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Pharmacological Telomerase Inhibition Can Sensitize Drug-Resistant and Drug-Sensitive Cells to Chemotherapeutic Treatment

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ABSTRACT

Effective strategies to reverse or prevent chemotherapeutic resistance are required before cancer therapies can be curative. Telomerase is the ribonucleoprotein responsible for de novo synthesis and maintenance of telomeres, and its activity is predominantly observed in cancer cells. The telomerase enzyme has been successfully inhibited or inactivated to sensitize cells to cellular stresses; however, no studies have determined yet the effect of combining a pharmacological inhibitor of telomerase catalysis and traditional chemotherapeutics for the treatment of drug-sensitive or drug-resistant cancers. Here, we describe the effect of 2-[(*E*)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid (BIBR1532), a small-molecule inhibitor of telomerase catalytic activity, on drug-resistant leukemia and breast cancer cells and their parental counterparts when treated in combination with chemotherapeutics. We observed that BIBR1532-treated cells show progressive telomere shortening, decreased proliferative capacity, and sensitization to chemotherapeutic treatment. These effects are telomere length-dependent, because cells insensitive to BIBR1532 or cells released from telomerase inhibition did not demonstrate changes in growth ability or drug sensitivity. Our novel observations suggest that pharmacological telomerase inhibition in combination therapy may be a valid strategy for the treatment of both drug-sensitive and drug-resistant cancers.

Chemotherapeutic drug resistance remains a major obstacle to the effective treatment and cure of almost all cancers. Numerous strategies to overcome drug resistance are currently being explored, but none have demonstrated success in the clinic. Sensitization of resistant tumors to drug treatment will probably require interference with multiple cellular processes. Therefore, the combination of resistant reversal strategies may have substantial effect on the overall survival of many cancers.

Telomerase is a reverse transcriptase enzyme; its primary function is the maintenance and de novo synthesis of telomeres at the ends of linear chromosomes (Cech, 2004). Telomerase activity is rarely present in normal somatic cells but is observed in ~85% of all cancer cells tested, making the telomerase enzyme an attractive target for anticancer therapeutics (Corey, 2002; Cech, 2004). Our lab and others have reported that telomerase activity is up-regulated in human cell lines upon treatment with DNA-damaging chemotherapeutics, suggesting that telomerase may play an active role in the response to DNA damaging agents (Moriarty et al., 2002; Klapper et al., 2003; Jeyapalan et al., 2004). Furthermore, telomerase expression in normal human fibroblasts enhances DNA repair activities (Shin et al., 2004). Telomerase has also been implicated in mediating other effects in addition to telomere maintenance, such as suppression of apoptosis, promotion of in vivo proliferative capacity, and protection against ischemia and *N*-methyl-D-aspartate–induced neurotoxicity (Cerone et al., 2004).

The consequence of telomerase inhibition in immortal hu-

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ABBREVIATIONS: ALT, alternative lengthening of telomeres; NT, nontreated; PD, population doubling; TRAP, telomeric repeat-amplification protocol; TRF, terminal restriction fragment; PCR, polymerase chain reaction; MCF-7/Adr^R, doxorubicin-resistant MCF-7 cell line; MCF-7/Mln^R, melphalan-resistant MCF-7 cell line; BIBR1532, 2-[(*E*)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid; HL60/MX2, etoposide-resistant HL60 cell line; WT, wild type; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; kb, kilobase; Rel, late-passage BIBR1532-treated HL60/MX2 cells released from treatment and allowed to proliferate for an additional 30 population doublings; Cont, control.

man cells is telomere shortening and eventual growth arrest and/or apoptosis (Hahn et al., 1999; Corey, 2002). Unfortunately, these effects are often observed only in cells with initially short telomeres and after prolonged antitelomerase treatment. Furthermore, strong selective pressure to maintain telomeres can lead to the reactivation of telomerase, either by transcriptional up-regulation or loss of the inhibitor (Zhang et al., 1999; Delhommeau et al., 2002) or the activation of an alternative lengthening of telomere (ALT) mechanism (Bechter et al., 2004). These observations suggest that telomerase inhibition alone may not be an effective strategy for cancer treatment (Corey, 2002). However, combining telomerase inhibition with chemotherapeutic treatment may prove more effective than either approach on its own. Indeed, studies have demonstrated that telomerase inhibition or inactivation generates increased cellular sensitivity to UV irradiation (Wong et al., 2000), DNA-damaging agents (Chen et al., 2003), and the tyrosine kinase inhibitor imatinib (Tauchi et al., 2002). On the other hand, telomerase overexpression confers cellular protection from apoptosis by serum deprivation and DNA-damaging agents (Cerone et al., 2004).

Telomerase inhibition is usually achieved via gene knockout, antisense oligonucleotides, or dominant-negative forms of the telomerase enzyme. Although these strategies are specific, one major drawback to these approaches is their clinical feasibility and/or usefulness (Lane, 2005). Therefore, we were interested in determining the effect of combining the treatment of a small-molecule pharmacological inhibitor of telomerase catalytic activity, BIBR1532, and traditional chemotherapeutics on paired drug-sensitive and drug-resistant cell lines. BIBR1532 is a highly selective noncompetitive, non-nucleoside pharmacological inhibitor of telomerase catalytic activity, demonstrating in vitro IC_{50} concentrations in the nanomolar range (Damm et al., 2001; Pascolo et al., 2002).

This is the first study to address the consequence of using a highly selective and potent pharmacological inhibitor of telomerase catalytic activity, BIBR1532, in combination with chemotherapeutics; moreover, there have been no reports addressing the feasibility of using such a pharmacological telomerase inhibitor to sensitize drug-resistant cells to traditional therapies. We hypothesized that BIBR1532 would inhibit telomerase and sensitize both drug-sensitive and drug-resistant cell lines to traditional chemotherapeutics such as etoposide, melphalan, or doxorubicin, three commonly prescribed DNA damage-inducing chemotherapeutics used for the treatment of cancers of the bone, lung, breast, brain, blood, and ovaries (http://www.nlm.nih.gov/medlineplus/druginformation.html). To test this, we first characterized basal telomerase activity and basal telomere length in drug-resistant human promyelocytic leukemia (HL60/MX2) and breast cancer (MCF-7/Mln^R and MCF-7/Adr^R) cell lines and their drug-sensitive parental (WT) counterparts (HL60/WT and MCF-7/WT, respectively). We examined telomere length, growth capacity, and chemotherapeutic sensitivity in parental and drug-resistant cell lines treated with BIBR1532. We found that BIBR1532 reduced growth capacity and enhanced chemotherapeutic sensitivity in both drug-sensitive and drug-resistant cell lines in a telomere length-dependent manner. These novel observations suggest that pharmacological telomerase inhibitors may aid in the treatment of both drug-sensitive and drug-resistant malignancies.

Materials and Methods

Cell Lines and Reagents. HL60/WT and etoposide-resistant HL60/MX2 cells (purchased from American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with antibiotics and 10% fetal bovine serum (WISENT, St. Bruno, QC, Canada) and at each passage were counted by hemacytometer and strictly maintained at a cell density between 2×10^5 and 1.2×10^6 cells/ml. MCF-7/WT, melphalan-resistant MCF-7/Mln^R (acquired from Dr. Moulay Alaoumi-Jamali, McGill University, Montreal, QC, Canada), and doxorubicin-resistant MCF-7/Adr^R (from Dr. Michael Pollack, McGill University) cells were grown in minimal essential medium supplemented with antibiotics and 10% fetal bovine serum (WISENT) and were routinely passed one in four upon reaching 80 to 90% confluence. Where indicated, cells were grown continuously in media containing 2.5 μM BIBR1532 (a gift from Dr. Jacques van Meel, Boehringer Ingelheim, Vienna, Austria) suspended in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO). Etoposide, melphalan, and doxorubicin were purchased from Sigma-Aldrich; dissolved in DMSO, 95% ethanol, or H_2O, respectively; and stored in aliquots at -20°C. Where indicated, vehicle refers to the respective solvent.

Telomerase Activity (TRAP) Assay. Cells were collected and stored at -80°C until extracts were prepared in Nonidet P-40 lysis buffer. Telomeric repeat-amplification protocol (TRAP) reactions were performed with 10 ng of protein extract per reaction (unless otherwise indicated), incubated at 30°C for 30 min, and telomerase elongation products were amplified by PCR (Moriarty et al., 2002). Telomerase activity was quantified relative to the internal PCR control, and this ratio was expressed relative to the same ratio calculated for the indicated reference samples.

To determine telomerase activity upon IC_{50} etoposide treatment, HL60/WT and HL60/MX2 cells were plated in six-well plates in a final volume of 5 ml and at a starting density of 2×10^5 cells/ml. Cells were treated with vehicle or IC_{50} concentrations of etoposide (0.35 μM for HL60/WT and 9.84 μM for HL60/MX2 cells). Twenty-four, 48, 72, and 96 h after treatment, cells were collected and processed for TRAP as described. To verify the effect of etoposide treatment, cells were plated in parallel for an MTT assay, which was performed at the 48-h time point. For in vitro determination of BIBR1532-mediated telomerase inhibition, 2.5 μM BIBR1532 or an equal volume of DMSO was added to TRAP reactions containing 40 or 20 ng of untreated MCF-7/WT and MCF-7/Adr^R cell extracts. Reactions were incubated at 30°C for 30 min and processed as described above.

Telomere Length Analysis. Telomere length was determined by terminal restriction fragment (TRF) analysis (Cerone et al., 2001). Genomic DNA was extracted by standard procedure and digested with HinfI and RsaI. Equal amounts of digested DNA were separated by pulse-field gel electrophoresis; gels were then partially dried, denatured and neutralized, and incubated for a minimum of 12 h with a [γ-^{32}P]ATP 5′-end–labeled telomeric probe. Telomeric signals were visualized after exposure of gels to a PhosphorImager screen or to X-ray film (Eastman Kodak, Rochester, NY). Hybridization signals were quantified with ImageQuant (Amersham Biosciences, Inc., Piscataway, NJ) from at least two independent DNA extracts subjected to electrophoresis on two separate gels (minimally), and mean telomere length was determined and expressed $\pm$ S.D. as described previously (Cerone et al., 2001).

Cell Viability (MTT) Assay. In 96-well, flat-bottomed microtest plates, HL60/WT and all MCF-7 cell lines were plated in triplicate, in a final volume of 200 μl of media, and at a cell density of 1×10^4 cells/well. HL60/MX2 cells were plated at a starting density of 2×10^4 cells/well. On the same day of plating for HL60 cell lines and the next day for MCF-7 cell lines, cells were treated in triplicate as indicated. Forty-eight hours after treatment, MTT assays were performed as described previously (Christodoulopoulos et al., 1999). All MTT assays were performed at least two independent times.

Proliferation Assay. In 24-well plates and in a final well volume of 1 ml, HL60/WT and HL60/MX2 cells were plated at a starting density of 4×10^5 cells/ml and treated with IC_{25} etoposide (0.117 μM for HL60/WT and for 3.21 μM HL60/MX2). Twenty-four hours after treatment, cells were diluted in a final volume of 5 ml of media (1:20 for HL60/WT, 1:10 for HL60/MX2), allowed to proliferate (5 days for HL60/WT, 6 days for HL60/MX2) and counted with trypan blue. Viable cells counts were normalized and expressed relative to that of the nontreated controls.

Colony-Forming Assay. MCF-7 cell lines were plated at a density of 2×10^5 cells/ well in six-well plates the day before treatment. MCF-7/WT cells and MCF-7/Mln^R cells were treated with 1 and 5 μM melphalan, respectively. MCF-7/WT and MCF-7/Adr^R cells were treated with 0.125 and 100 μM doxorubicin, respectively. Twenty-four hours after treatment, cells were diluted (1:500 for non–chemotherapy-treated cells or 1:10 for chemotherapy-treated cells) into 10-cm^2 plates and allowed to proliferate until control cells had grown into clearly visible colonies (1–3 weeks, depending on cell lines and treatments). Plates were then stained with crystal violet, and colonies were counted and normalized to the number observed for controls. At least two plates of the same treatment were counted for each colony-forming assay, and graphs represent experiments from at least two independent experiments. For accuracy, only colony-forming assays that gave greater than 20 colonies in control plates were used when calculating averages.

Statistical and Data Analysis. Data were analyzed and graphed using Microsoft Excel (Microsoft, Redmond, WA). Statistical analysis was performed by *t* test using the online statistical software GraphPad Quickcalcs (http://www.graphpad.com/quickcalcs/ttest1.cfm). Statistical probability in figures is expressed as $\star p < 0.05$ and $\star\star p < 0.01$.

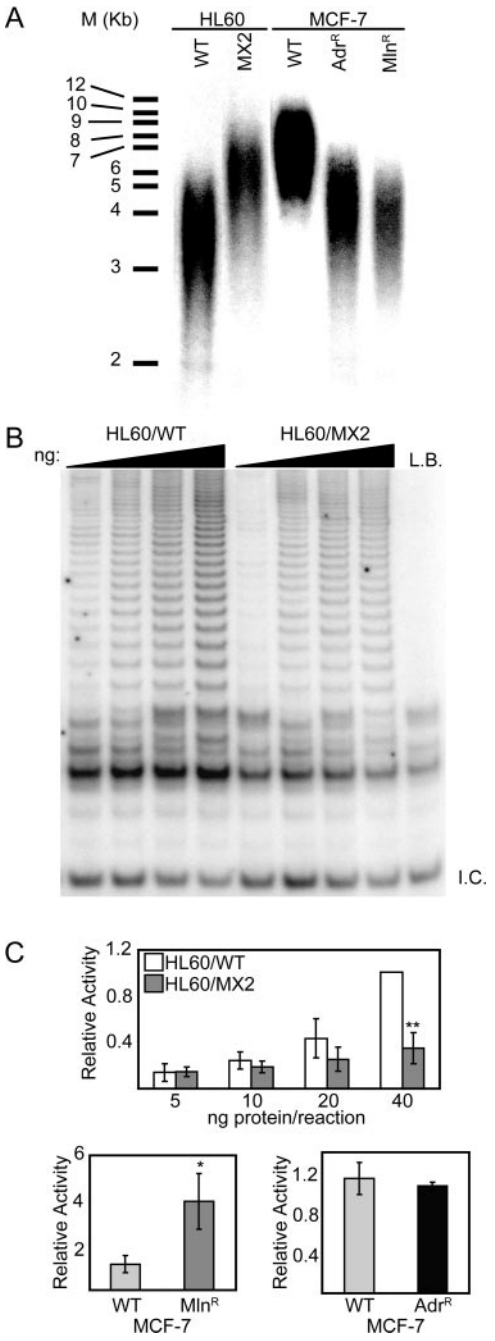


Fig. 1. Basal telomere length and telomerase activity of paired drug-resistant and -sensitive cell lines. A, TRF analysis of basal telomere length. M, DNA marker (in kilobases). B, representative gel showing basal telomerase activity of 5, 10, 20, or 40 ng (◢) of HL60/WT or HL60/MX2 protein extract per TRAP reaction. L.B., lysis buffer; I.C., internal PCR control. C, quantification of basal telomerase activity from three independent extract preparations expressed relative to the telomerase activity/PCR internal control ratio of the 40 ng reaction, ± S.D. ⋆, $p < 0.05$, and ⋆⋆, $p < 0.01$ compared with relative activity of parental cell line.

Results

Drug-Resistant and Drug-Sensitive Cell Lines Display Different Basal Telomere Lengths and Basal Telomerase Activities. A number of reports have indicated that drug-resistant cell lines have different levels of telomerase activity or exhibit changes in telomere length compared with drug-sensitive controls (Park et al., 1998; Kuranaga et al., 2001; Kim et al., 2002; Incles et al., 2003; Deschatrette et al., 2004). Therefore, we characterized basal telomere length

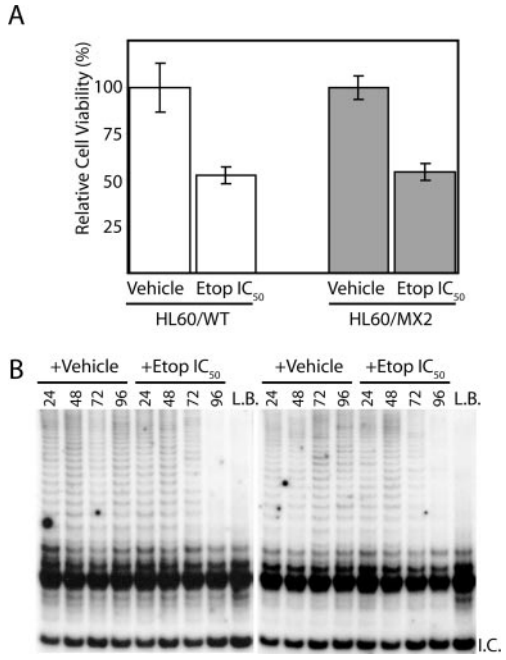
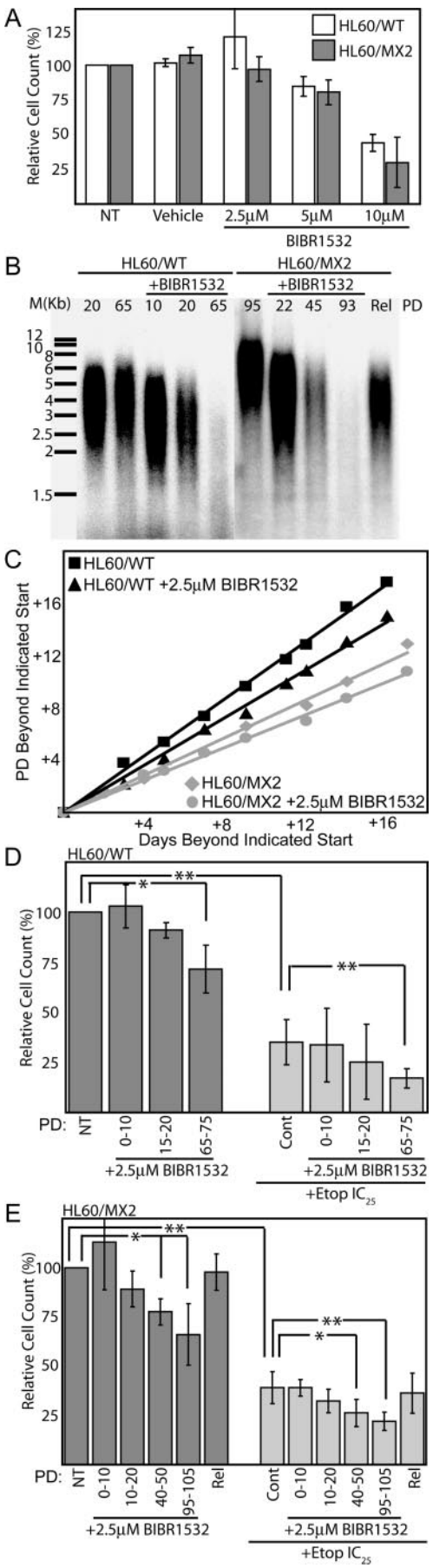


Fig. 2. HL60/WT and HL60/MX2 telomerase activity after treatment at IC_{50} etoposide concentrations. A, cellular viability is inhibited by 50% 48 h after etoposide treatment (0.35 μM for HL60/WT cells, 9.84 μM for HL60/MX2 cells) as measured by MTT assay. Graph represents data from three independent treatments ± S.E. B, representative data showing HL60/WT and HL60/MX2 telomerase activity 24 to 96 h after vehicle or IC_{50} etoposide treatment. L.B., lysis buffer; I.C., internal PCR control.



and telomerase activity in parental and drug-resistant cell lines. HL60/MX2 cells displayed 28-fold resistance to etoposide compared with parental HL60/WT cells (Fig. 2A) (Harker et al., 1989). MCF-7/Mln^R cells and MCF-7/Adr^R cells are 5-fold and more than 200-fold resistant to melphalan (Yen et al., 1995) and doxorubicin (Alaoui-Jamali et al., 1993), respectively. In all cases, we observed differences in basal telomere length between parental and drug-resistant cells (Fig. 1A). HL60/MX2 cells have longer telomeres (mean TRF length, 5.73 ± 0.15 kb) compared with those of HL60/WT cells (mean TRF length, 3.66 ± 0.29 kb). On the other hand, telomere lengths were shorter in both MCF-7/Mln^R and MCF-7/Adr^R (mean TRF length, 4.15 ± 0.21 and 4.66 ± 0.68 kb, respectively) compared with those of MCF-7/WT (mean TRF length, 7.06 ± 0.6 kb). Likewise, basal telomerase activity of HL60/MX2 and MCF-7/Mln^R cells differed from that of the WT cell lines as measured by TRAP. HL60/MX2 showed lower basal telomerase activity, MCF-7/Mln^R cells demonstrated higher basal telomerase activity, but MCF-7/Adr^R cells did not display any change in activity compared with that of the parental cell line (Fig. 1, B and C).

HL60/MX2 Cells Demonstrate No Significant Difference in Telomerase Activity Compared with HL60/WT Cells When Treated with IC_{50} Concentrations of Etoposide. A number of studies have reported that telomerase activity increases upon cellular treatment with certain chemotherapeutic drugs (Moriarty et al., 2002; Klapper et al., 2003; Jeyapalan et al., 2004). Therefore, we assessed telomerase activity of HL60/WT and HL60/MX2 cells at different time points after treatment with IC_{50} concentrations of etoposide (Fig. 2A). In both HL60/WT and HL60/MX2 cell lines, we observed a decrease in telomerase activity 72 and 96 h after treatment (Fig. 2B), but we observed no statistically significant differences between cell lines. Finally, at the etoposide concentrations and time points analyzed, we observed no statistically significant up-regulation of telomerase activity after treatment.

BIBR1532 Inhibits Telomerase Activity, Induces Telomere Shortening, and Reduces Proliferative Capacity in Both Drug-Sensitive HL60/WT and Drug-Resistant HL60/MX2 Cell Lines. Telomerase inhibition or inactivation has been reported to increase sensitivity to chemotherapeutic treatment (Corey, 2002). However, there have been no studies to date that have addressed the usefulness of a pharmacological inhibitor of telomerase catalytic activity in

Fig. 3. BIBR1532 (2.5 μM) inhibits telomerase, induces telomere shortening, reduces proliferative capacity, and sensitizes HL60/WT and HL60/MX2 to etoposide (Etop) treatment. A, cellular growth 72 h after treatment with 2.5, 5, or 10 μM BIBR1532. Next, 2×10^5 cells/ml were plated, treated as indicated, and counted by hemocytometer and trypan blue exclusion method 72 h later. Cell counts were normalized to those of NT controls. Graphs represent three independent experiments ± S.D. B, TRF analysis of HL60/WT and HL60/MX2 cells untreated or treated with 2.5 μM BIBR1532 for the indicated number of PDs. Rel, late-passage BIBR1532-treated HL60/MX2 cells released from treatment and allowed to proliferate for an additional 30 PD; M, DNA marker (in kilobases). C, growth analysis of cells in mass culture at the time of proliferation assays. Graph shows HL60/WT BIBR1532-treated cells starting from PD 60 and HL60/MX2 BIBR1532-treated cells starting from PD 93. D and E, proliferative capacity of HL60/WT (D) and HL60/MX2 (E) ± IC_{25} etoposide treatment as measured by 5- or 6-day proliferation assay. PD, the number of population doublings the cells were treated with BIBR1532 before experiments were performed. Bars represent values from at least three independent proliferation assays ± S.D. *, $p < 0.05$, and **, $p < 0.01$ compared with the indicated control.

combination strategies. BIBR1532 is an effective small-molecule inhibitor of the human telomerase enzyme, demonstrating high potency and selectivity in vitro (Damm et al., 2001). We first determined whether telomerase inhibition sensitizes HL60 cell lines to etoposide treatment. Both HL60/WT and HL60/MX2 cell lines were treated with 2.5 μM BIBR1532; higher concentrations inhibited cell growth (Fig. 3A), probably because of nonspecific cytotoxicity. We observed progressive telomere shortening upon prolonged and continued growth of HL60/WT and HL60/MX2 cells in the presence of BIBR1532 (Fig. 3B). This effect was reversible, because late population doubling (PD) BIBR1532-treated HL60/MX2 cells released from telomerase inhibition and allowed to proliferate an additional 30 PDs (hereafter referred to as HL60/MX2 Rel) displayed lengthened telomeres (Fig. 3B, Rel). Despite progressive telomere shortening, we observed no major defect in cellular proliferation of mass cultures, as shown by similar slopes of growth curves (Fig. 3C). However, when HL60/WT and HL60/MX2 cells were diluted to low densities (1:20 or 1:10, respectively) and allowed to proliferate for longer periods of time (5 or 6 days, respectively) without allowing the cultures to become overconfluent, we observed decreased proliferative capacity of late PD BIBR1532-treated cells (Fig. 3, D and E). Proliferative capacity progressively declined as the duration of BIBR1532 treatment increased. We observed that the reduced proliferative capacity of telomerase-inhibited cells was reversible and returned to baseline levels observed for controls after release from BIBR1532 treatment [Fig. 3E: compare nontreated (NT) and Rel samples]. Next, we treated both HL60/WT and HL60/MX2 cells with IC_{25} etoposide for 24 h and performed proliferation assays to analyze the effect of combining BIBR1532 and etoposide treatment. We observed that IC_{25} etoposide treatment significantly inhibited cellular proliferation (Fig. 3, D and E: compare NT and Cont samples). Finally, we observed that BIBR1532 pretreatment further sensitized both drug-sensitive and drug-resistant HL60 cell lines to IC_{25} etoposide treatment [Fig. 3, D and E: compare Cont (treated with etoposide only) with samples treated with both etoposide and BIBR1532].

BIBR1532 Inhibits Telomerase Activity and Induces Telomere Length Shortening and Chemotherapeutic Sensitization in MCF-7/WT and MCF-7/Mln^R but Not in MCF-7/Adr^R. As for the HL60 cell lines, treatment of MCF-7 cell lines with BIBR1532 doses of greater than 2.5 μM impaired short-term cell viability, as measured by the MTT assay (Fig. 4A); therefore, cells were treated with 2.5 μM BIBR1532. After continuous treatment with BIBR1532, we observed no difference in cellular growth of treated versus untreated cultures (Fig. 4C), but we did observe telomere shortening in MCF-7/WT and MCF-7/Mln^R cell lines but not in MCF-7/Adr^R cells (Fig. 4B). BIBR1532, when used alone, significantly inhibited the colony-forming ability of MCF-7/WT cells and reduced the number of MCF-7/Mln^R colonies counted (Fig. 5A). It is interesting that only MCF-7/WT and MCF-7/Mln^R cells demonstrated decreased colony-forming ability after continuous growth in BIBR1532 followed by combination treatment with melphalan or doxorubicin (Fig. 5, B and C). This effect was progressive and dependent on the duration of BIBR1532 treatment. Sensitization to chemotherapeutics occurred in both drug-sensitive MCF-7/WT and drug-resistant MCF-7/Mln^R BIBR1532-treated cell lines (Fig. 5, B and C). MCF-7/Adr^R cells did not show telomere shortening (despite prolonged growth in BIBR1532) (Fig. 4B), nor did they demonstrate any sensitization toward doxorubicin treatment (Fig. 5C).

This apparent difference between MCF-7/WT, MCF-7/Mln^R, and MCF-7/Adr^R cells with respect to BIBR1532 sensitivity prompted us to ask whether the telomerase enzyme of

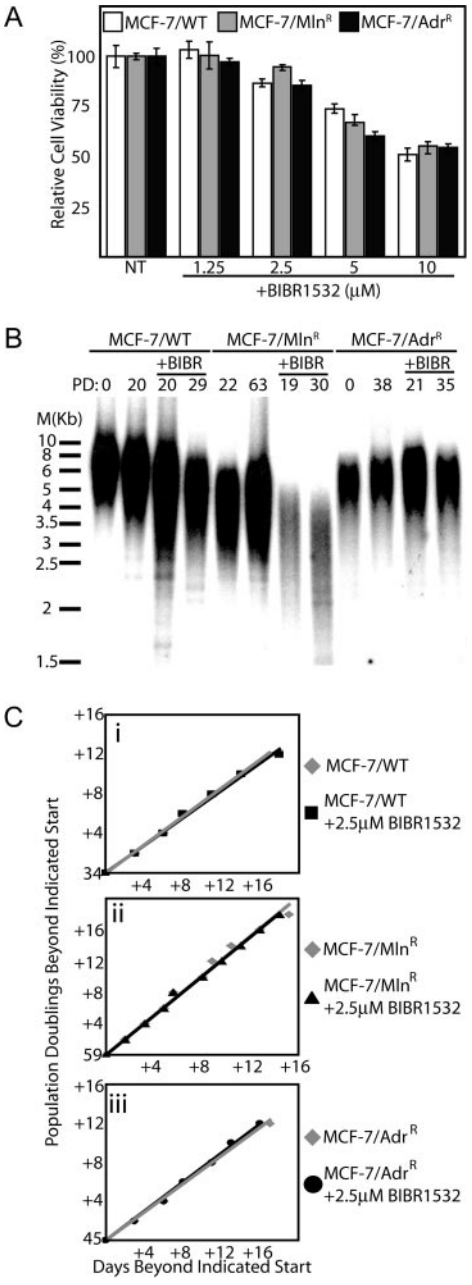


Fig. 4. BIBR1532 (2.5 μM) inhibits telomerase and induces telomere shortening in MCF-7/WT and MCF-7/Mln^R-treated cells without affecting mass culture growth. A, cell viability 72 h after treatment of MCF-7 cells with 1.25, 2.5, 5, or 10 μM BIBR1532, as measured by MTT assay. Values were normalized to those of NT controls, and graphs represent two independent experiments ± S.E. B, TRF analysis of MCF-7 cells untreated or treated with 2.5 μM BIBR1532 (+BIBR) for the indicated number of PDs. M, DNA marker (in kilobases). C, growth analysis of cells in mass culture at the time of colony-forming assays. Graphs show MCF-7/WT and MCF-7/WT BIBR1532-treated cells starting from PD 34 (i), MCF-7/Mln^R and MCF-7/Mln^R BIBR1532-treated cells starting from PD 59 (ii), and MCF-7/Adr^R and MCF-7/Adr^R BIBR1532-treated cells starting from PD 45 (iii).

the MCF-7/Adr^R cells was resistant to BIBR1532. We assessed the sensitivity of the telomerase enzyme in vitro by adding 2.5 μM BIBR1532 to TRAP reactions of untreated MCF-7/WT and MCF-7/Adr^R cell extracts. We observed similar inhibition of telomerase activity from extracts of both cell lines upon the addition of BIBR1532, indicating that the telomerase enzyme in MCF-7/Adr^R cells is not resistant to BIBR1532-mediated catalytic inhibition (Fig. 6).

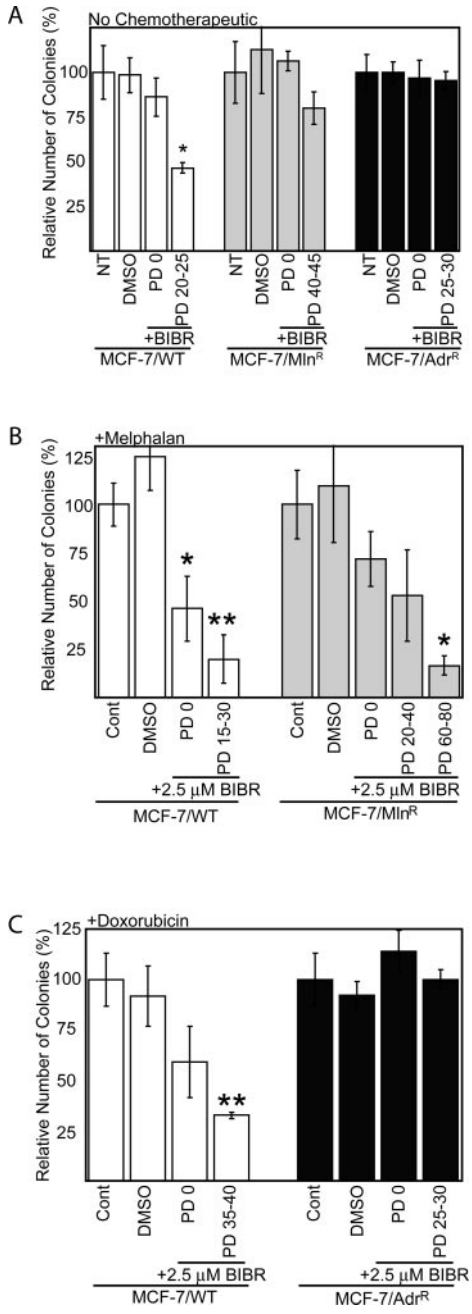


Fig. 5. BIBR1532 (2.5 μM) treatment reduces colony-forming ability and sensitizes MCF-7/WT and MCF-7/Mln^R cells to melphalan and doxorubicin, or melphalan treatment, respectively, in a telomere length-dependent fashion. Colony-forming assays of Cont, DMSO-, or BIBR1532-treated MCF-7 cells. PD, the number of population doublings cells were pretreated with 2.5 μM BIBR1532 (+BIBR). A, colony-forming assays without chemotherapeutic cotreatment. B, colony-forming assays of MCF-7/WT and MCF-7/Mln^R with 1 and 5 μM melphalan treatment, respectively. C, colony-forming assays of MCF-7/WT and MCF-7/Adr^R with 0.125 and 100 μM doxorubicin treatment, respectively. Bars represent the relative number of colonies counted after treatment compared with controls ± S.E. ⋆, $p < 0.05$, and ⋆⋆, $p < 0.01$ compared with control (NT or Cont) of same cell line.

Discussion

In previous studies, telomerase has been inhibited or inactivated via gene knockout, antisense, or dominant-negative strategies to induce growth arrest or apoptosis and to sensitize cells to various cellular stresses (Hahn et al., 1999; Corey, 2002). In addition, two studies reported that treatment with antisense oligonucleotides targeting either hTERT or the hTR template sensitized human bladder and prostate cancer cells, respectively, to chemotherapeutic treatment (Chen et al., 2003; Kraemer et al., 2004). Whereas gene therapy and antisense strategies hold great potential for the treatment of many human diseases, the feasibility of gene therapy and clinical usefulness of most antisense compounds remain to be proven (Lane, 2005). A potentially more practical strategy for antitelomerase therapy in patients is the use of small-molecule inhibitors of telomerase catalytic activity, such as BIBR1532. Unfortunately, because of the time lag between the start of antitelomerase treatment and growth arrest or apoptosis, telomerase inhibitors alone may not be effective anticancer agents (Corey, 2002).

We observed differences in both basal telomere length and telomerase activity of drug-resistant cells versus drug-sensitive parental cells. Other studies observed different telomere lengths and basal telomerase activity in drug-resistant cells compared with drug-sensitive controls (Park et al., 1998; Kuranaga et al., 2001; Kim et al., 2002; Incles et al., 2003; Deschatrette et al., 2004). It is noteworthy that one study that tracked telomere lengths and drug sensitivities of rat hepatoma cells reported that periods of drug-resistance to methotrexate or cisplatin correlated with either shortened or elongated telomeres, respectively (Deschatrette et al., 2004). Another reported that the expression of multidrug resistance genes, telomere length, and telomerase activity were all increased upon long-term treatment of human colorectal carcinoma cells with cisplatin and 5-fluorouracil (Kuranaga et al., 2001). On the other hand, others observed that increased sensitivity of human cell lines to chemotherapeutics correlated with higher basal telomerase activity (Asai et al., 1998;

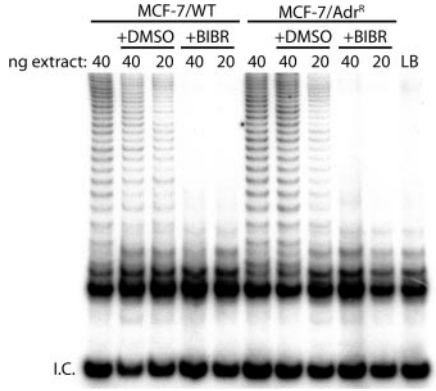


Fig. 6. Telomerase enzyme from MCF-7/Adr^R protein extracts is sensitive to BIBR1532 in vitro. TRAP assay of MCF-7/WT and MCF-7/Adr^R cell extracts without or with the in vitro addition of DMSO or 2.5 μM BIBR1532 to TRAP reactions, which were incubated for 30 min at 30°C and processed as described under *Materials and Methods*. ng extract, nanograms of protein extract per reaction; L.B., lysis buffer; I.C., internal PCR control.

Lin et al., 2001). These opposing correlations may reflect differences in cell type, drug type, or levels of drug resistance between studies. It is tempting to speculate that changes in basal telomere length or telomerase activity, in general, may contribute to the development or maintenance of chemotherapeutic resistance. However, we cannot rule out the possibility that the observed differences in telomere length and basal telomerase activity in drug-resistant cells versus parental cells are caused by clonal selection and expansion during the process of in vitro drug-resistance development. Indeed, when subclones of Hela and 293 cells were isolated and analyzed, the clones showed a range of mean telomere lengths and telomerase activity, and some differed dramatically from those of the mass culture (Bryan et al., 1998).

Previous studies have reported a time- and concentration-dependent up-regulation of telomerase activity in human leukemia, neuroblastoma, and pancreatic cells after treatment with etoposide (Moriarty et al., 2002; Klapper et al., 2003; Jeyapalan et al., 2004). Other studies report no up-regulation of telomerase activity after treatment but share the observation that at later times, after treatment, telomerase activity is decreased (Park et al., 1998; Lin et al., 2001). As far as we are aware, no studies have compared telomerase activity in drug-resistant and parental cell lines after treatment with equivalent concentrations of drug. Two studies have reported that telomerase activity in doxorubicin-resistant cells is increased or maintained after doxorubicin treatment compared with drug-sensitive controls (Ishikawa et al., 1999; Yoon et al., 2003). However, equal doxorubicin concentrations were used to treat drug-sensitive and drug-resistant cell lines in these experiments that did not take into account differences in drug sensitivities. We treated HL60/WT and HL60/MX2 cells with equivalent and clinically relevant concentrations of etoposide (Moriarty et al., 2002). We observed no significant difference in telomerase activity between the two cell lines after treatment, nor did we observe any significant up-regulation of telomerase activity. For HL60/WT cells, the results are consistent with our previous observation that 0.5 μM etoposide treatment does not significantly alter telomerase activity within a 48-h time course (Moriarty et al., 2002).

BIBR1532 is a potent, selective, and reversible non-nucleoside inhibitor of human telomerase catalytic activity that exhibits in vitro IC_{50} concentrations in the nanomolar range (Damm et al., 2001). In the original reports, proliferation was almost completely arrested in telomerase-positive cell lines after prolonged (PD >120) treatment with 10 μM BIBR1532 (Damm et al., 2001). BIBR1532 treatment should only inhibit telomerase activity, and telomerase inhibition and telomere shortening should be tolerated until telomeres become short and dysfunctional. Treatment of leukemia cells with high concentrations (30–80 μM) of BIBR1532 causes short-term cytotoxicity in both telomerase-positive and telomerase-negative cells (El-Daly et al., 2005). These results suggest that short-term antiproliferative effects of BIBR1532 are probably telomerase-independent. We performed our experiments using 2.5 μM BIBR1532 after the initial observation that higher concentrations inhibited cell growth or viability in a relatively short time frame (72 h). Continuous 2.5 μM BIBR1532 treatment did not affect mass population growth but inhibited telomerase and induced telomere shortening in all cell lines except for MCF-7/Adr^R. This effect was reversible, progressive, and dependent on the duration of treatment. It is important to note that when BIBR1532-treated HL60 cell lines were tested for growth ability over a 5- or 6-day period, we observed decreased proliferative capacity of cells with short telomeres. When treated in combination with etoposide, cells with short telomeres demonstrated a further reduction in proliferative capacity compared with controls. This effect was probably telomere length-dependent, because proliferative capacity declined with the increasing number of population doublings in the presence of BIBR1532 and as release from BIBR1532 treatment restored growth ability to control levels.

Colony-forming assays of MCF-7/WT and MCF-7/Mln^R cells revealed the decreased colony-forming ability of BIBR1532-treated cells. When a combination of BIBR1532 and chemotherapeutic treatment was used, both drug-sensitive MCF-7/WT and drug-resistant MCF-7/Mln^R cell lines exhibited a dramatically reduced capacity to recover from drug treatment. Again, this effect is most probably telomere length-dependent, because continuous BIBR1532 treatment progressively sensitized MCF-7/WT and MCF-7/Mln^R cells to chemotherapeutics. MCF-7/Adr^R cells did not demonstrate any change in telomere length (despite prolonged BIBR1532 treatment), nor did they show any alteration in colony-forming ability with or without high-dose doxorubicin treatment. These results are consistent with recent observations showing that telomerase-mediated resistance to genotoxic stress is telomere length-dependent, probably because of the ability of telomerase to elongate short, presumably dysfunctional, telomeres (Rubio et al., 2004). DNA damage responses are activated when telomeres become short or experimentally uncapped (Gire et al., 2004). We hypothesize that telomere shortening and DNA damage are the consequences of continued BIBR1532 treatment and provide an explanation for the telomere length-dependent reduction of HL60 proliferative capacity and MCF-7/WT and MCF-7/Mln^R colony-forming ability.

MCF-7/Adr^R cell-insensitivity to telomerase inhibition by BIBR1532 is probably telomerase-independent. When BIBR1532 was added in vitro to cell extracts, telomerase activity of both MCF-7/WT and MCF-7/Adr^R cells was effectively inhibited. Therefore, cellular mechanisms involving BIBR1532 influx, efflux, or metabolism may be responsible for MCF-7/Adr^R resistance to pharmacological telomerase inhibition.

To our knowledge, this report is the first to show that a pharmacological inhibitor of telomerase catalytic activity can sensitize cells to traditional drug treatment in a telomere length-dependent fashion. Incles et al. (2003) demonstrated previously that a G-quadruplex–interacting agent (BRACO-19) sensitized parental and flavopiridol-resistant human colon carcinoma cell lines over a 10- to 20-PD treatment period. Whereas a longer time is required for BIBR1532 to exert its effects, the mechanism of action between the two pharmacological agents is also different. It is proposed that G-quadruplex–interacting agents induce and/or stabilize the formation of non–Watson-Crick G-quadruplex DNA structures within G-rich sequences, such as telomeres (Hurley, 2002). G-quadruplex structures are predicted to sequester single-stranded telomeric DNA that typically serves as a substrate for telomerase (Hurley, 2002). Fifteen days of subcytotoxic BRACO-19 treatment induced telomere shortening, cellular senescence,

complete proliferative arrest, and decreased hTERT expression in a human uterus carcinoma cell line, demonstrating that BRACO-19 can target telomeres and inhibit telomerase (Burger et al., 2005). However, a number of other studies have demonstrated that G-quadruplex–interacting agents affect telomere stability and exert cytotoxic effects in the absence of telomere shortening or telomerase expression (notably in telomerase-negative ALT cell lines) (Pennarun et al., 2005). Furthermore, G-quadruplex–interacting agents can also inhibit transcription by stabilizing G-quartet structures in nontelomeric DNA such as the *c-myc* oncogene (Hurley, 2002; Siddiqui-Jain et al., 2002). In contrast, BIBR1532 is a highly selective and potent inhibitor of telomerase that targets the enzyme's catalytic activity and demonstrates very little enzymatic inhibition against a panel of DNA and RNA polymerases, including human immunodeficiency virus-1 reverse transcriptase, and no growth inhibition of telomerase-negative normal human fibroblasts or SA0S-2 ALT cells (Damm et al., 2001). Therefore, the only known mechanism of action for BIBR1532 is through telomerase inhibition and telomere shortening. In conclusion, the results presented in this study suggest that pharmacological inhibition of telomerase catalytic activity may be a highly selective strategy of anticancer therapy that might aid in the treatment of both drug-resistant and -sensitive malignancies.

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