Telomerase inhibition potentiates the effects of genotoxic agents in breast and colorectal cancer cells in a cell-cycle specific manner.

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Abstract

Previous studies have shown that telomerase facilitates DNA-damage repair and cell survival following stress. It is not clear how telomerase promotes DNA repair, or whether short-term telomerase inhibition, combined with genotoxic stress, can be exploited for cancer therapy. Here, we show that transient inhibition of telomerase activity by the specific inhibitor GRN163L increases the cytotoxicity of some, but not all, DNA-damaging agents. Such synergistic inhibition of growth requires the use of DNA-damaging agents that are toxic in S/G2 phase of the cell cycle. Notably, inhibition of ATM kinase, together with telomerase inhibition, synergistically increases the cytotoxicity induced by the G2-specific topoisomerase II inhibitor etoposide. By varying the timing of telomerase inhibition, relative to the timing of DNA damage, it is apparent that the pro-survival functions of telomerase occur at early stages of DNA damage recognition and repair. Our results suggest that the protective role of telomerase in cell-cycle-restricted DNA damage repair could be exploited for combined anti-cancer chemotherapy.

Introduction

Telomerase is a specialized reverse transcriptase that maintains telomeres, nucleoprotein structures that cap the ends of linear chromosomes. Somatic inhibition of telomerase activity functions as a tumor suppression mechanism important in long-lived organisms, limiting indefinite proliferation and thus decreasing the likelihood of accumulating multiple mutations essential for carcinogenesis (1, 2). Surveys of cancer-patient samples established that more than 85% of human tumors have high telomerase activity, primarily through the transcriptional up-regulation of the telomerase reverse transcriptase (hTERT) gene (3). Solid tumors reactivate telomerase in the late initiation phase of tumorigenesis (4). Accordingly, telomerase activity and *in situ* hTERT detection have been used as diagnostic and prognostic markers in tumor pathology studies (5-7).

The prevalence of telomerase activation in human cancers makes the targeting of telomerase an appealing therapeutic option. Current rationale for the use of telomerase inhibitors in anti-cancer therapies is based on the knowledge that these agents disrupt telomere length homeostasis in cancer cells, eventually leading to telomere depletion after a sufficient lag time (8). Previously, it was shown that faster cancer cell death resulted from combining other cytotoxic agents with telomerase inhibition, by chemical inhibitors or genetic manipulations such as RNA interference or expression of dominant-negative hTERT (9, 10). These reports concluded that telomere attrition is essential for treatment efficacy (9), but this conclusion may have been influenced by the lag time required to genetically manipulate and generate stable cell lines to achieve telomerase inhibition. GRN163L is a thiophosphoamidate oligomer with 13 bases complementing the template region of telomerase RNA. GRN163L competitively inhibits the binding of the telomerase holoenzyme to its substrate (11, 12). GRN163L-mediated telomerase inhibition has a synergistic effect when combined with breast cancer treatments

such as ionizing radiation (13), and the authors again concluded that these effects were dependent on telomere length attrition. New studies of GRN163L with other anticancer agents such as paclitaxel, a microtubule stabilizer (14), and trastuzumab, a monoclonal antibody against HER2 positive cancers (15), showed cooperativity between telomerase inhibition and these treatments. GRN163L treatment in these combination studies was over longer periods, and the effects of the combinations were evaluated immediately after the cessation of treatment, with cell viability assays such as the trypan blue exclusion method.

Pro-survival functions of telomerase, unrelated to telomere length maintenance, could contribute to cancer growth. Matsutomi *et al*. have shown that reduced endogenous expression of telomerase in primary fibroblasts abrogates DNA damage repair by changing chromatin architecture and ATM activation (16). The ability of telomerase to protect against DNA damage may explain the strong preference of telomerase activation over ALT in most tumors. Here we investigate whether telomerase-positive cancer cells better tolerate the genotoxic effects of common chemotherapeutic agents with or without concurrent short-term telomerase inhibition.

Materials and Methods

Cell lines and Reagents

Human breast adenocarcinoma cell line MCF-7 (ATCC# HTB-22) and human colorectal carcinoma cell lines HT29 (ATCC# HTB-38) and LS180 (ATCC#CL-187) were obtained from the American Type Culture Collection (ATCC) at the commencement of this work. Identities of all three cell lines were authenticated at the conclusion of experiments (STR analysis by RADIL service at the University of Missouri in August 2010). All cell culture media, antibiotics and other cell culture reagents are commercially available from Invitrogen/Gibco unless otherwise noted. Etoposide, irinotecan and oxaliplatin were obtained from Sigma/Aldrich. Bleomycin and ATM

inhibitor KU55933 were obtained from Calbiochem. GRN163L (Imetelstat) was obtained from Geron, resuspended in PBS, and stock concentration was determined before each experiment using UV-spectrophotometer absorbance.

TELOMERASE INHIBITION, DNA DAMAGE INDUCTION AND COLONY FORMING UNIT ASSAYS

Prior to DNA damage induction, MCF-7 or HT29 cells were treated for 24h with 10μM GRN163L, a dose determined with a dose-response TRAP experiment described below. Following treatment, cells were incubated for an additional 24h with a 2-fold serial dilution of etoposide (dose ranges: MCF: 0.025-12.8μM; HT29: 0.05-12.8μM), bleomycin (MCF-7: 0.01-10.24μg/ml; HT29: 0.02-20.48μg/ml), irinotecan (MCF-7: 0.1-102.4μM; HT29: 0.1-102.4μM) or oxaliplatin (MCF-7: 0.1-102.4μM; HT29: 0.1-102.4μM) in the presence or absence of 10μM GRN163L. Experiments requiring ATM inhibition were carried out in an identical manner with one modification: the ATM inhibitor KU55933, was added at a concentration of 10μM in combination with the DNA-damaging agents (17).

The Colony Forming Unit Assay was performed essentially as described (16). Cells were incubated for 2 weeks at 37ºC and 5% CO_2 to allow single colonies to develop. The minimum threshold for inclusion required colonies to be >50μm in diameter. Each combination treatment curve was normalized to its own control group: cells receiving the same dose of GRN163L or ATM inhibitor KU55933, but no DNA-damaging agent. Each experiment was repeated at least three times. LD_{50}s for each experiment were obtained using the software GraphPad Prism. Student's t-test was used for statistical comparison.

TELOMERASE ACTIVITY ASSAYS

Telomerase activity was measured using whole cell extracts and a modified TRAP assay (18, 19). TRAP activity was measured for each of the combination experiments to confirm the efficient inhibition of telomerase activity by GRN163L treatment (Supplementary Figure 2).

Western blot assays

Whole cell lysate from treated cells was electrophoresed on SDS-PAGE gel, transferred and subsequently blotted with p53 (Cell Signalling) and ß-actin (Sigma), as previously described (20). The membranes were immunoblotted with the appropriate HRP-conjugated secondary antibody, followed by ECL Plus (Amersham) for detection on Kodak X-ray films.

Immunofluorescent analysis

Cells were seeded onto glass cover slips and treated with the same chemotherapeutic regimens described above. At the conclusion of chemical treatment, cells were fixed in 3.7% paraformaldehyde, solubilized with 0.1% Triton X-100 in 1xPBS supplemented with 1% fetal calf serum. Fixed cells were immunostained with γH2AX antibodies (Millipore) and Texas Red-labeled secondary antibodies against mouse IgG (Molecular Probes). DNA was counterstained with the nucleic acids dye Yo-PRO-1 and mounted using Fluoromount G (Southern Biotech). Immunofluorescent signals were observed with a Zeiss Pascal confocal microscope, fitted with the appropriate lasers and filters (UBC BioImaging Centre).

Results

Efficient Telomerase Inhibition by GRN163L in breast cancer and colorectal cancer cell models

To achieve efficient short-termed inhibition of telomerase activity in cultured cancer cell models, we used the specific telomerase inhibitor, GRN163L (Imetelstat, Geron Corporation). We chose

three different cancer cell lines for our experiments: MCF-7, a breast cancer cell line previously reported to respond well to telomerase inhibition by GRN163L (21), and HT-29 and LS180, two colorectal cell lines that are highly telomerase-positive with different p53 status and microsatellite stabilities (22-24). Cells were cultured with increasing doses of GRN163L for 24h. Cell extracts from each treatment group were assayed for telomerase activity with the TRAP activity assay.

TRAP profiles showed that a dose of 8μM GRN163L resulted in a 25 to 125-fold inhibition of telomerase activity in MCF-7 cells (Figure 1A). Compare the lack of telomere repeat activity in extracts treated with 8μM GRN163L to the control profiles, where weak TRAP activity was observed at 125-fold dilution. We found very similar dose-response relationships of GRN163L and TRAP activity in HT-29 and LS180 colorectal cancer cells (Figure 1B and 1C). QPCR analysis of mean telomere length in these three cell lines did not reveal any significant changes with GRN163L treatments for 24h or 48h (Supplementary Figure 1). We concluded that a 24h treatment with 10μM GRN163L is sufficient to inhibit telomerase function as assayed with TRAP in all three cancer cell models.

TELOMERASE INHIBITION BY GRN163L SYNERGISTICALLY INCREASED THE CYTOTOXICITY OF S/G2-SPECIFIC DNA-DAMAGING AGENTS

If telomerase activation facilitates DNA-damage repair, telomerase inhibition at the time of genotoxic stimulus should cause increased cytotoxicity in cancer cells. We measured the pharmacological and dose-response profiles of common DNA-damaging agents, in combination with GRN163L for telomerase inhibition. To study the genotoxicity of DNA-damage events in breast and colon cancer cells, we performed dose-response experiments using a colony forming unit (CFU) assay. In contrast to most cell viability assays, such as the colorimetric MTT assay or

the other flow-cytometry based protocols, CFU measures long-term recovery and proliferation of cultured cells following cytotoxic stress induction.

Using the breast cancer cell model MCF-7, we observed a significant shift of the dose-response curves for the S/G2-specific double-strand DNA-damaging agent etoposide, in the presence of GRN163L. Etoposide induces double-stranded DNA breaks in S and G2 cell cycle phase-specific manner (25, 26). Addition of GRN163L reduced the lethal dose of etoposide by 2.8-fold, with a significant p-value at 0.005 (unpaired t-tests, GraphPad Prism. Figure 2A). The parallel shift of the dose-response curves suggests a conserved cytotoxic mechanism. Next, we measured the LD_{50} of etoposide in the colorectal cancer cell line HT-29. Consistent with our observations in MCF-7 cells, the addition of GRN163L telomerase inhibition significantly increased the cytotoxicity of etoposide in HT-29 cells (Figure 2B). Adding telomerase inhibition reduced the lethal dose of etoposide by 2.5-fold in HT-29 colorectal cancer cells, with a significant p-value of 0.006.

To corroborate this observation, we combined telomerase inhibition with another cell-cycle dependent DNA-damaging agent. Irinotecan is converted to its active metabolite SN-38 by intracellular hydrolysis, resulting in the inhibition of topoisomerase I. Inhibition of topoisomerase I by these compounds results in the generation of DNA nicks in replication- and transcription-active cells (27). These single-stranded DNA nicks are converted into double-strand breaks by DNA replication machinery as they pass through the unrepaired loci during the DNA synthesis phase of the cell cycle (28). Co-administration of irinotecan with telomerase inhibitor GRN163L in breast cancer cell line MCF-7 resulted in an increased cytotoxicity of the DNA-damaging agent (Figure 2C). The addition of telomerase inhibitor reduced the lethal dose of irinotecan in MCF-7 cells by 2.1-fold, with a significant p-value at 0.02. A similar increase in the cytotoxicity

of irinotecan by the coadministration of telomerase inhibitor GRN163L was observed in the colorectal cell line HT-29 (Figure 2D). We observed a 1.7-fold increase in the cytotoxicity of irinotecan in HT-29 cells concurrently treated with the telomerase inhibitor GRN163L. This difference in LD50 is significant with a p-value of 0.02. Notably, the combination of irinotecan with GRN163L is less effective than etoposide with GRN163L.

These experiments indicate that telomerase activity conferred a survival advantage in DNA-damaged human cancer cells. Transient loss of telomerase activity induced by a specific enzyme inhibitor is sufficient to reverse this protection. Administration of telomerase inhibition at the time of DNA-damage induction resulted in synergistic increases of cytotoxicity.

TELOMERASE INHIBITION BY GRN163L HAS NO EFFECTS ON THE CYTOTOXICITY OF CELL CYCLE NON-SPECIFIC DNA-DAMAGING AGENTS

Telomerase activity in human cells is regulated in a manner that is specific to the cell cycle (19, 29, 30). Since both etoposide and irinotecan are cytotoxic at the S and G2 phases of the cell cycle, we next tested whether the synergy between telomerase inhibition and DNA- damaging agents was dependent on the cell cycle timing of the genotoxic agent action.

We treated MCF-7 breast cancer cells with the double-strand DNA-damaging agent bleomycin. Bleomycin's cytotoxicity is attributed to its ability to cleave nucleic acids, leaving lesions that are difficult to repair (31). Bleomycin cytotoxicity is not cell-cycle dependent, although similar to other DNA-damaging agents, actively cycling cells exhibit increased sensitivity to bleomycin treatment. Unlike cell-cycle stage specific DNA-damaging agents, MCF-7 cells did not exhibit any increased sensitivity to bleomycin in the presence of telomerase inhibitor GRN163L (Figure 3A, p=0.90). In parallel, addition of GRN163L slightly increased the cytotoxicity of bleomycin in

colorectal cancer cell line HT-29 by 1.2-fold (Figure 3B). However, this small difference in LD_{50} was not statistically significant for our assay parameters ($p = 0.28$).

We next tested the effect of oxaliplatin, another cell-cycle stage non-specific DNA-damaging agent. Oxaliplatin is a platinum-containing alkylating agent that forms direct cross-links with DNA by attacking nucleophilic sites on the base, with cytotoxicity correlated to the number of inter-strand crosslinks, leading to double-strand DNA breaks (32). In agreement with our observations of the other non cell-cycle specific DNA-damaging agent bleomycin, combining oxaliplatin with GRN163L did not result in an increase in its cytotoxicity (Figure 3C, $p = 0.76$). This observation was corroborated in the colorectal cell line HT-29 (Figure 3D). Paradoxically, addition of telomerase inhibitor showed a small protective effect against oxaliplatin cytotoxicity, but this difference was not statistically significant for our assay parameters ($p = 0.29$).

Co-administration of GRN163L and DNA-damaging agents with broad genotoxicity induction timing did not show the same potentiation seen with the previous combination treatments. Adding telomerase inhibitor to these cell-cycle non-specific DNA-damaging agents caused no increase in cytotoxicity. We concluded that concurrent telomerase inhibition only benefits a select group of DNA-damaging agents that have a defined window of cytotoxic activity within the cell cycle.

GRN163L TELOMERASE INHIBITION AND ATM INHIBITION SYNERGISTICALLY INCREASE THE CYTOTOXICITY OF CELL-CYCLE SPECIFIC DNA-DAMAGING AGENTS

Our data demonstrated that telomerase activity protects against the cytotoxicity of double-stranded DNA-damaging agents active in S/G2 of the cell cycle. Telomerase inhibition in primary human fibroblasts by small hairpin RNA targeting hTERT was associated with a loss of

ATM kinase activity. As a result, there is a significant loss of phosphorylation of ATM targets following the induction of DNA damage, compared to telomerase positive cells (16). Since the ATM-mediated homologous recombination DNA repair mechanism is particularly important at S/G2 phases of the cell cycle, we subsequently tested the influence of combining GRN163L and ATM inhibition on DNA-damage-induced cytotoxicity.

We tested three cancer cell lines with concurrent administration of specific ATM inhibitor KU55933, with GRN163L and S/G2 specific double-strand DNA-damage inducer etoposide. ATM inhibition was confirmed with the loss of phosphorylation of ATM targets p53 and γH2AX, normally evident following the induction of dsDNA damage (Figure 4 insets and Supplementary Figure 3). Treatment with KU55933 or GRN163L separately, in combination with etoposide, increased the cytotoxicity of the DNA-damaging agent in MCF-7 cells, compared to the cells treated with topoisomerase II inhibitor alone (Figure 4A, $p = 0.001$ and 0.007 respectively). Combining three therapeutic agents together reduced the lethal dose of etoposide even further (Figure 4A, $p = 0.0004$). The increased cytotoxicity of the triple combination, compared to the GRN163L/etoposide and KU55933/etoposide double combinations, suggest that telomerase and ATM inhibition have an additive effect, and that inhibiting these two prospectively related pathways did not result in the diminished therapeutic efficiency of either agent.

We repeated this triple-agent treatment with the colorectal cell lines HT-29 and LS180. Addition of specific ATM inhibitor KU55933 or GRN163L to etoposide increased the cytotoxicity of the DNA-damaging agent in both colorectal cancer cell lines, compared to cells treated with etoposide alone (Figure 4B and 4C). Combining three therapeutic agents together further reduced the lethal dose of etoposide in HT-29 cells and LS180 cells, with highly significant p-values of 0.007 and <0.001 respectively (Figure 4B and 4C).

TIMING OF THE GRN163L INHIBITION OF TELOMERASE ACTIVITY AFFECTS ITS POTENTIATION OF THE G2-SPECIFIC DNA-DAMAGING AGENT ETOPOSIDE

To further probe how telomerase inhibition potentiates etoposide's cytotoxicity, we performed order-of-addition experiments. We compared MCF-7 breast cancer cells in four treatment groups. One group received etoposide alone, and the other three groups received etoposide doses with GRN163L, respectively administered 24h before, concurrent with or 4.5h after the introduction of the G2-specific DNA-damaging agent.

The order-of-addition experiments illustrated that the pre-treatment of cells with GRN163L is required for optimal synergy with etoposide. Pre-treatment with GRN163L for 24h before the onset of DNA damage substantially reduced the viability of MCF-7 cells ($p = 0.0008$, Figure 5). Addition of GRN163L concurrently with and after the introduction of etoposide resulted in lethal doses of the DNA-damaging agent only slightly different from that of the cells receiving etoposide alone ($p = 0.02$ and 0.03 respectively, Figure 5), indicating that addition of GRN163L concurrently or after the induction of DNA damage minimally potentiates the effects of etoposide. These findings suggest that telomerase must be effectively inhibited by the time DNA damage is induced, for the optimal potentiation of cytotoxicity. This experiment also showed that decreased CFU growth in GRN163L treatment is not likely due to post-treatment effects of residual intracellular GRN163L. Otherwise, all three groups receiving GRN163L would show similar post-treatment effects.

Discussion

High telomerase activity is consistently observed in primary breast and colon cancer samples (3, 33, 34). In addition to its canonical role providing telomere length maintenance and the

associated proliferative immortality, we and others have proposed that high telomerase activity confers an additional growth advantage to cancer cells by promoting survival in the presence of cellular stresses (reviewed in (35) and (36)). A prior report, using short hairpin RNA knockdown of telomerase expression in primary fibroblasts (16), provided us with the impetus to inhibit this pro-survival function of telomerase in cancer cells as an additional anti-cancer therapeutic application for telomerase inhibition. Unlike long-term telomerase inhibition for the goal of telomere attrition, telomerase inhibition to reduce cell survival in conditions of DNA damage could be used as a short-term therapeutic application.

Short-termed, concurrent administration of the telomerase inhibitor GRN163L sensitized breast and colorectal cancer cells to the cytotoxic effects of cell-cycle stage specific DNA-damaging agents, but it showed minimal potentiation of other non-cell-cycle stage specific agents. It remains possible that GRN163L administration can contribute to the cytotoxic mechanism of most DNA-damaging agents, including the bleomycins and the platinum compounds under some conditions. However, the potentiation effect of concurrent GRN163L administration seems to be confined to the subpopulation of cells in S/G2 phase, thereby reducing the contribution of this mechanism's effect in the overall cytotoxicity of the non-cell cycle specific DNA-damaging agents. The small contribution of increased cytotoxicity in S/G2 cells may not be sufficient to increase cytotoxicity in the overall cell population. While off-target effects have been reported with therapeutic oligonucleotides (37), GRN163L treatment in telomerase-negative human cells, and mismatched control oligomer in cancer cells, produced minimal toxicities (38, 39). Accordingly, we did not detect any significant loss of CFU growth in the three cancer cell models treated with short-term GRN163L, either alone or in combination with non-cell cycle specific genotoxic agents. GRN163L is currently in multiple phase I and phase II clinical trials in breast cancer patients, to assess its efficacy and toxicity. (12).

Based on our drug order-of-addition studies, telomerase inhibition at the time of genotoxic stimulus is essential for the optimal cytotoxic potentiation of the DNA-damaging agent etoposide. We found that the concurrent addition of telomerase inhibitor GRN163L, or addition 4.5h after the introduction of etoposide, showed a much smaller potentiation of the DNA-damaging agent than that observed with cells receiving GRN163L pretreatment. This observation suggests that the enzyme plays a role in DNA damage recognition and/or early repair events. Considering the mechanism of etoposide action, we put forward that in a non-synchronized population of MCF-7 cells, only a fraction of cells would be in G2 at the time of etoposide administration. The delay for cells to reach G2, and hence etoposide toxicity, increases the time for GRN163L to fully inhibit telomerase actions, before the onset of cytotoxicities.

Addition of ATM inhibitor KU55933 to GRN163L/etoposide combination treatment increased cytotoxicity further, as observed in MCF-7 breast cancer cells. A previous model proposed that telomerase acts upstream from ATM activation and that telomerase inhibition prevents ATM activation through the structural regulation of chromatin (16). Our data suggest the possibility that ATM and telomerase work independently and augment each other in the DNA damage repair process, because inhibiting ATM and telomerase activity together provided an additive effect on cytotoxicity. Alternately, this synergy could also reflect combined partial inhibition of two steps in the same pathway. The synergy of ATM and telomerase inhibition to increase the cytotoxic effects of etoposide observed in MCF-7 cells was also observed in colorectal cancer cells LS180, but not in HT-29 colorectal cancer cells. Comparison of LS180 and HT-29 revealed two notable differences. First, LS180 cells are microsatellite unstable, with defects in the mismatch repair mechanism, whereas HT-29 cells exhibit normal microsatellite stability,

indicating that their mismatch repair is functional (23). Second, whereas LS180 cells preserve wild type p53 function, HT29 cells are reported to express a DNA-binding deficient form of p53 (R273H) (22, 40). This dominant-negative form of p53 reduces wild type p53 binding to its cognate DNA binding sites and results in the loss of tumor suppression activities. Future studies will be required to determine if these differences underlie the disparity in the role of active telomerase in cell survival following DNA damage.

While our data suggests that telomerase provides survival advantages to counter S/G2 specific DNA damage, in a manner unrelated to telomere length, the actions of telomerase at telomeres may play a significant role in this protective mechanism. Extremely short telomeres known as T-stumps occur with low frequency in transformed cells and cancer cells (41). T-stumps are protected by TERT and the abundance of these structures may be manipulated by changing TERT levels (41, 42). Transient inhibition of telomerase by GRN163L could potentially destabilize T-stumps and activate DNA damage response pathways, in S/G2, coinciding with the time of telomere synthesis. In addition, recent data showed that both the ATR and ATM DNA damage signal pathways are involved in normal DNA replication through telomeres. Coordinated activation of these two kinases was proposed for a two-phase model of telomere structural resolution and reformation during S/G2 of the cell cycle (43, 44). Inhibition of telomerase could prolong this DNA damage signal activation at the telomeres, specific to S/G2. Additional DNA damage induced at this time could result in the observed increase in cell death. Finally, topoisomerase II alpha is implicated in the resolution of replication-related topology strains at the telomeres. There is an inverse relation between the requirement for topoisomerase action and available TRF2, with its associated nuclease Apollo, at the telomeres (45). It is conceivable that transient inhibition of telomerase by GRN163L could induce kinetic or structural changes at telomeres, tipping the balance towards reliance on topoisomerase

activities. This could explain the synergistic induction of cytotoxicity with combined topoisomerase II alpha inhibition and telomerase inhibition.

In previous studies, synergies between GRN163L and various anti-cancer treatments such as ionizing radiation, microtubule inhibition, and inhibition of oncogenic signals (HER2) were considered to be dependent on longer-term changes associated with telomere length (13) and chromatin status (14). Our experiments were designed to examine whether telomerase actions other than the long-term maintenance of telomere length and chromatin may play an important role in response to genotoxic stimuli. Our data imply that this is indeed the case, and that the contribution of telomerase occurs in a manner that is cell-cycle stage specific: telomerase activity can increase cell survival by mitigating the deleterious impact of genotoxic agents predominantly active in G2 phase of the cell cycle. This is the first mechanism-based study combining telomerase inhibition and genotoxic agents in colorectal cancer cell models. Our data demonstrates that when DNA damage is induced in S/G2 phase of the cell cycle, telomerase inhibition increases the cytotoxicity of a genotoxic stimulus. These findings encourage the design of new chemotherapeutic regimens to exploit the pro-survival function of active telomerase.

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Figure Legend

Figure 1. GRN163L inhibition of telomerase activity in breast and colorectal cancer cells is dose dependent. TRAP activity profiles showing the inhibition of telomerase activities by GRN163L in (A) MCF-7, (B) HT29 and (C) LS180 cells. Addition of standard levels of internal control DNA to the TRAP activity assay obstructed detection of residual telomerase activity (data not shown). Smaller quantities of internal control DNA were used for these samples, to allow accurate detection of residual telomerase activity in GRN163L treated extracts.

Figure 2. Telomerase inhibition by GRN163L increases the cytotoxicity of the S/G2-specific DNA-damaging agents etoposide and irinotecan, in MCF-7 breast cancer and HT29 colorectal cancer cells. Dose-response curves show the loss of colony forming units in treated cells. Etoposide combination treatments were tested in (A) MCF-7 and (B) HT29 cells; irinotecan combination treatments were tested in (C) MCF-7 and (D) HT29 cells. E = etoposide, G = GRN163L, I = irinotecan.

Figure 3. Telomerase inhibition by GRN163L has no additional effect on the cytotoxicity of cell-cycle non-specific DNA damaging agents bleomycin (A and B) and oxaliplatin (C and D), in MCF-7 (A and C) and HT29 (B and D) cells. None of the comparison groups showed a significant difference between the LD_{50}s of the single agent and LD_{50}s obtained from cells treated with GRN163L combinations. B = bleomycin, G = GRN163L, O = oxaliplatin.

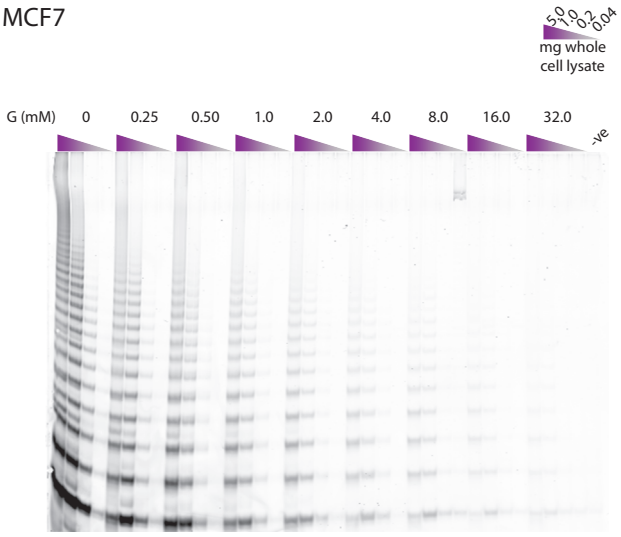
Figure 4. Addition of ATM kinase inhibitor KU55933 further increased the cytotoxicity of etoposide and GRN163L combinations in (A) MCF-7 cells. To confirm that ATM inhibition by KU55933 was effective, we examined the stability of p53 following DNA damage induction.

Corresponding bar graphs showed the comparisons between mean LD_{50}s from at least three different experiments. (B) HT29 cells. Dose-response curves show the loss of colony forming units in cells treated with etoposide and the specified combinations. The presence of a non-specific band occluded the p53 protein signal in the HT29 whole cell extract Western blots. To confirm the effectiveness of ATM inhibition, we performed immunocytochemistry analysis of another ATM target, phosphorylated H2AX. Nuclear γH2AX foci were evident following etoposide treatment, and ATM inhibition reduced the number and the intensity of γH2AX foci formation. Corresponding bar graphs compare mean LD_{50}s from a minimum of three different experiments. (C) LS180 cells. Dose-response curves show the loss of colony forming units in cells treated with etoposide and the specified combinations. Western analysis of p53 protein signals indicated that ATM inhibition was effective. Corresponding bar graphs compare mean LD_{50}s from a minimum of three different experiments. E = etoposide, G = GRN163L, KU = KU55933.

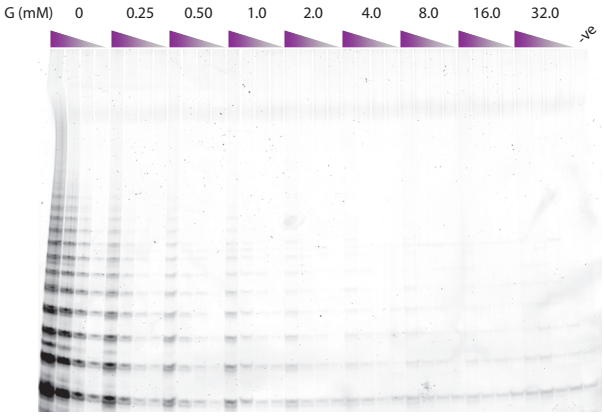
Figure 5. Telomerase inhibition at the time of genotoxic insult is necessary for a synergistic increase in cytotoxicity. A timeline of the order of addition experiment is shown. Corresponding bar graphs compare mean LD_{50}s from a minimum of three independent experiments, in the presence or the absence of GRN163L. E = etoposide, G = GRN163L.

Figure 1

A) MCF7



B) HT29



C) LS180

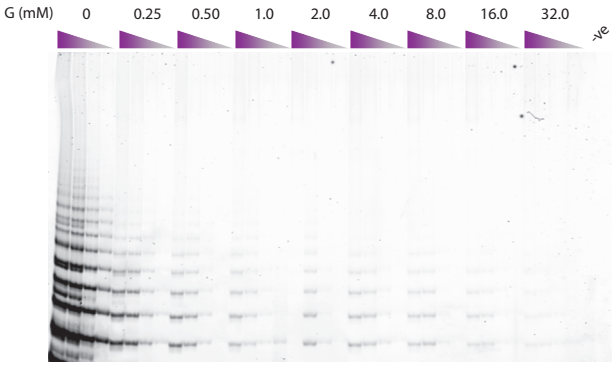


Figure 2

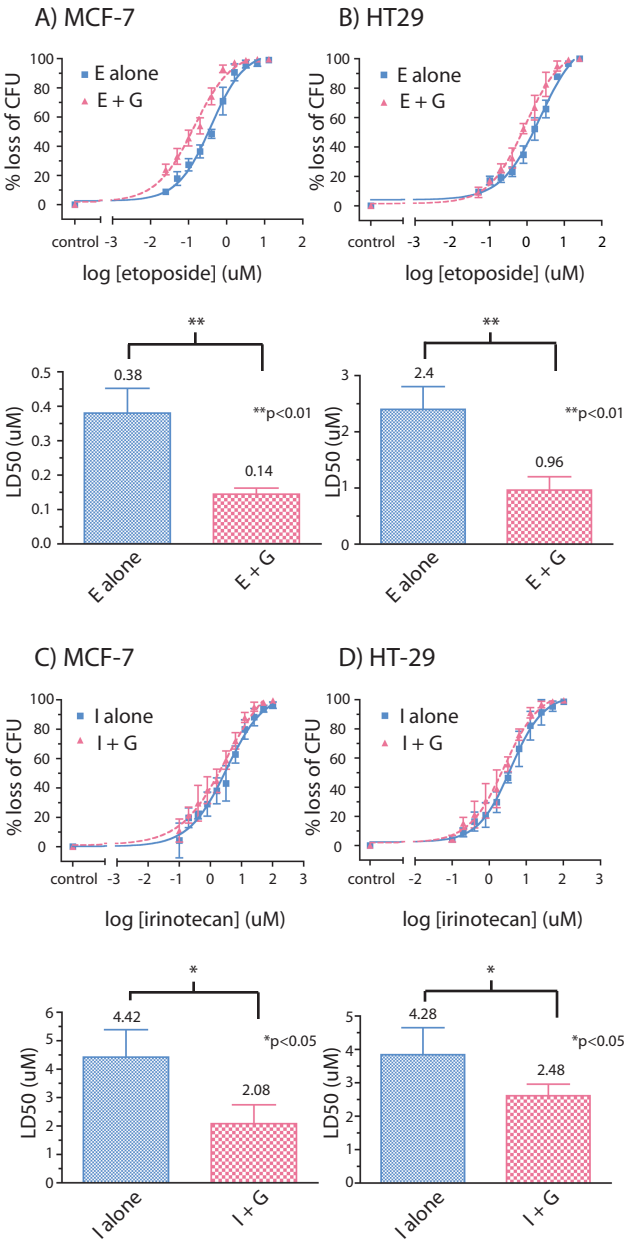


Figure 3

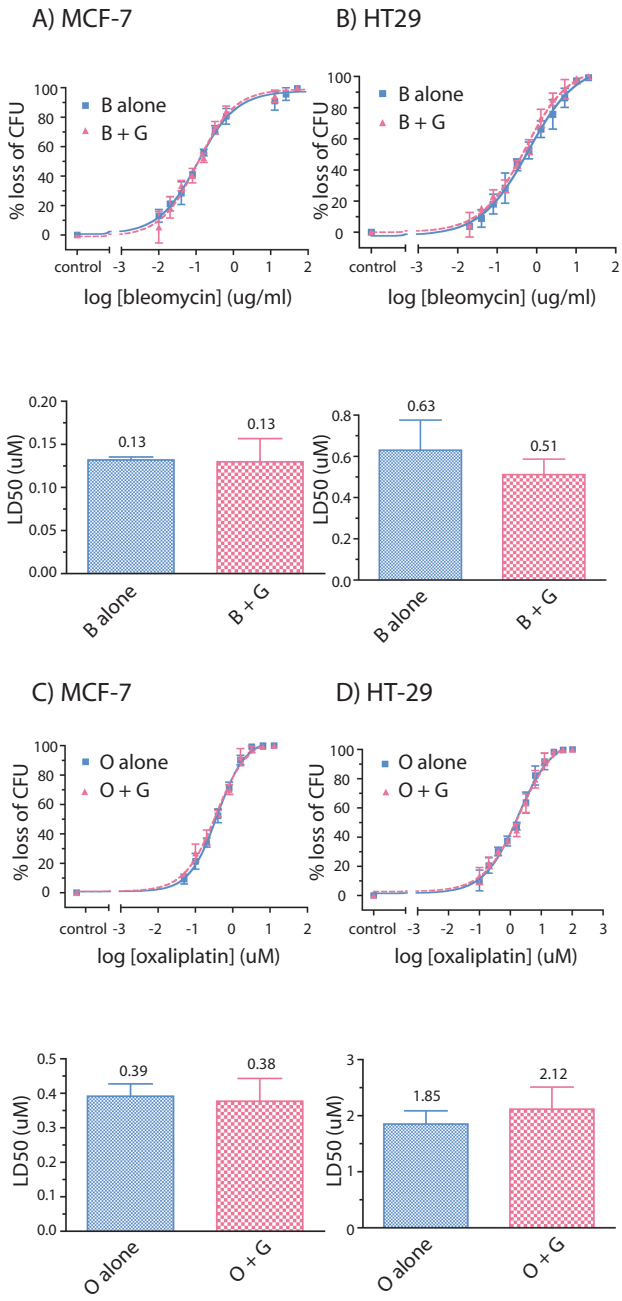
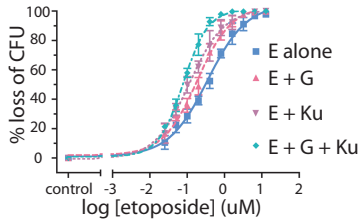
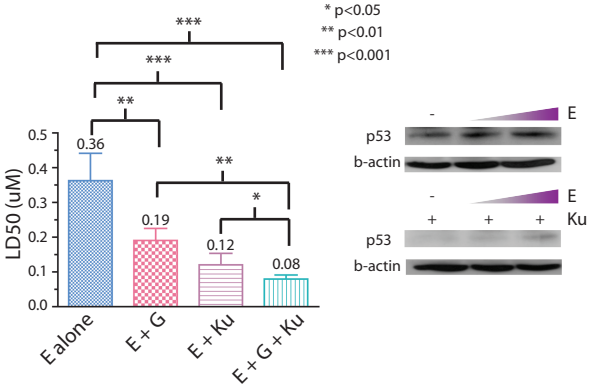


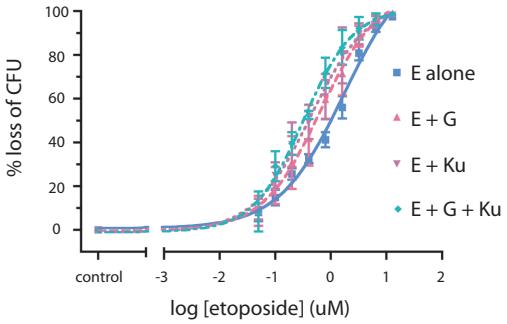
Figure 4

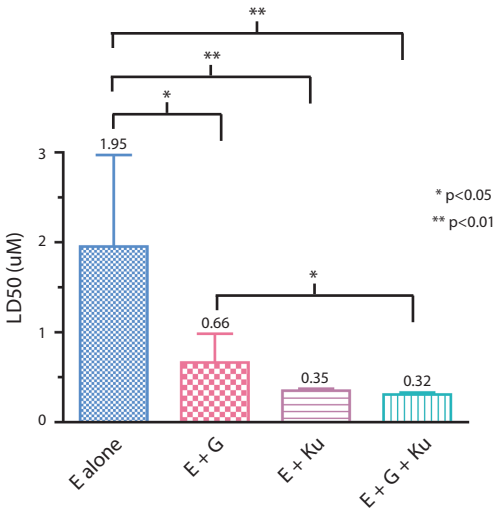
A) MCF-7



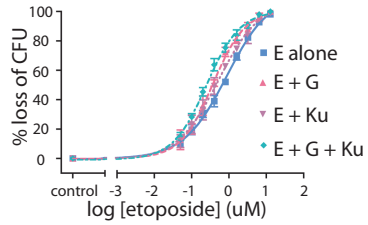


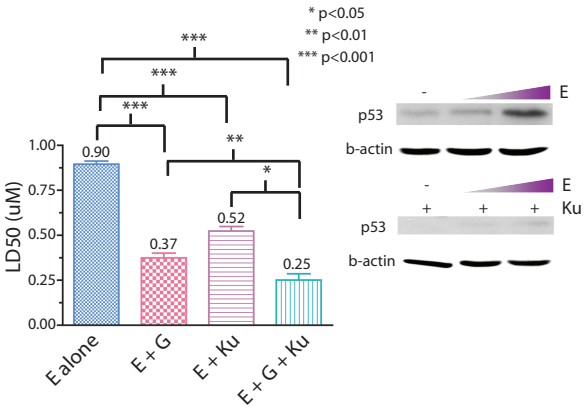
B) HT29





C) LS180





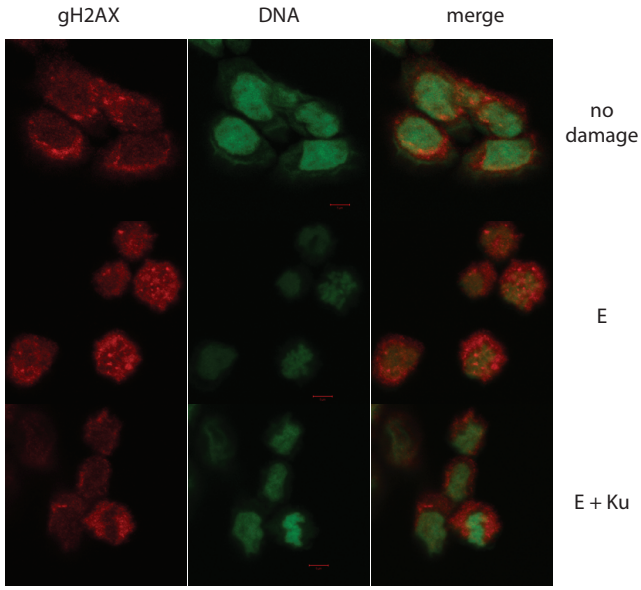
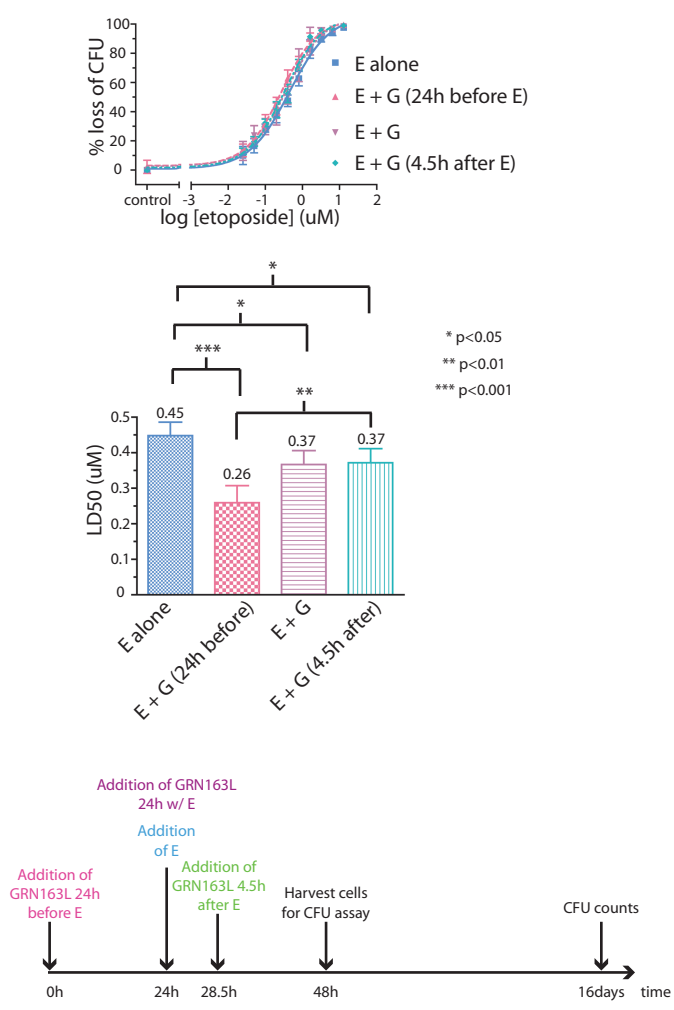


Figure 5

MCF-7



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