Targeted Covalent Inhibition of Telomerase

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Telomerase is a ribonucleoprotein complex responsible for maintaining telomeres and protecting chromosomal integrity. The human telomerase reverse transcriptase (hTERT) is expressed in ~90% of cancer cells where it confers the capacity for limitless proliferation. Along with its established role in telomere lengthening, telomerase also serves non-canonical extra-telomeric roles in oncogenic signaling, resistance to apoptosis, and enhanced DNA damage response. Here, we report a new class of natural product-inspired covalent inhibitors of telomerase that target the catalytic active site. We developed rationally designed probe compounds that modulate both the telomeric and extra-telomeric activities of telomerase and present new opportunities to investigate the diverse functions of this unique molecular machine.

The ends of chromosomes in human cells are capped by telomeres, 5- to 10-kb tandem arrays of 5'-TTAGGG-3' sequence that, with the cognate six-protein shelterin complex,¹ protect chromosome ends against recognition as DNA damage.^{2,3} As telomeres progressively shorten with each cell division cycle, one or more may reach a critical length; the resulting DNA damage response blocks further proliferation and promotes apoptosis or cellular senescence.⁴⁻⁶ Stem cells, embryonic cells, and germ cells overcome this proliferation limit by expressing telomerase, a ribonucleoprotein (RNP) complex that counteracts telomere shortening by synthesizing TTAGGG repeats at chromosome 3' ends.⁷ The human telomerase core is composed of an RNA-dependent DNA polymerase, the human telomerase reverse transcriptase (hTERT)⁸, and the human telomerase RNA (hTR)⁹ which serves as the template for telomere repeat synthesis. While hTR is broadly expressed, hTERT is normally silenced in somatic cells. However, reactivation of hTERT is observed in ~90% of human malignancies and has been directly linked to cancer cell immortality, making hTERT a compelling target for inhibition. In addition to telomere maintenance, genetic studies of hTERT have identified multiple extra-telomeric activities that may promote malignancy including deregulation of oncogenic signaling pathways, resistance to apoptosis and enhanced DNA repair.¹⁰⁻¹² In recent decades, diverse strategies have been pursued to target telomerase in cancer, including the non-competitive small-molecule inhibitor BIBR1532,¹³ antisense oligonucleotide hTR binders such as imetelstat,¹⁴ and G-quadruplex stabilizers that block telomerase from extending telomeres.¹⁵ While these agents have contributed significantly to our understanding of telomerase and telomere biology, none provide a means to covalently block hTERT catalytic activity or its telomere-independent functions. Our goal in these studies was to discover and develop natural product-inspired, synthetically accessible covalent inhibitors of telomerase as new tools to study both the telomere-lengthening and extra-telomeric functions of human telomerase (Fig. 1).

Among the diverse natural products that have been examined as candidate telomerase inhibitors, chrolactomycin (**1**), a tetrahydropyran macrolide antibiotic isolated from *Streptomyces* sp. 569N-3, was reported to display an IC₅₀ of 0.5 μ M in *in vitro* telomerase assays.¹⁶ Notably, chrolactomycin was hypothesized to act as a suicide substrate via conjugation of an exomethylene group to an undetermined hTERT nucleophile. Efforts by several groups toward *de novo* synthesis of the closely related macrolide okilactomycin have been successful, but chrolactomycin has not been accessed by synthesis to date and the length of these routes leaves structure-activity studies essentially unexplored.^{17,18}

Despite concerns of off-target reactivity, small-molecule covalent inhibitors can have clear advantages over non-covalent therapeutics.¹⁹ Their irreversible binding decreases dependence on affinity or drug levels and can raise the barrier to drug resistance.²⁰ The clinical success of ibrutinib,²¹ a first-generation covalent inhibitor of Bruton's tyrosine kinase, has led to multiple second generation investigational agents with increased specificity, establishing the value of combining rational compound design and proteomic validation to accelerate development of targeted covalent inhibitors. Drawing on our laboratory's previous work on similar tetrahydropyran macrolides,²² we hypothesized that applying rational compound design to chrolactomycin might lead to synthetically tractable, small-molecule analogs that preserve the covalent mechanism and specificity for hTERT, providing a novel chemical path toward tool development targeting telomerase.

To validate a covalent mechanism for telomerase inhibition by chrolactomycin, we examined effects on the well-studied TERT protein from *Tribolium castaneum*, tcTERT, and its template RNA, tcTR. Chrolactomycin purified from the actinomycete *Actinospica* could completely and irreversibly block telomerase activity of recombinant tcTERT in the Telomere Repeat Amplification Protocol (TRAP)⁴ assay with an apparent IC₅₀ of 320 nM. Proteolysis and tandem mass spectrometry of tcTERT treated with 1 μ M **1** yielded a single modified peptide, HPQDEIPY**C**GK (Fig. S14-15). Based on the tcTERT crystal structure, the modified residue, C390 (C931 in hTERT), lies within the putative active site.²³ Consistent with our hypothesis that the exomethylene group of **1** mediates its covalent binding, an analog lacking the electrophilic double bond (saturated ketone **3**, Fig. 2A) displayed reduced activity compared to **1** in MCF7 ER⁺ human breast cancer cell lysates (IC₅₀ of 12 μ M and 157 nM, respectively). Lacking a high resolution structure for hTERT to model chrolactomycin binding,²⁴ tcTERT was utilized for our *in silico* studies.²³ Docking of **1** in the active site of tcTERT was examined using the Schrödinger suite (Glide module). By placing the terminal carbon atom of the α,β enone of chrolactomycin in close proximity (3.3 Å) to the C390 sulfur atom in the active site, several non-bonding interactions could be observed with potential to stabilize binding and facilitate delivery of the electrophilic warhead (Fig. 2B). In turn, the docking revealed few if any contacts between tcTERT and the chrolactomycin macrolide loop. Drawing on these insights, we designed simplified analogs that might maintain probe-target interactions but could be obtained via a straightforward, modular synthetic sequence. To commence, bicyclic dioxinone precursors were obtained by a Lewis acid-catalyzed Prins reaction with enantioenriched β -hydroxydioxinones formed via ketoreductase reduction and

aldehydes.²² Conversion to these β -ketoesters by dioxinone opening and subsequent trapping by an alcohol nucleophile, followed by α -methylation and then methylenation yielded the target structures (Scheme S1). These simplified analogs could be accessed in 4 steps vs. >25 steps required for the syntheses of chrolactomycin and okilactomycin.¹⁷ With this strategy in hand, >150 compounds were synthesized and compared in a medium-throughput TRAP assay.⁴ Initial SAR analysis identified that substituents larger than methyl at the C6 position and a lack of H-bonding at the C2/C3 positions led to significantly decreased potency. We then focused on increasing H-bonding interactions at C2/C3 by targeting three structure classes poised for late-stage diversification: reductive amination (aldehydes), amide bond formation (carboxylic acids), and [3+2] cycloadditions (alkynes) to facilitate rapid library development (Fig. 2C). Further optimization led to lead compound **NU-1** that displayed an IC₅₀ of 90 nM in TRAP assays in MCF7 cell lysate and an IC₅₀ of 270 nM in MCF7 cells in culture. These results were confirmed in four other telomerase-positive human cancer cell lines, A549 lung cancer, ACHN renal carcinoma, MDA-MB-231 triple negative breast cancer and HeLa cervical cancer (Fig. S1). Like des-exomethylene forms of **1** and other analogs, **2** displayed no appreciable inhibition, indicating a covalent mechanism remained critical for telomerase inhibition (Fig. 2 and Fig. S2). **NU-1** displayed satisfactory performance in plasma and microsomal stability assays, and showed favorable lipophilicity and CYP450 inhibition profiles. While **NU-1** displayed moderate thiol reactivity when challenged with N-acetyl cysteine or N-acetyl lysine, no ketone-Schiff base formation was observed with other amino acids (Fig. S14). When **NU-1** was incubated with tcTERT, proteolysis and mass spectrometry yielded a single modified peptide HPQDEIPY**C**GK, localizing the site of modification to the active site C390 and confirming a shared mechanism of action with chrolactomycin (Fig. S16).

To further evaluate **NU-1**, inhibition of telomerase activity was assessed by direct primer extension assay. Human telomerase complexes purified from telomerase-overexpressing HEK293T cells²⁵ were treated with **1**, **NU-1** or **2**. **NU-1** and **1** inhibited telomere repeat synthesis in the low-micromolar range, while **2** displayed no inhibition (Fig. 3A-B), verifying the critical role of the enone warhead. Incubation of purified telomerase with 100 μ M **NU-1** displayed irreversible inhibition kinetics with a $t_{1/2}$ of ~15 min at 23 °C, with complete inhibition observed after ~6 h (Fig. 3C). We then assessed inhibition of *endogenous* telomerase in MCF-7 and A549 cells after culture in the presence of 10 μ M **NU-1** or **2** control for 24 h. **NU-1** com-

pletely eliminated endogenous telomerase activity, whereas **2** was indistinguishable from the DMSO (vehicle) control (Fig. 3D).

To evaluate the impacts of telomerase inhibition on cell proliferation, MCF7 cells were cultured in media containing **1**, **NU-1** or **2**. Both exomethylene-bearing compounds conferred similar dose-responsive loss of clonogenicity (IC_{50} of 21 μ M and 27 μ M respectively), while **2** had no effect (Fig. S3A). A similar pattern was observed in A549, ACHN, MDA-MB-231, and HeLa telomerase-positive cells (Fig. S3B-E). By contrast, like DMSO control or **2**, treating the telomerase-negative cancer cell lines Saos-2 or Wi-38²⁶ with up to 100 μ M **1** or **NU-1** did not affect viability (Fig. 3E, Fig. S4). Consistent with a covalent mechanism, washing the treated cells to remove free **NU-1** and then incubation in fresh culture media did not restore viability or telomerase activity even after 24 h (see Fig. S7,S8 for details). Comparing **NU-1** to the reversible telomerase inhibitors BIBR1532 (IC_{50} ~0.1 μ M) or MST-312 (IC_{50} ~1 μ M) in MCF7 cells revealed qualitatively similar effects on clonogenicity, though **NU-1** displayed the lowest apparent IC_{50} (Fig. S6). However, when the treated MCF7 cells were washed and allowed to recover for 4 h, telomerase activity was restored in cells treated with BIBR1532 or MST-312 (Fig. S7) but remained blocked in cells treated with **NU-1**.

Along with its canonical role in telomere maintenance, hTERT has been ascribed non-canonical functions that modulate the DNA damage response, raising the possibility that telomerase inhibition might provide a route to sensitizing cancer cells to chemo- or radiotherapy.²⁷ Thus, we examined effects of treating MCF7 cells with **NU-1**, **2**, BIBR1532 or MST-312 along with clinical chemotherapy agents. Incubating cells with telomerase inhibitors for 24 h conferred substantial sensitization to the topoisomerase I poison irinotecan. The IC_{50} shifted from 310 nM irinotecan in cells pre-treated with DMSO to 82 nM for **NU-1** (Fig. 4A). Compound **2** was similar to DMSO at 325 nM while BIBR1532 and MST-312 had more modest effects, yielding IC_{50} values of 168 nM and 151 nM. A similar pattern was observed in A549 cells (Fig. S10) but not in telomerase negative Saos-2 cells (Fig. 4B). Notably, no chemosensitization was observed when **NU-1** was either coadministered with irinotecan or added after irinotecan (Fig. S9), suggesting that the telomerase activity must be depleted prior to inducing DNA damage to mediate sensitization. A similar pattern was observed with the topoisomerase II poison etoposide, the spindle poison paclitaxel and the DNA damaging agent doxorubicin (Figs. S11-S13). Moreover, loss of cellular proliferation was observed in combination with irradiation in MCF-7 cells (Fig. 4C).

To further examine how targeting hTERT activity may impact DNA damage response, we monitored the formation and resolution of ionizing radiation-induced foci (IRIF), domains of altered chromatin that form at chromosomal double strand breaks. Immunofluorescence analysis to track the histone modification γ H2AX or recruitment of the adaptor protein 53BP1 each revealed persistent IRIF 24 h after irradiation in MCF7 cells pre-treated for 2 h with **1** or **NU-1**, compared to DMSO or **2** (Fig. 4D-E). Treatment with **NU-1** conferred little or no IRIF formation to unirradiated MCF7 controls and IRIF persistence was not observed after irradiation in telomerase-negative Saos-2 cells (Fig. 4F-G).

Telomerase inhibition can drive otherwise immortal cancer cells into replicative senescence,²⁸ an effect commonly ascribed to progressive telomere erosion resulting in a persistent DNA damage response and irreversible cell cycle checkpoint arrest. However, that **NU-1** delayed IRIF resolution raised the question whether an altered DNA damage response upon telomerase inhibition²⁹ might similarly potentiate therapy-induced senescence after irradiation. Indeed, when cells treated with **NU-1** were irradiated and maintained in culture for 4 days, they arrested cell proliferation and developed characteristic features of senescence including large, flattened cells that expressed senescence-associated beta-galactosidase (SA- β Gal, Fig. 4H-I). MCF7 cells treated with **NU-1**, **1**, **2** or DMSO for 2 h prior to 6 Gy irradiation displayed 65, 43, 40 and 32% SA- β Gal⁺ cells after 4 days. **1** and **NU-1** did not induce SA- β Gal activity on their own, suggesting a telomere-independent effect, and **NU-1** did not increase the fraction of SA- β Gal⁺ cells over DMSO in irradiated telomerase-negative Saos-2 cells (59 and 60%, respectively, Fig. 4J-K).

Along with effects on telomere maintenance and DNA damage response, hTERT has been implicated in regulation of gene expression that affects proliferation, inflammation, cell survival and other pathways linked to malignancy.³⁰ To examine how hTERT inhibition with **NU-1** affects cellular gene expression, the transcriptomes of MCF-7 cells treated with 0.5 μ M **NU-1** or DMSO for 48 h were compared, revealing ~ 4,000 differentially expressed genes (< or > 1.5 fold compared to control). Ingenuity pathway analysis (IPA) indicated that **NU-1** treatment significantly affected multiple cancer-associated pathways (Table S1), indicating a role for hTERT catalytic activity in gene regulation.

A new and effective class of telomerase inhibitors has been developed inspired by the natural product chrolactomycin, a covalent inhibitor of human telomerase reverse transcriptase (hTERT) catalytic activity. Rational compound design resulted in the lead compound **NU-1**, a simplified analog accessible in four synthetic steps that binds to hTERT and directs an exomethylene group to react with an active-site

cysteine, leading to irreversible enzyme inhibition. **NU-1** blocks both telomeric and non-canonical functions of hTERT to target cancer cell proliferation and DNA damage response, conferring sensitization to chemotherapy and radiation in telomerase-positive cancer cells. Despite concerns about potential reactivity with cellular nucleophiles, **NU-1** had negligible effects on telomerase-negative cancer cells. This study provides proof-of-principle for sensitive, covalent chemical probes to dissect telomerase functions in cells and lead compounds to validate hTERT as a target to enhance conventional genotoxic cancer therapies.

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Author Contributions: R.C.B., S.J.K. and K.A.S. conceived and directed the project. R.C.B. conducted all organic synthesis, TRAP telomerase activity assays and cell viability assays. S.B.C. conducted all direct primer-extension assays. Y.L. conducted other cell-based studies. D.W. conducted analysis of transcriptome data. R.K.M. developed the computational docking model for compound design with tcTERT. R.C.B., S.B.C., S.J.K and K.A.S. prepared the manuscript.

Fig. 1: A natural product platform for rational design of small molecule probes of telomerase. A) Extra-telomeric activities of telomerase, all independent on telomeric activity. B) Workflow for rational design of chrolactomycin inspired chemical probes.

Fig. 2: Chrolactomycin as a model for the development of novel telomerase inhibitors. A) Confirmation that chrolactomycin inhibits telomerase through a covalent mechanism B) Cheminformatics model for rational design of chrolactomycin inspired chemical probes. C) Workflow for probe synthesis and identification of **NU-1**, a first-in-class chemical probe for telomerase

Fig. 3: Chrolactomycin and **NU-1** inhibit telomerase by direct primer extension assay. A-B) HEK293T cells overexpressing telomerase show that chrolactomycin and **NU-1** inhibit telomerase, where **2** does not. C) Time-course measurement of telomerase inhibition by **NU-1** shows a time-dependent inhibition profile. D) A549 and MCF-7 cells expressing endogenous telomerase show complete loss of telomerase activity when dosed with **NU-1**. E) Cell Viability measurement of **NU-1** across a panel of telomerase positive and telomerase negative cell lines

Fig. 4. Telomerase inhibition as a strategy in chemo- and radiosensitization A) **NU-1** demonstrates sensitization to the DNA DSB inducer irinotecan in telomerase positive cells (MCF-7) B) but not telomerase negative (Saos-2). C) Telomerase inhibition in combination with irradiation impacts cellular proliferation. D-E) Telomerase inhibition causes decreased DNA repair capabilities upon exposure to irradiation in telomerase positive cells (MCF-7) F-G) but not telomerase negative (Saos-

2). Bar, 10 μm . H-I) Telomerase inhibition causes an increase in the onset of senescent cellular profiles with irradiation in telomerase positive MCF-7 cells J-K) but not telomerase negative (Saos-2). Bar, 20 μm .

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Supporting Information

Targeted Covalent Inhibition of Telomerase

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General Information for Chemistry

All reactions were carried out under an argon or nitrogen atmosphere in flame-dried glassware with magnetic stirring. Solvents used in reactions were purified by passage through a bed of activated alumina. Unless stated otherwise, reagents were purified prior to use following the guidelines of Perrin and Armarego.¹ Purification of reaction products was carried out by flash chromatography on Biotage Isolera 4 systems with Ultra-grade silica cartridges. Analytical thin layer chromatography was performed on EM Reagent 0.25 mm silica gel 60-F plates. Visualization was accomplished with UV light. Infrared spectra were recorded on a Bruker Tensor 37 FT-IR spectrometer. ¹H NMR spectra were recorded on an AVANCE III 500 MHz spectrometer with direct cryoprobe (500 MHz) and Bruker Avance III 600 MHz (151 MHz) system. Spectra are reported in ppm using solvent as an internal standard (CHCl₃ at 7.26 ppm). Peak multiplicities are reported as (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad; coupling constant(s) in Hz; integration.) Proton-decoupled ¹³C NMR spectra were recorded on an AVANCE III 500 MHz with direct cryoprobe (125 MHz) spectrometer or Bruker Avance III 600 MHz (151 MHz) system. These are reported in ppm using solvent as an internal standard (CDCl₃ at 77.16 ppm). Low-resolution mass spectra were obtained on WATERS Acquity-H UPLC-MS with a single quad detector (ESI) Varian 1200 Quadrupole Mass Spectrometer. High-resolution mass spectra were obtained using an Agilent 6120A LC-time of flight mass spectrometer. Gas chromatography experiments were run on Agilent 7890A/5975C GC/MS System.

General Procedure for Synthesis of NU-1

2,2-Dimethyl-6-(2-oxo-2-methylethyl)-4H-1,3-dioxin-4-one (S1): yield, 61%.

Prepared according to literature precedent.² Dioxinone (25 g, 176 mmol) in THF (75 mL) was added dropwise to LHMDS in THF (1.0M; 250 mL, 250 mmol) and THF (165mL) at -20 °C, and after 45 min, diethylzinc in hexanes (1.0M; 250 mL, 250 mmol) was added over 2 h. After a further 30 min, the reaction mixture was allowed to warm up to -10 °C and N-acetylimidazole (27.5 g, 250mmol) was added portionwise over 15 min. After 3.5 h, H₂O:THF (1:9; 75 mL) was added dropwise, followed by aqueous HCl (100 mL) and EtOAc (250 mL). The pH was adjusted to pH 1-2 using aqueous HCl (265 mL), and

the layers were separated. The aqueous layer was extracted with EtOAc (250 mL), and the combined organic extracts were washed with brine (250 mL), dried (MgSO₄), rotary evaporated, and chromatographed (hexanes:EtOAc 3:2) to give **S1** as pale yellow crystals.

Analytical Data: ¹H NMR spectroscopy (500 MHz, CDCl₃) δ 5.35 (s, 1H), 3.37 (s, 2H), 2.25, (s, 3H), 1.72 (s, 6H); ¹³C NMR spectroscopy (125 MHz, CDCl₃) δ 200.9, 164.4, 160.7, 107.2, 96.6, 47.9, 30.2, 25.0. LCMS (ESI): Mass calculated for C₉H₁₂O₄ [M+H]⁺, 185, Found 185. All physical data for this product corresponds with literature values.³

(R)-6-(2-hydroxypropyl)-2,2-dimethyl-4H-1,3-dioxin-4-one (S2): yield, 99%.

Prepared according to literature precedent.⁴ 160 mL of 0.1 M phosphate buffer (pH 7.0) was added to a 500 mL 3 neck round bottom flask equipped with an overhead stirrer, a nitrogen inlet, and a septum. 100 mg of KRED-P01-C01 was then added followed by 50 mg of NADPH. In a separate 100 mL round bottom flask, 20.0 g of **S1**, 20 mL isopropanol (IPA) and 20 mL cyclopentylmethyl ether (CPME) were added. Upon the β-ketodioxinone substrate completely dissolving (required slight heating and stirring), this solution was added to the solution containing KRED-P01-C01. The reaction was stirred for 72 hours at 30 °C. Upon reaction completion, solid NaCl was added to the reaction mixture. The solution was then filtered, extracted with ethyl acetate (5 x 150 mL), dried with MgSO₄, filtered, and then concentrated *in vacuo* to obtain >99% pure β-hydroxydioxinone **S2**

Analytical Data: ¹H NMR spectroscopy (500 MHz, CDCl₃) δ 5.33 (s, 1H), 4.16-4.08 (m, 1H), 2.38 (1H, d, *J* = 2.8), 2.37 (s, 1H), 1.70 (s, 6H), 1.27 (3H, d, *J* = 6.2). ¹³C NMR spectroscopy (125 MHz, CDCl₃) δ 169.3, 161.3, 106.6, 94.9, 65.1, 43.2, 25.2, 24.9, 23.5. LCMS (ESI): Mass calculated for C₉H₁₄O₄ [M+H]⁺, 187, Found 187

Enantiomeric ratio determined by chiral phase HPLC (Chiracel OD-H, i-PrOH:hexanes, 10:90, 1.0 mL/min). All physical data for this product corresponds with literature values³

(5S,7R)-5-(but-3-yn-1-yl)-2,2,7-trimethyl-7,8-dihydro-4H,5H-pyrano[4,3-d][1,3]dioxin-4-one (S3):
yield, 74%

A flask was charged with 4Å MS (2:1 by wt), pent-4-ynal (2.93 g, 35.7 mmol) and **S2** (1.661 g, 8.92 mmol). Dichloromethane (Volume: 44.6 ml) was added and the reaction was cooled to -78°C . TMS-OTf (3.55 ml, 19.63 mmol) was added dropwise and stirred for 5 h. The reaction was quenched at -78°C with water and allowed to warm to room temperature. The suspension was then filtered through a Biotage Isolute phase separator and then concentrated. The crude product was purified by flash chromatography (0 to 10% ethyl acetate/hexanes) to yield **S3**.

Analytical data: ^1H NMR (500 MHz, Chloroform- d) δ 4.47 (dq, $J = 7.6, 2.3$ Hz, 1H), 3.70 (ddq, $J = 12.1, 6.1, 3.1, 2.3$ Hz, 1H), 2.34 (qd, $J = 2.6, 1.7$ Hz, 2H), 2.23 – 2.08 (m, 2H), 1.93 (t, $J = 2.4$ Hz, 1H), 1.90 – 1.81 (m, 1H), 1.54 (s, 2H), 1.28 (d, $J = 6.2$ Hz, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 168.0, 156.3, 108.6, 104.4, 84.3, 71.9, 70.8, 68.8, 37.9, 33.1, 25.8, 23.3, 19.2. LCMS (ESI): Mass calculated for $\text{C}_{14}\text{H}_{19}\text{O}_4$ $[\text{M}+\text{H}]^+$, 251, Found 251

methyl (2*S*,3*R*,6*R*)-2-(but-3-yn-1-yl)-6-methyl-4-oxotetrahydro-2*H*-pyran-3-carboxylate (S4):

yield, 91%

In a microwave vial **S3** (0.250 g, 1.00 mmol) was dissolved into toluene (Volume: 5.0 ml, 0.2 M). Dry methanol (0.64 ml, 10.0 mmol, 10 equiv) was added, the reaction vial was capped, and the reaction heated in a Biotage microwave reactor at 150 °C for 40 m. After the vial was cooled to room temperature, the solution was concentrated. The crude product was purified on flash chromatography (20% EtOAc/hexanes) to yield **S4**.

Analytical data: ¹H NMR (500 MHz, Chloroform-*d*) δ 5.29 (d, *J* = 2.1 Hz, 1H), 5.01 (t, *J* = 2.6 Hz, 1H), 4.61 (d, *J* = 3.8 Hz, 1H), 3.90 (ddd, *J* = 10.1, 6.2, 3.5 Hz, 1H), 3.74 (s, 3H), 2.79 – 2.66 (m, 1H), 2.57 (ddd, *J* = 16.9, 9.9, 2.1 Hz, 1H), 2.38 – 2.25 (m, 2H), 2.10 – 2.00 (m, 1H), 1.78 – 1.60 (m, 1H), 1.29 (d, *J* = 6.1 Hz, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 205.6, 173.9, 84.2, 78.3, 70.8, 69.8, 60.2, 51.7, 49.3, 34.9, 21.2, 19.7.

LCMS (ESI): Mass calculated for C₁₂H₁₇O₄ [M+H]⁺, 225, Found 225

methyl (2*S*,3*R*,6*R*)-2-(but-3-yn-1-yl)-3,6-dimethyl-4-oxotetrahydro-2*H*-pyran-3-carboxylate (S5):

yield, 85%

S4 (0.150 g, 0.669 mmol) was dissolved in acetonitrile (Volume: 3.34 ml, 0.2 M) in a vial with stir bar. To the solution was added potassium carbonate (0.388 g, 2.81 mmol, 4 equiv) and methyl iodide (0.418 ml, 6.69 mmol, 10 equiv). The reaction mixture was heated to 70 °C for 2 hours before recooling to room temperature and quenching with saturated aqueous ammonium chloride. The mixture was diluted

with dichloromethane, filtered through a Biotage Isolute phase separator and concentrated. The crude product was purified by flash chromatography (10% EtOAc/hex) to yield **S5**.

Analytical data: ^1H NMR (500 MHz, Chloroform- d) δ 4.12 (dd, J = 10.4, 1.9 Hz, 1H), 3.82 (ddd, J = 10.2, 6.1, 4.4 Hz, 1H), 3.76 (s, 3H), 2.53 – 2.40 (m, 1H), 2.40 – 2.28 (m, 3H), 1.84 – 1.72 (m, 1H), 1.36 (s, 3H), 1.32 (d, J = 6.1 Hz, 3H), 0.15 (s, 9H); ^{13}C NMR (126 MHz, CDCl_3) δ 206.20, 171.23, 83.35, 78.78, 73.33, 68.74, 61.84, 52.34, 45.09, 29.82, 21.92, 15.50, 14.28; LCMS (ESI): Mass calculated for $\text{C}_{13}\text{H}_{19}\text{O}_4$ $[\text{M}+\text{H}]^+$, 239, Found 239

methyl (2*S*,3*R*,6*R*)-2-(but-3-yn-1-yl)-3,6-dimethyl-5-methylene-4-oxotetrahydro-2*H*-pyran-3-carboxylate (S6): yield, 71%

Prepared according to literature precedent.⁵ To a solution of **S5** (0.239 g, 1.00 mmol) in (Volume: 5.00 ml, 0.2 M) microwave vial, CH_2Br_2 (6.00 mmol) and Et_2NH (12.00 mmol) were added. The container was sealed and the reaction heated in a Biotage microwave reactor at 100 °C for 1 hour. After the vial was cooled to room temperature, the reaction solution was concentrated. Diethyl ether was added to the crude reaction mixture to precipitate out the ammonium salts. The mixture was filtered, and the filtrate was concentrated. The crude product was purified by flash chromatography (10% EtOAc/hex) to yield **S6**.

Analytical data: ^1H NMR (500 MHz, Chloroform- d) δ 6.19 (dd, J = 2.2, 0.8 Hz, 1H), 5.41 (dd, J = 2.1, 0.8 Hz, 1H), 4.54 (dt, J = 6.3, 2.2 Hz, 1H), 4.29 (dd, J = 10.4, 2.0 Hz, 1H), 3.74 (s, 3H), 2.45 – 2.27 (m, 2H), 1.96 (t, J = 2.6 Hz, 1H), 1.46 (d, J = 6.2 Hz, 3H), 1.34 (s, 3H); ^{13}C NMR (126 MHz, CDCl_3) δ 204.33, 170.67, 146.76, 122.78, 105.93, 83.93, 78.85, 74.83, 68.96, 68.40, 62.77, 60.03, 52.63, 51.95, 29.61, 22.93, 21.98, 19.39; LCMS (ESI): Mass calculated for $\text{C}_{14}\text{H}_{19}\text{O}_4$ $[\text{M}+\text{H}]^+$, 251.13, Found 251

methyl (2S,3R,6R)-2-(2-(1-(2,4-difluorobenzyl)-1H-1,2,3-triazol-4-yl)ethyl)-3,6-dimethyl-5-methylene-4-oxotetrahydro-2H-pyran-3-carboxylate (NU-1): yield, 85%

1-(Azidomethyl)-2,4-difluorobenzene (3.13 mg, 0.018 mmol, 1.0 equiv) and **S6** (5.12 mg, 0.020 mmol, 1.0 equiv) were dissolved in t-BuOH (Volume: 0.140 ml) room temperature. To this, a solution of copper(II) sulfate pentahydrate (0.907 mg, 3.63 μ mol, 0.12 equiv) and sodium (R)-2-((S)-1,2-dihydroxyethyl)-4-hydroxy-5-oxo-2,5-dihydrofuran-3-olate (1.439 mg, 7.26 μ mol, 0.25 equiv) in water (Volume: 0.140 ml) was added. The reaction mixture was stirred at 40 °C for 5 h. After completion, the reaction mixture was extracted with EtOAc (3 x). The organic layer was dried over sodium sulfate and the excess solvent was removed under reduced pressure. The crude product was purified by column chromatography to yield **NU-1**.

Analytical data: ^1H NMR (500 MHz, Chloroform- d) δ 7.41 – 7.29 (m, 6H), 5.47 (dd, J = 8.1, 4.3 Hz, 2H), 4.97 (d, J = 14.9 Hz, 1H), 4.37 (d, J = 6.0 Hz, 1H), 4.32 (s, 3H), 4.18 – 3.91 (m, 3H), 2.91 (s, 4H), 2.78 (dd, J = 14.8, 11.6 Hz, 1H), 2.43 (dd, J = 14.8, 2.8 Hz, 1H), 2.12 – 1.95 (m, 2H), 1.42 – 1.29 (m, 6H), 1.29 – 1.21 (m, 3H), 1.18 – 1.08 (m, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 193.6, 172.8, 158.6, 158.5, 140.1, 126.8, 115.6, 78.4, 74.6, 57.5, 51.7, 30.0, 20.6, 20.1.

LCMS (ESI): Mass calculated for $\text{C}_{21}\text{H}_{23}\text{F}_2\text{N}_3\text{O}_4$ $[\text{M}+\text{H}]^+$ 420, Found 420.

tcTERT Covalent Docking.

All modeling was conducted using the Schrodinger Suite, Version 11.2 (Schrodinger). The crystal structure of tcTERT (PDB: 3DU5) was obtained from the Protein Data Bank. **NU-1** was built in Maestro and

prepared for docking using LigPrep with protonation states assigned using Epik. Covalent docking was performed using the default settings and the ligand and surrounding residues were further minimized using MacroModel and the OPLS3 forcefield.

Protease Digestion and Mass Spectrometry Analysis

Chrolactomycin, NU-1 or DMSO-treated tcTERT (50 µg, ~600 pmol) was diluted with ammonium bicarbonate buffer (pH 8.0), then reduced for 30 min at 56 °C with 10 mM DTT. After cooling for 5 min, the protein was alkylated with 25 mM iodoacetamide for 30 min at room temperature in the dark, and digested overnight with 2.5 µg of trypsin at 37 °C. Digested peptides (~2 pmol) were injected onto a self-packed precolumn (4 cm POROS10R2) and eluted into the mass spectrometer (LTQ OrbitrapVelos, Thermo Fisher). Peptides were subjected to MS² by CAD (electron multiplier detection, relative collision energy 35%, $q = 0.25$) as well as HCD (image current detection, resolution at m/z 400 = 7,500, relative collision energy 35%).

Cell Lines

MCF-7 (ATCC HTB-22), MDA-MB-231 (ATCC HTB-26), HeLa (ATCC CCL-2), ACHN (ATCC CRL-1611), A549 (ATCC CCL-185), Wi-38 (ATCC CCL-75) and Saos-2 (ATCC HTB-85) cells were obtained from ATCC. All cell lines were cultured according to the manufacturer's instructions at 37 °C in 5 % CO₂. Mycoplasma tests were performed every six months.

Telomeric Repeat Amplification Protocol Assay

The telomeric repeat amplification protocol (TRAP) assay was performed using the TRAPeze RT Telomerase Detection Kit (EMD Millipore) and iTaq DNA polymerase (Bio-Rad).

Preparation of cell extracts for measurement of telomerase activity in cell lysates: MCF-7 cells were cultured in DMEM media supplemented with 10% Fetal Bovine Serum (FBS), 1% pen/strep and with 5%

CO₂ and 37 °C. Cells were plated in 2 x T75 flasks and cultured for 3 days. Flasks were rinsed twice with PBS and treated with 0.05% trypsin-EDTA (Thermo Fisher) to detach cells. A sample of detached cells was diluted 1:1 with Trypan Blue 0.4% solution (Thermo Fisher) and counted with a hemocytometer. The cell suspension was washed twice in PBS and the cell pellet was resuspended in 200 µl of 1X CHAPS Lysis Buffer (10 mM Tris-HCl, pH 7.5; 1 mM MgCl₂; 1 mM EGTA; 0.1 mM benzamidine; 5 mM β-mercaptoethanol; 0.5% CHAPS; 10% glycerol)/10⁶ cells, and incubated on ice for 30 min. Cell extracts were transferred to microcentrifuge tubes, 200 µl/tube, and centrifuged at 12,000 x g for 20 min at 4 °C to remove cell debris. A cell pellet of 1 x 10⁷ telomerase-positive cells supplied with the kit as a positive control was also extracted as described above.

Preparation of compounds for assay: A stock solution of each compound was made in DMSO with a concentration of 100 mM and vortexed for 1 min to ensure complete dissolution. A 6-fold dilution was performed in DMSO in a 96-well plate to produce compound solutions of 100, 10, 1, 0.1, 0.01, and 0.001 mM (1000X of the final concentration).

Preparation of a TSR8 standard curve: A series of samples were prepared using serial dilutions of the included TSR8 control template. These were diluted with CHAPS Lysis Buffer to obtain TSR8 concentrations of 20 amol/µL, 2 amol/µL, 0.2 amol/µL, 0.02 amol/µL.

Compound treatment for measurement of telomerase activity in cell lysates: The 1000X stock solutions of the compound samples were transferred to a 96-well plate using the LabCytte Echo 550 and then diluted with the cell extracts to yield final compound concentrations of 100, 10, 1, 0.1, 0.01, and 0.001 µM. The plate was covered and shaken at room temperature for 30 min.

Compound treatment in whole cells: MCF-7, HeLa, ACHN, MDA-MB231 and A549 cells were cultured in DMEM media supplemented with 10% Fetal Bovine Serum (FBS), 1% pen/strep and with 5% CO₂ and 37 °C. Cells were plated in 96 well plates and allowed to attach for 24 h. Media was removed, and fresh media containing various concentrations of compound was added, and the plate was incubated for

24 h. Media was removed, the cells were washed 2 times with PBS, and the cells were lysed in 20 μ l of 1X CHAPS Lysis Buffer (10 mM Tris-HCl, pH 7.5; 1 mM MgCl₂; 1 mM EGTA; 0.1 mM benzamidine; 5 mM β -mercaptoethanol; 0.5% CHAPS; 10% glycerol). The cell extract was then centrifuged at 12,000 x g for 20 min at 4 °C to remove cell debris.

Compound treatment in whole cells with compound washout: MCF-7 cells were plated in 96 well plates and allowed to attach for 24 h. Media was removed, and fresh media containing various concentrations of compound was added, and the plate was incubated for 24 h. Media was removed, cells were washed 2 times with PBS, and fresh media without compound was added. Cells were incubated at 37 °C for an additional “X” hours. Media was removed, the cells were washed 2X with PBS, and the cells were lysed in 20 μ l of 1X CHAPS Lysis Buffer (10 mM Tris-HCl, pH 7.5; 1 mM MgCl₂; 1 mM EGTA; 0.1 mM benzamidine; 5 mM β -mercaptoethanol; 0.5% CHAPS; 10% glycerol). The cell extract was then centrifuged at 12,000g for 20 min at 4 °C to remove cell debris.

For TRAPeze reactions, a “master mix” was prepared according to the TRAPeze protocol, containing 5 μ l of the TRAPeze reaction mix; 0.4 μ l Taq polymerase; 17.6 μ l PCR grade, nuclease-free water. The master mix and treated cell extracts were transferred using the TTP Labtech mosquito Crystal liquid handling system into separate wells in a white, 364-well RT-PCR plate. Each well contained 1.2 μ l of the master mix and 1.2 μ l of the treated cell extract to give a final volume of 2.4 μ l, with two duplicates for each sample. The RT-PCR plate was then sealed and centrifuged for 5 min. A sample containing only CHAPS Lysis Buffer was included as negative control. The TSR8 standards were included to generate a standard curve. Samples were amplified in a BioRad CFW384 RT-PCR detection system with PCR parameters of 30 min at 30 °C for 1 cycle, 2 min at 95 °C for 1 cycle, and then 45 cycles of 15 s at 94 °C, 60 s at 59 °C, and 10 s at 45 °C. Real-time fluorescent data was obtained during the 10 s at 45 °C step. The number of cycles at which the fluorescence level reached a threshold (Ct) was averaged between the duplicates for each sample. Using a calibration curve obtained from the TSR8 standards, a lin-

ear equation was obtained to convert the Ct values into arbitrary telomerase activity units which were plotted as a function of compound concentration to construct dose-response curves and calculate IC50.

Cell Viability Measurements

Compound treatment for measurement of cell viability in whole cells: MCF-7, HeLa, MDA-MB231 and A549 cells were cultured in DMEM media supplemented with 10% Fetal Bovine Serum (FBS), 1% pen/strep and with 5% CO₂ and 37 °C. Cells were plated in 96 well plates and allowed to attach for 24 h. Media was removed, and fresh media containing various concentrations of compound was added, and the plate was incubated for 24 h. The plate was removed from the incubator and allowed to equilibrate to room temperature for 30 min. 50 µl of freshly prepared CellTiter-Glo (Promega) reagent was added, and the plate was mixed on an orbital shaker for 10 min to induce cell lysis and stabilize luminescent signal. The luminescence was recorded on a PerkinElmer Enspire multimode plate reader.

Compound treatment for measurement of cell viability in whole cells with compound washout: MCF-7 cells were plated in 96 well plates and allowed to attach for 24 h. Media was removed, and fresh media containing various concentrations of compound was added, and the plate was incubated for 24 h. Media was removed, cells were washed 2 times with PBS, and fresh media without compound was added. Cells were incubated at 37 °C for an additional “X” h and analyzed as above.

Pre-Dosing Chemosensitization Studies

MCF-7, A549 and Saos-2 cells were plated in 96 well plates and allowed to attach for 24 h. Media was removed, and fresh media containing set concentrations of DMSO control, **NU-1, 2**, MST-312 and BIBR1532 was added, and the plate was incubated for 4 h. The plate was removed from the incubator; DMSO stock solutions of chemotherapies (irinotecan, etoposide, paclitaxel and doxorubicin) were added

to the culture media. The plate was placed on an orbital shaker for 10 minutes to ensure mixing of DMSO chemotherapy solution. The plate was then incubated at 37 °C for 24 h and analyzed as above.

Co-Dosing Chemosensitization Studies

MCF-7 cells were plated in 96 well plates and allowed to attach for 24 h. Media was removed, and fresh media containing set concentrations of DMSO control, **NU-1** and **2**, as well as DMSO stock solutions of varying doses of irinotecan. The plate was placed on an orbital shaker for 10 min to ensure mixing. The plate was then incubated at 37 °C for 24 h and analyzed as above.

Post-Dosing Chemosensitization Studies

MCF-7 cells were plated in 96 well plates and allowed to attach for 24 h. Media was removed, and fresh media containing varying concentrations of irinotecan was added, and the plate was incubated for 4 h. The plate was removed from the incubator and set concentrations of DMSO control, **NU-1** and **2** were added to the culture media. The plate was placed on an orbital shaker for 10 minutes to ensure mixing. The plate was then incubated at 37 °C for 24 h. The plate was removed from the incubator and allowed to equilibrate to room temperature for 30 minutes. 50 µl of freshly prepared CellTiter-Glo (Promega) reagent was added, and the plate was mixed on an orbital shaker for 10 minutes to induce cell lysis and stabilize luminescent signal. The luminescence was recorded on a plate reader.

Primer-Extension Telomerase Activity Assays

Telomerase activity assays were performed as previously described.⁶ Experiments in Figures **3A** and **3B** utilized telomerase immunopurified with the hTERT antibody as described in the reference above; telomerase stock solution was at ~10 nM in “telomerase buffer”: 50 mM HEPES-KOH (pH 8); 300 mM KCl; 2 mM MgCl₂; 10% v/v glycerol; 0.1% v/v Triton X-100; 1 mM DTT.

For Figure 3A, extension reactions were performed in a total volume of 100 μL . The compounds **NU-1** or **2** were prepared in DMSO at 20 $\times$ final concentration (2000 μM , 200 μM , or 20 μM). 5 μL of DMSO or the respective compound/DMSO dilution was placed in a tube, followed by 45 μL of a solution containing: 2.2 mM MgCl_2 ; 1.1 mM DTT; 2.2 mM dTTP, 2.2 mM dATP, 22 μM dGTP, ~ 5 μCi α - ^{32}P -dGTP (Perkin Elmer), and the DNA substrate 5'-biotin-CTAGACCTGTCATCA(TTAGGG) $_3$ -3' (2.2 μM). Reactions were initiated by addition of 50 μL telomerase solution, providing final concentrations of: ~ 5 nM telomerase; 25 mM HEPES-KOH; 150 mM KCl; 2 mM MgCl_2 ; 0.05% v/v Triton X-100; 5% v/v glycerol; 1 mM DTT; 5% v/v DMSO; 1 mM dATP/dTTP; 10 μM dGTP. Reactions were incubated at 37 $^\circ\text{C}$ for 8 h, then quenched with addition of 400 μL STOP solution: 10 mM Tris-HCl (pH 7.5); 2 M NaCl; 1 mM EDTA; $\sim 5,000$ cpm 5'- α - ^{32}P -(CTAGACCTGTCATCA) $_2$ -biotin-3' (recovery/loading control). Solutions were incubated with 30 μL Dynabeads M-280 Streptavidin suspension (Thermo-Fisher, suspension used as is) with rotation overnight. Beads were recovered by Dyna-magnet and washed twice with 500 μL [10 mM Tris-HCl (pH 7.5); 2 M NaCl; 1 mM EDTA]; followed by 300 μL [10 mM Tris-HCl (pH 7.5); 1 mM EDTA]. Beads were suspended in 30 μL [9:1 v/v Formamide/TBE:D-biotin solution (5 mM in TE)]. Samples were denatured at 80 $^\circ\text{C}$ for 10 min, and the product solutions recovered on the Dyna-magnet. Products (6 μL) were electrophoresed over a 10% denaturing acrylamide/8 M urea gel at 75 Watts until bromophenol blue was $\sim 3/4$ down the gel (~ 50 min).

For Figure 3B, two 500 μL binding reactions were prepared, each containing a 25 μL aliquot of either **NU-1** or **2** (2 mM stock in DMSO = 100 μM final concentration) and ~ 10 nM telomerase in telomerase buffer. Immediately upon mixing ($t = 0$), and at $t = 10, 20, 40, 60, 120, 240$, and 360 min, 50 μL of each reaction was combined with 50 μL extension mix [2 mM MgCl_2 , 1 mM DTT, 2 mM dTTP, 2 mM dATP, 20 μM dGTP, ~ 5 μCi α - ^{32}P -dGTP (Perkin Elmer), and the DNA substrate 5'-biotin-

CTAGACCTGTCATCA(TTAGGG)₃-3' (2 μ M)]. All reactions were incubated at 37 °C overnight, and then processed as described above.

For Figure 3C, the control or treated cells were prepared and collected as pellets of 5×10^6 cells each at NW and pellets were shipped to CMRI on dry ice. Frozen cell pellets were thawed on wet ice and suspended in 1 mL telomerase buffer supplemented with 1 mM PMSF. Endogenous telomerase was immunopurified and assayed as described.^{6a}

Ionizing radiation-induced foci imaging

MCF7 or Saos2 cells were seeded on cover glass at 2.5×10^4 per well in 24 well plates and cultured overnight. Cells were treated either with DMSO, **1** (0.05 μ M), **NU-1** (0.05 μ M for MCF-7 cells, 2.5 μ M for Saos-2 cells), or **2** (0.05 μ M) for 2 h before 6 Gy irradiation. Non-irradiated cells but with the same treatment serves as a control. For immunocytochemical staining, cells were fixed with 4% PFA 24 h after irradiation and permeabilized with 10% Triton-X 100 for 10 min. After blocking with 5% BSA, primary antibodies for γ H2AX (Millipore, 05-636, 1:1000) or 53BP1 (Novus, NB100-304, 1:1000) were then incubated on cell slides overnight at 4° C. Following PBS washes, fluorescent secondary antibodies (Jackson ImmunoResearch) were applied for 1 h at room temperature. Cell slides were mounted with ProLong Gold (Thermo) after PBS washes. Foci images were captured on an Zeiss Axiovert 40CFL with a 40x/0.75 Plan-NeoFluar objective and pseudo-colored using ImageJ.⁷ Two or more replicates were performed.

SA- β Gal assay

Cells were seeded at 3×10^4 per well in 6 well plates and treated with inhibitors for 2 h prior to 6 Gy irradiation. Cells were fixed after 4 or 5 days and assayed for SA- β Gal activity as described.⁸ Images were captured on a Zeiss Axiovert 200M microscope with 20x/0.5 Plan-NeoFluar objective and AxioCam digital camera controlled by OpenLab software. SA- β Gal positive and negative cells were counted in mul-

multiple fields, yielding an average percentage indicated on each SA- β Gal image as mean $\pm$ SEM. Two or more replicates were performed.

Time-lapse live cell analysis

MCF7 or Saos2 cells were seeded in a 6 well plate at 3×10^4 cells per well and cultured overnight. Cells were then treated with inhibitors and/or irradiated with 6 Gy. The plates were then analyzed by time-lapse in an IncuCyteS3 (Sartorius) live cell imaging system. Phase contrast images were acquired at 20 X magnification with scanning every 4 h for 5 d. More than 25 non-overlapping fields were captured for each well. Quantitative analysis of cell confluency was performed using IncuCyteS3 2018 software.

RNA-seq Analysis

3×10^4 MCF-7 cells were seeded in a T25 flask, cultured overnight and treated with 0.5 μ M **NU-1** or DMSO for 48 h. Media was removed, cells were washed 2 times with PBS, followed by chemical dissociation with TrypLe (Thermo Fisher). Media was added to quench the TrypLe, the cell suspension was centrifuged at 2000 rpm for 5 min and pellets were shipped to Applied Biological Materials for RNA isolation and total RNA sequencing. RNA was isolated using Trizol (Thermo Fisher). The quality of the RNA extraction was assessed by gel electrophoresis. All the samples passed internal QC for library preparation. Sequencing libraries were prepared using Illumina TruSeq Stranded mRNA Library Preparation kit following manufacturer's recommendations. The quality of the libraries was assessed using Qubit DNA assay, Agilent Bioanalyzer and qPCR. All libraries passed internal QC. Sequencing was performed with an Illumina NextSeq system.

To carry out the transcriptomic study, pair-end reads were aligned with the hg38 human reference genome by Hisat2. The abundance of genes was quantified by Htseq-count. The differentially expressed genes (DEGs) between **NU-1** treated vs. control cells were performed with EdgeR in R studio, with dis-

persion value estimated as 0.01. Genes were considered significantly upregulated if they displayed fold change >1.5 and P value <0.1 and significantly downregulated if fold change <-1.5 and P value <0.1, comparing **NU-1**-treated and control cells. The DEG lists were subjected to Ingenuity Pathway Analysis (IPA, Qiagen Bioinformatics) to study the enriched pathways.

***In Vitro* Drug-Like Properties**

***In vitro* drug-like properties**
Human Plasma Stability ($t_{1/2}$): 41 min
Mouse Liver Microsomes ($t_{1/2}$): 31 min
N-acetyl cysteine kinetics ($t_{1/2}$): 42 min
logD: 2.8
CYP1A2 inhibition: 13% (@ 10 μ M)
PAMPA (pH 5.0, 6.2, 7.4, Log(Pe)): -3.6, -3.8, -3.7

CYP450 Inhibition Assay

Assay was conducted using the commercially available Vivid CYP1A2 Screening Kit (Thermo Fisher) according to the manufacturer's protocol. α -naphthoflavone and DMSO were used as a positive and negative control respectively based on the manufacturer's protocol.

Non-Cell-Based Assay for Drug Transport (PAMPA assay)

Assay was conducted using the commercially available parallel artificial membrane permeation assay (PAMPA, Millipore Sigma) according to the manufacturer's protocol.

Liver Microsomal Stability Assay

Assay was conducted using the Corning UltraPool HLM 150 Mixed Gender microsomal kit (Corning Life Sciences) according to the manufacturer's protocol.

N-Acetyl Cysteine and N-Acetyl Lysine Kinetic Studies

Assay was conducted as described with minor modifications.⁹ Briefly, a 1 mM stock solution of **NU-1** with internal standard (phenacetin) in DMSO was added to a 50 mM solution of either N-acetyl cysteine

or N-acetyl lysine in 67 mM phosphate buffer (pH 7.4), as well as added to a solution of 67 mM phosphate buffer (pH 7.4) control. Samples were taken every 2 min for 2 h and quenched immediately with MeOH and centrifuged upon sampling. Samples were placed on a Waters Acquity UPLC-MS system and measured for the ratio of **NU-1** to internal standard intensity to calculate the amount of **NU-1** present at each time point relative to the amount of **NU-1** at the first injection of the compound in buffer. Assay was conducted in triplicate.

Kinetic Solubility Measurements of **NU-1**

Solubility readings were taken on a Synergy HTX plate reader using a 384 well plate format. Total volume of solution 50 μ l with a final DMSO concentration at 1 % v/v in PBS. Solubility curve was established using concentration ranges from 1-500 μ M that were prepared by serial dilution of **NU-1** (50 mM in DMSO). Measurements were made in triplicate.

	Conc X	50X	DMSO	Stock solution	Final remaining volume
A	1	50	9	1 of C	10
B	5	250	9	1 of E	10
C	10	500	8	2 of E	9
D	25	1250	5	5 of E	10
E	50	2500	10	10 of F	12
F	100	5000	18	2 of 50 mM	10
G	250	12500	5	5 of H	10
H	500	25000	10	10 of 50 mM	10

	Conc X	50X	DMSO	Stock solu- tion	Final remaining vol- ume
A	1	50	49	1 of 50X	10
B	5	250	49	1 of 50X	10
C	10	500	49	1 of 50X	10
D	25	1250	49	1 of 50X	10
E	50	2500	49	1 of 50X	10
F	100	5000	49	1 of 50X	10
G	250	12500	49	1 of 50X	10
H	500	25000	49	1 of 50X	10

Supplemental Figures

Fig S1: TRAPeZe assay measurement of NU-1 across various telomerase positive cells

Fig S2: TRAPeze assay measurement of 2 across various telomerase positive cell lines

Fig S3: Cell Viability of 1, 2 and NU-1 across various telomerase positive cell lines

Fig S4: Cell Viability measurement of 1, 2 and NU-1 across various telomerase negative cell lines

Fig S5: Comparison of cell viability measurement of NU-1 against known telomerase inhibitors BIBR1532 and MST-312 in MCF-7 cells

Fig S6: Comparison of telomerase inhibition against known telomerase inhibitors BIBR1532 and MST-312 in MCF-7 cells 4 hours after compound washout. Cells were incubated with compound for 24 hours prior to compound washout.

Fig S7: Comparison of telomerase inhibition with NU-1 at various time-points post-compound washout in MCF-7 cells. Cells were incubated with NU-1 for 24 hours prior to compound washout.

Fig S8: Comparison of cell viability measurement with NU-1 at various time-points post-compound washout in MCF-7 cells. Cells were incubated with NU-1 for 24 hours prior to compound washout.

A) Co-administer Telomerase Inhibitor

B) Post-administer Telomerase Inhibitor

Fig S9: Comparison of cell viability measurement with various dosing of irinotecan against set doses of NU-1, BIBR1532 and MST-312 in MCF-7 cells. A) Telomerase inhibitor and irinotecan were co-dosed at the same time point. B) Irinotecan was dosed at $t = 0$ h, telomerase inhibitor was dosed at $t = 4$ h

A) Irinotecan Sensitization (A549)

Fig S10: Comparison of cell viability measurement with various dosing of irinotecan against set doses of NU-1 and 2 in A549 cells. DMSO was used as control.

A) Etoposide Sensitization (MCF-7)

B) Etoposide Sensitization (A549)

C) Etoposide Sensitization (SAOS-2)

Fig S11: Comparison of cell viability measurement with various dosing of etoposide against set doses of NU-1 and 2 in MCF-7, A549 and SAOS-2 cells. DMSO was used as control.

A) Paclitaxel Sensitization (MCF-7) **B) Paclitaxel Sensitization (A549)** **C) Paclitaxel Sensitization (SAOS-2)**

Fig S12: Comparison of cell viability measurement with various dosing of paclitaxel against set doses of NU-1 and 2 in MCF-7, A549 and SAOS-2 cells. DMSO was used as control.

A) Doxorubicin Sensitization (MCF-7)

B) Doxorubicin Sensitization (A549)

C) Doxorubicin Sensitization (SAOS-2)

Fig S13: Comparison of cell viability measurement with various dosing of doxorubicin against set doses of NU-1 and 2 in MCF-7, A549 and SAOS-2 cells. DMSO was used as control.

Fig S14: HPQDEIPYCGK Peptide of DMSO control treated tcTERT.

Fig S15: HPQDEIPYCGK Peptide of chrolactomycin (1) control treated tcTERT.

qe3_2017jul20_07_90i20_uc170718_rb2-2-#9792 RT: 23.25 A¹ : 6.31E4
T: FTMS + p NSI d Full ms2 710.3058@hcd28.00 [140.0000-1470.0000]

Fig S16: HPQDEIPYCGK Peptide of NU-1 control treated tcTERT.

Table S1. Most significantly altered pathways identified by IPA analysis of DEGS induced by treatment with **NU-1**

Ingenuity Canonical Pathways	-log(p-value)
Interferon Signaling	5.07
NER Pathway	4.46
Hereditary Breast Cancer Signaling	4.41
NRF2-mediated Oxidative Stress Response	4.24
Cell Cycle Control of Chromosomal Replication	4
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	3.95
Protein Ubiquitination Pathway	3.91
Triacylglycerol Biosynthesis	3.49
Role of BRCA1 in DNA Damage Response	3.45
Aldosterone Signaling in Epithelial Cells	3.43
Pancreatic Adenocarcinoma Signaling	3.35
Unfolded protein response	3.17
Pyrimidine Ribonucleotides De Novo Biosynthesis	3.12
Sirtuin Signaling Pathway	3.08
Adipogenesis Pathway	3.02

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