDH	GGCAGTGATGGCATGGACTG	CGGGAAGCTTGTGATCAATGG
hTERT	GCCAAGTTCCTGCACTGGCTGATG	GTTCTGGGGTTTGATGATGCTGGCG
Pot1	TTCAGATGTTATCTGTCAATCAGAACCTG	GAACACTGTTTACATCCATAGTGATGTATTGTTCC
TEP1	TCAAGCCAAACCTGAATCTGAG	CCCGAGTGAATCTTTCTACGC
TRF1	GCACGAATTCAACATGGCGGAGGATGTTTC	GCACTCTAGATCAGTCTTCGCTGTCTGAGG
hTR	TCTAACCCTAACTGAGAAGGGCGTAG	GTTTGCTCTAGAATGAACGGTGGAAG

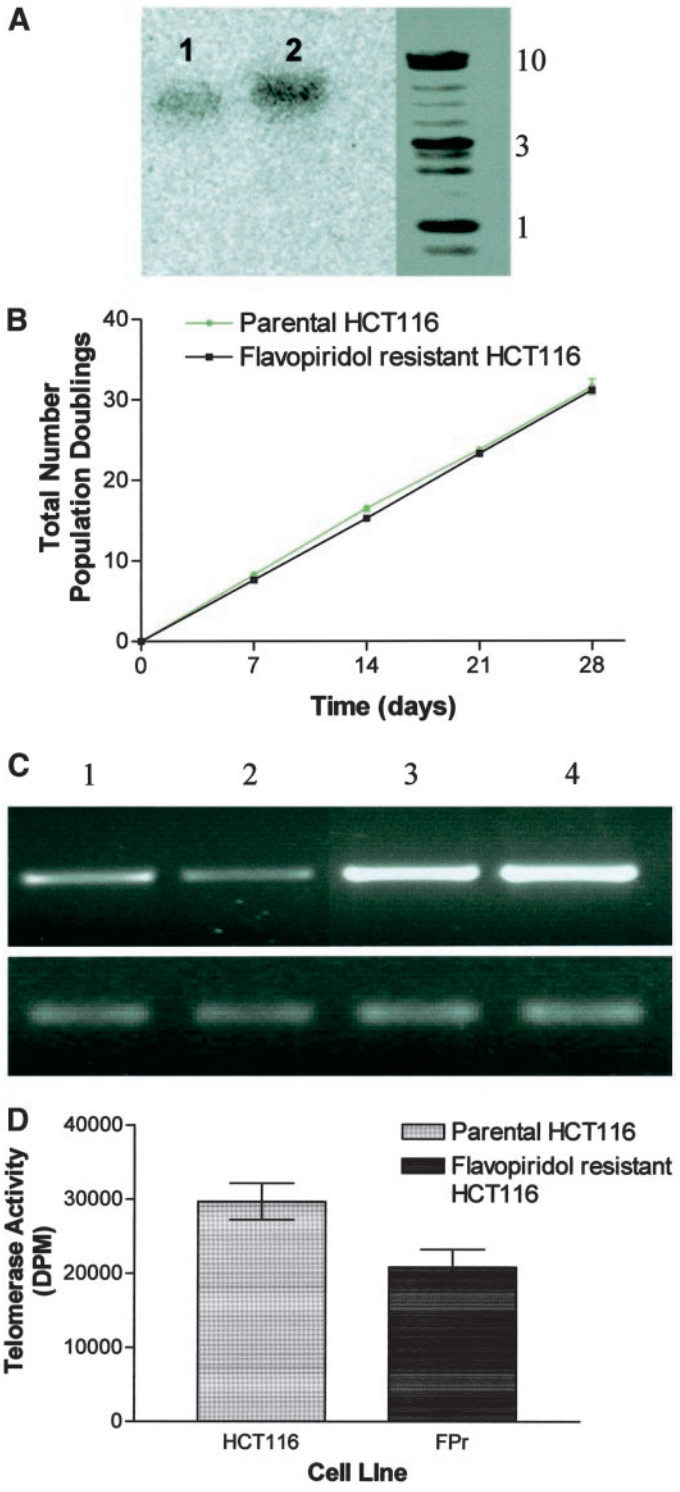

Fig. 1. a, Southern blot measurement of telomere length in (lane 1) HCT116 and (lane 2) HCT116 flavopiridol-resistant cells. Molecular weights are labeled as determined using a 10-kb molecular ruler (Abgene, Epsom, UK). b, graph comparing the growth of flavopiridol-resistant HCT116 and wild-type HCT116 cells. The population doubling times for the flavopiridol-resistant and the parental cells were 1.02 and 0.96 days, respectively ($n = 3$). c, RT-PCR for hTERT (top) and GAPDH (bottom) in HCT116-1 (lane 1), HCT116-2 (lane 2), HCT116-flavopiridol resistant-1 (lane 3), and HCT116-flavopiridol resistant-2 (lane 4). d, graphical representation of telomerase activity in HCT116 and HCT116 flavopiridol-resistant cells. Telomerase activity is plotted as disintegrations per minute counted ($n = 3$) ($p = 0.009$).

pared the growth rates of both the WT and flavopiridol-resistant HCT116 cells. The growth curve (Fig. 1b) demonstrates that both the WT and flavopiridol-resistant HCT116 cells displayed identical growth kinetics. Hence, flavopiridol resistance does not seem to be a result of any changes in the rate of cellular growth.

RT-PCR against hTERT revealed that the mRNA for this protein is overexpressed in the resistant cells at a level 2.5-fold greater than that of the parental cells (Fig. 1c). However, TRAP assay analysis revealed that despite these observations, telomerase activity in the resistant cells was slightly decreased compared with that of the parental cells (Fig. 1d). To rule out any inhibitory effect of flavopiridol on telomerase activity, TRAP assays were also performed in its presence. No effect on telomerase activity was observed at concentrations below 20 μM (data not shown). Additional RT-PCR was performed against the telomerase RNA component (hTR) and the telomerase associated protein TEP1. Neither protein demonstrated any changes in mRNA expression (data not shown).

In addition to screening for any changes in hTERT expression, we examined the cells to ascertain whether there were any changes with regard to telomere stability in the resistant cells. We hypothesized that because of their roles in telomere maintenance and in the *cis*-regulation of telomerase activity, the telomere-associated proteins TTAGGG repeat binding factor 1 (TRF1; Broccoli et al., 1997; van Steensel and de Lange, 1997), TRF2 (van Steensel et al., 2000), and Pot1 (Baumann and Cech, 2001) may be differently expressed in the resistant cells. Western blotting demonstrated that there was no change in the expression of TRF2. TRF1 mRNA expression was also unchanged. RT-PCR for Pot1, however, revealed that mRNA for this protein is overexpressed in the resistant cells (Figs. 2, a and b).

Flavopiridol-Resistant Cells Exhibit Decreased Expression of p53 and p21 with No Change in Cyclin D1 Expression. The finding that the resistant cells possess elongated telomeres despite the lack of change in telomerase activity suggested that telomere elongation may be taking place via other means. It has previously been shown that non–telomerase-mediated telomere elongation, or alternative lengthening of telomeres, is preceded by increases in cyclin D1 and a decrease in p53 expression and that p53 status is associated with telomere stability and drug resistance (Lee et al., 2000; Opitz et al., 2001). To examine whether this was the case in the flavopiridol-resistant cells, we performed Western blots for p53, p21, and cyclin D1. As shown in Fig. 3, the resistant cells displayed a reduction in the expression of both p53 and p21, but no change in cyclin D1 expression compared with the parental cells.

Expression of the P-TEFb Subunits CDK9, Cyclin T1, and Cyclin T2a/b Are Unchanged in Flavopiridol-Resistant Cells. Because of the potent and specific inhibition of P-TEFb by flavopiridol (Chao and Price, 2000), we performed Western blotting and RT-PCR to investigate whether up-regulation of P-TEFb was responsible for the acquisition flavopiridol resistance. The flavopiridol-resistant cells did not exhibit any change in the expression of mRNA or protein for CDK9, cyclin T1, or cyclin T2a or T2b (Fig. 4). In addition, we were unable to detect any changes in the expression of CDK2 in the resistant cells.

Combinations of BRACO-19 and Flavopiridol Prevent the Cellular Recovery That Occurs with Either Compound Alone. The potential link between flavopiridol resistance and the telomerase/telomere pathways suggested that by using combinations of flavopiridol and a telomerase inhibitor, we might be able to prevent the growth of cells that are resistant to flavopiridol alone. We began by treating HCT116 cells with 2 μM BRACO-19 alone, 40 nM flavopiridol alone (at the IC_{25} concentration), or a combination of both compounds. Treatment of HCT116 cells with BRACO-19 alone led to a slight decrease in cell growth (Fig. 5a). Treatment with flavopiridol alone also initially reduced cell growth, but this was followed by a slow recovery of growth rate as resistance began to develop. However, treatment with the BRACO-19 + flavopiridol combination led to a complete inhibition of cell growth after 21 days of treatment. This data indicates that the presence of BRACO-19 prevents the emergence of cells with resistance to flavopiridol.

It is also clear that the converse is true, where treatment with the combination prevents cells growing through BRACO-19 treatment. Because flavopiridol has been shown to bind to DNA, we hypothesized that the combination might be influencing the binding affinity of BRACO-19 at the telomere. In addition, because flavopiridol-resistant cells possess elongated telomeres, telomere elongation may result in an increased number of DNA binding sites for flavopiridol. Hence, in a population of cells with increased telomere length, BRACO-19 would be essentially sequestered by the telomeric DNA and less would be available to interact with its target. To examine whether this was the case, we performed FRET assays in the presence of flavopiridol, BRACO-19, or a combination of the two. The FRET probe used is designed to resemble the human 3′ telomeric single-stranded DNA overhang and has been shown to form G-quadruplexes under the conditions employed in the assay.

The melting curve obtained using BRACO-19 alone is exactly as one would expect for a G-quadruplex–stabilizing compound, where rapid increases in melting temperature are

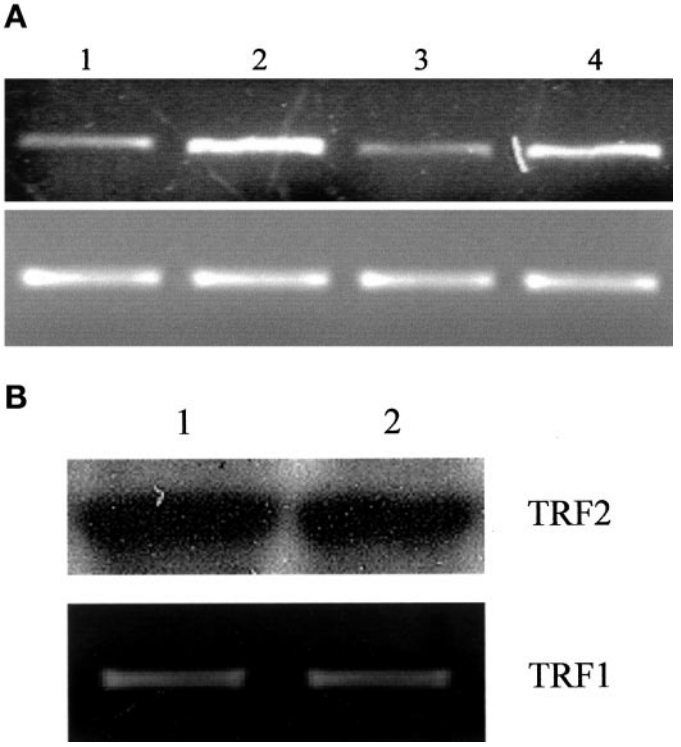

Fig. 2. a, RT-PCR analysis of Pot1 (top) and GAPDH (bottom) expression in wild-type HCT116 cells (lanes 1 and 3) and in HCT116 flavopiridol-resistant cells (lanes 2 and 4). Each reaction contained 900 ng of total RNA. b, top, Western blot analysis of TRF2 expression in wild-type HCT116 cells (lane 1) and HCT116 flavopiridol-resistant cells (lane 2). Bottom, RT-PCR for TRF1. Amounts used were 15 μg and 900 ng of protein and RNA, respectively.

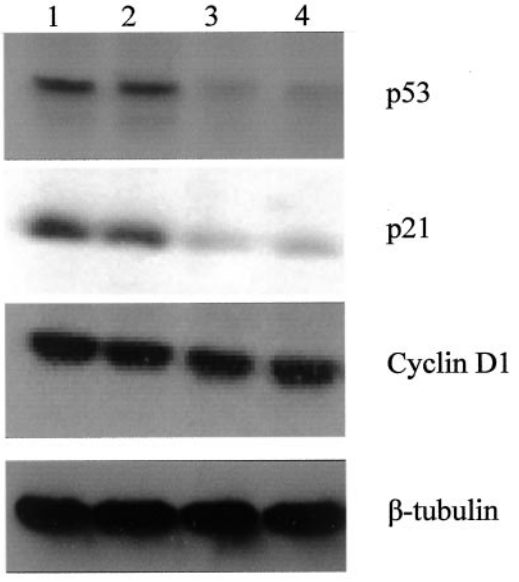

Fig. 3. Western blot analysis of p53, p21, cyclin D1, and β-tubulin protein from) wild-type HCT116 (lanes 1 and 2 and HCT116 flavopiridol-resistant total cell lysates (lanes 3 and 4).

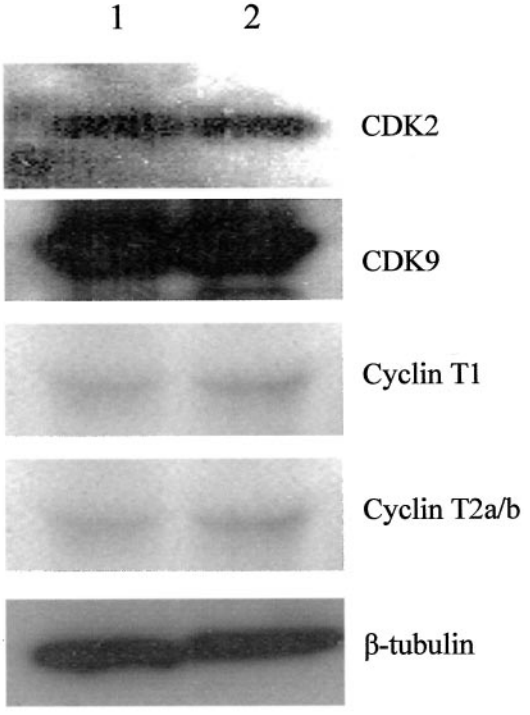

Fig. 4. Western blots for CDK2, CDK9, cyclin T1, and cyclin T2a/b. In each, lane 1 contains wild-type HCT116 cell protein and lane 2 contains HCT116 flavopiridol-resistant cell protein. Both lanes contain 15 μg of protein.

evident with small increases in concentration (Fig. 5b). This shows that the presence of BRACO-19 is stabilizing the secondary structure formed. Using flavopiridol in the same assay, however, we were unable to detect any changes in DNA stability, even at concentrations of 12 μM. Moreover, when BRACO-19 and flavopiridol were added to the reaction in combination (at a ratio of 5:1), no changes were observed compared with the melting profile obtained using BRACO-19 alone. This demonstrates that flavopiridol alone is not binding to G-quadruplex DNA and flavopiridol does not influence the DNA binding characteristics of BRACO-19.

The behavior of cells treated with the BRACO-19 + flavopiridol combination demonstrates that the combination prevents recovery from treatment with either compound alone. Although the basis for this synergy is not totally clear, the observation suggests that a factor(s) that mediates the recovery with BRACO-19 would prevent flavopiridol resistance and vice versa. To examine whether this is the case, we treated flavopiridol-resistant HCT116 cells with 2 μM BRACO-19. As Fig. 5c shows, compared with treatment with a vehicle control (DMSO) or flavopiridol alone, BRACO-19 significantly inhibits the growth of the resistant cells. The extent of the growth inhibition by BRACO-19 treatment is comparable with the response of these cells to treatment with the BRACO-19 + flavopiridol combination.

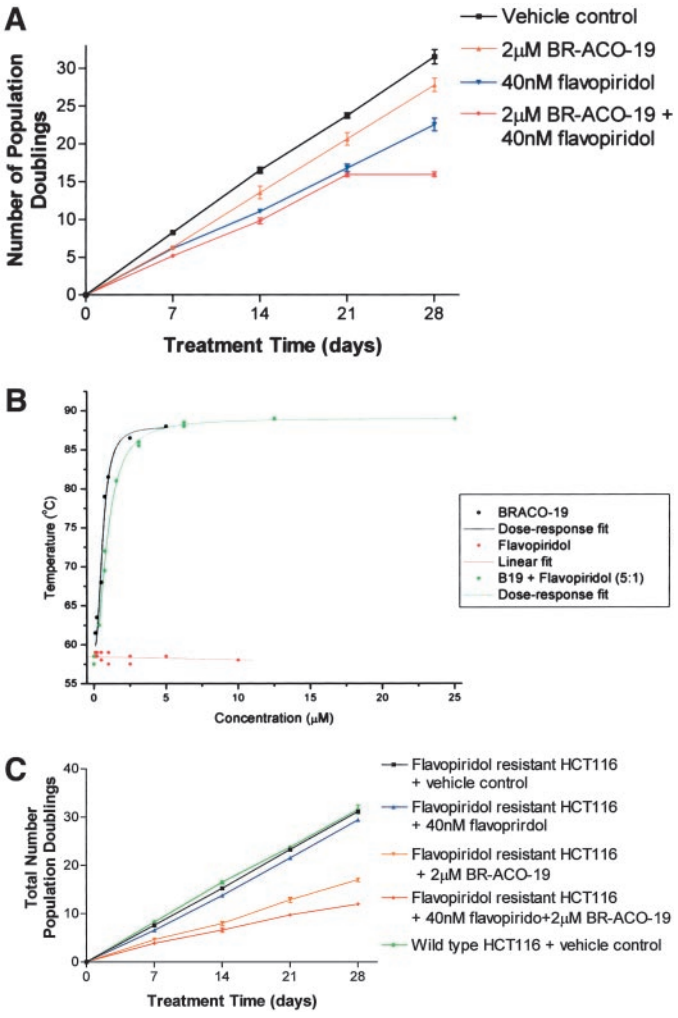

Fig. 5. a, graph showing the response of wild-type HCT116 cells to treatment with 2 μM BRACO-19, 40 nM flavopiridol, or a combination of the two. Cells (1×10^5) were seeded initially; the experiment was terminated when less than 1×10^5 were counted ($n = 4$). b, FRET-based analysis of the melting of quadruplex DNA in the presence of BRACO-19, flavopiridol, or a combination of the two. The melting curves for BRACO-19 alone and with flavopiridol are statistically identical. Flavopiridol alone did not produce quadruplex stabilization. c, the effect of treatment with 2 μM BRACO-19, 40 nM flavopiridol, or a combination of the two on the growth of flavopiridol-resistant HCT116 cells ($n = 3$).

Discussion

We have characterized an HCT116 cell line in this study with an acquired 8-fold resistance to the CDK inhibitor flavopiridol. This resistant line shows increased telomerase activity and telomere length compared with the parental line. A previous study on this resistant cell line detected few molecular changes, although small increases in cyclin E expression and associated kinase activities were found (Smith et al., 2001). Changes in telomere-associated features were not examined.

Because flavopiridol is a potent and specific inhibitor of P-TEFb, we initially examined the expression of this protein; up-regulation of target proteins is frequently associated with resistance to a number of chemotherapeutic agents, including thymidylate synthase inhibitors (Welsh et al., 2000). However, because we were unable to detect any changes in the expression of the P-TEFb subunits CDK9, cyclin T1, and cyclin T2, we conclude that changes in the expression of P-TEFb are not involved in the maintenance of flavopiridol resistance in HCT116 cells. Although expression of various ATP-binding pumps is a mechanism of resistance to several drugs, including paclitaxel (Kamazawa et al., 2002) and doxorubicin (Dolfini et al., 1997), no change in the expression of this protein was detectable in the cell line used in this study (Smith et al., 2001). This suggests that, in the HCT116 cell line at least, flavopiridol resistance is achieved by other mechanisms that are likely to be based on the cell cycle.

Expanding on recent studies indicating that telomere stabilization is involved in resistance to several anticancer drugs, we characterized the resistant line in terms of telomere length. Telomere shortening and telomere dysfunction increase cellular sensitivity to some DNA double-strand break– inducing agents (Lee et al., 2000; Kuranaga et al., 2001). We have detected a significant increase in mean telomere length in the resistant cells that was approximately 2 kb greater than that in parental cells. In addition, mRNA levels for the telomere-associated proteins hTERT and Pot1 were elevated, and expression of p53 and p21 proteins was decreased. These findings show that telomere stabilization is a characteristic of the resistance phenotype, although not necessarily a functional requirement. The capping of free telomeric ends by proteins such as hTERT and Pot1 protects telomeres from recognition as double-strand breaks and the induction of cell cycle arrest. It has also been suggested that some cells with very short telomeres escape senescence by means of telomere end-capping (Karlseder et al., 2002). The slight decrease in telomerase activity observed here in the resistant cells agrees well with this, because the presence of hTERT is more important than telomerase activity per se. This is likely to be controlled post-translationally or may represent a splice variant of hTERT. Several other proteins are thought to have roles in maintaining telomere organization, including RAD50, ATM, and TRF2 (Blackburn, 2001),

although we observed little change in TRF2 expression in the resistant cells. Sufficient TRF2 may be present at basal levels to facilitate telomere stabilization in the resistant cells.

Telomeres have also been postulated to be storage sites for DNA damage-response proteins (Wright et al., 1996). Hence, a mechanism by which telomere elongation leads to resistance to double-strand break–inducing agents may be via sequestration of the proteins that induce apoptosis or simply by allowing the cells to tolerate greater insults by reducing the chances of senescence/apoptotic pathways occurring. Although flavopiridol has been shown to bind to duplex DNA (Bible et al., 2000a), this occurred only at relatively high concentrations of the compound ($>$ 500 nM) and is unlikely to contribute to the mechanism of telomere stabilization as part of the resistance phenotype. Our own observations agree with this, because flavopiridol was shown neither to bind to telomeric DNA nor to effect G-quadruplex stability in vitro.

A clue to the mechanism by which telomere elongation occurs in these cells is provided by the observed decreases in p53 and p21 expression in the resistant cells. P53 has recently been implicated in the control of telomere length, where it has been shown to bind to the single-stranded telomeric overhang and is required for activation of the senescence pathways triggered by telomere shortening/uncapping (Stansel et al., 2001; Harrington and Robinson, 2002). Furthermore, inactivation of p53 is associated with alternative lengthening of telomeres, which is believed to occur via recombination events (Dunham et al., 2000). Although our resistant cells did not exhibit the characteristically long heterologous telomeres associated with alternative lengthening of telomeres, we cannot rule out some degree of homologous recombination occurring. Indeed, this would fit in well with the observation that telomerase activity in the resistant cells is unchanged despite the increases in telomere length. Interestingly, it has been reported recently that there is an increase in telomere length together with a decrease in telomerase activity in doxorubicin-resistant stomach cancer cells (Kim et al., 2002). These data suggest that telomere elongation/stabilization may be occurring in resistance to a large variety of drugs.

Therefore, if the mechanism underlying flavopiridol resistance is cell-cycle related, a downstream effect may be an alteration in p53 expression that indirectly leads to the changes we have seen in telomere length and capping. On the other hand, telomere lengthening and stabilization may be a prerequisite for the development of flavopiridol resistance in these cells. Whichever may be the case, these results provide a good rationale for treatment of cancer cells using combinations of telomerase inhibitors/telomere interacting compounds and cell-cycle inhibitors such as flavopiridol.

In support of this, our data also show that a combination of flavopiridol and the G-quadruplex telomerase inhibitor BRACO-19 (Read et al., 2001; Gowan et al., 2002) has a strongly synergistic effect on the inhibition of tumor cell growth. Long-term treatment of the wild-type HCT116 cells with flavopiridol alone results in a decrease in growth, which reaches a plateau at ~50% growth inhibition, after which a gradual recovery is eventually observed (data not shown). These cells do not respond to BRACO-19 alone. The combination (with BRACO-19 at a subcytotoxic dose) results in complete inhibition of cellular growth. Furthermore, we have shown that treatment of flavopiridol-resistant cells with BRACO-19 alone leads to rapid inhibition of cellular growth, which is not observed in the WT cells. The flavopiridol-resistant cells also responded to treatment with the combination by rapid growth arrest. It is currently unclear whether this growth arrest is reversible, although if the effect of BRACO-19 is to cause growth arrest by telomere shortening/uncapping, then it is unlikely that cells will recover. It is notable that growth-arrest in the resistant line occurs rapidly after administration and there is no indication of the extended time lag before response that is characteristic of classic catalytic-site telomerase inhibitors (Pascolo et al., 2002).

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