International Journal of Drug Development and Research

Sridhar et al., Int J Drug Dev & Res 2019, 11:4

10.36648/0975-9344.11.3.144

Research Article Open Access

Qualitative Analysis of the Biological Testing of Novel Telomerase Inhibitors

Suganthi Sridhar[*1], **Katie Uhl**[2], **Basma Khudur**[3], **Bikash Mishra**[3], **Robert Smart**[4] **and William Schroeder**[4]

[1]*Department of Biological Sciences, University of South Florida, St. Petersburg, FL, USA*

[2]*Department of Cell and Molecular Biology, School of Graduate Studies, Grand Valley State University, Allendale, MI, USA*

[3]*Department of Biomedical Sciences, College of Liberal Arts and Sciences, Grand Valley State University, Allendale, MI, USA*

[4]*Department of Chemistry, Grand Valley State University, Allendale, MI, USA*

[*]**Corresponding author:** Dr. Suganthi Sridhar, Associate Professor, Department of Biological Sciences, University of South Florida, 220 Davis Hall, 140 7th Ave S, St. Petersburg, FL 33701, USA, Tel: +1 (727)873-4776; E-mail: suganthi@mail.usf.edu

Received date: November 09, 2019; **Accepted date:** November 30, 2019; **Published date:** December 06, 2019

Abstract

Cancer is often referred to as being "immortal" because of high telomerase activity in these cells, which allows them to divide an infinite amount of times. BIBR-1532, (2-{[(2E)-3-(2Naphthyl)-2-butenoyl]amino}benzoic acid) is a known telomerase inhibitor that has undergone preclinical trials for the treatment of cancer. Based on the structure of BIBR-1532, three novel N- cinnamoyl substituted anthranilic acid derivatives (2-{[(2E)-3-(4 "X" phenyl)-2-propenoyl]amino}benzoic acids), similar to BIBR-1532 were synthesized. Their efficacies were tested against proliferative activity in metastatic prostate cancer cells, along with the known telomerase inhibitor BIBR-1532. Telomerase Repeat Amplification Protocol (TRAP) assays were performed on the compounds to confirm their anti-telomerase activity. The results showed all three newly synthesized cinnamic derivatives have antiproliferative qualities equivalent to or better than BIBR-1532 ($p=0.027$) and overall all compounds were significantly lower than the control ($p<0.0001$). TRAP assays further confirmed that the compounds also exhibited anti-telomerase activity like BIBR-1532. The newly synthesized cinnamic acid derivatives of BIBR- 1532 all showed antiproliferative and anti-telomerase activity. While these compounds were all tested against metastatic prostate cancer cells, these potential treatments have application in all types of cancers demonstrating high telomerase activity.

Keywords: Telomerase inhibitors; Cinnamic acid derivatives; BIBR-1532; Anthranilic acid derivatives and prostate cancer

Introduction

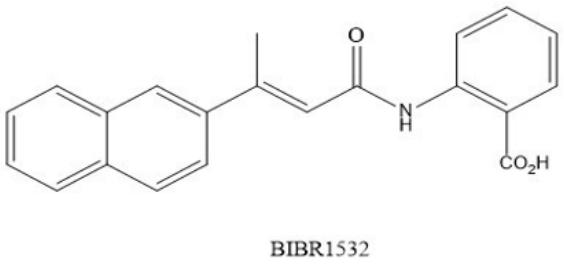
As of 2018, cancer is the second leading cause of death in the United States, second only to heart disease according to the American Cancer Society statistics. Excluding non-melanoma skin cancer, the four most prevalent cancers currently are lung, breast, prostate, and colorectal cancer. Among these, prostate cancer is the second leading cause of cancer deaths among men in the US and breast cancer in women. Based on calculations carried out by Rahib and colleagues, incidences of other cancers such as thyroid, melanoma, and uterine cancer are predicted to increase [1]. It is calculated predictions like these that stress the importance of clinical research. Research into the mechanisms involved in the conversion of normal cells into cancerous ones, continue to enhance the efficacy of treatments. Improvements in early detection and novel treatments have been linked directly to the decline in cancer-related deaths [2].

One of the trademark characteristics of cancerous cells is their ability to evade apoptosis signalling, with resultant immortality. One of the main mechanisms by which cancer cells achieve immortality is through activation or up-regulation of the telomerase enzyme. In normal cells, apart from stem and germ cells [3], telomerase expression is normally switched off soon after embryonic differentiation. During normal cell division, the non-coding segment of the DNA at the end of the chromosome known as the telomere begins to degrade or shorten. With each subsequent cell division, the telomere shortens until the cell reaches the Hay flick limit and undergoes apoptosis. By the activation or up-regulation of the telomerase enzyme, cancer cells are capable of replacing the telomere lost and thus divide indefinitely without undergoing apoptosis. Up-regulation or reactivation of telomerase is a critical feature identified in 90% of the cancers i.e., increased telomerase activity has been observed in 80-95% of a wide variety of cancers [3] including cancers of the oral cavity (75%), lung (80%), prostate (84%), breast (93%), colorectum (95%), bladder (98%) and neuroblastoma (94%) [4].

Telomerase is an enzyme that is a reverse transcriptase, creating complementary DNA using an RNA template. Telomerase is responsible for adding on DNA segments to the ends of chromosomes but is silent in almost all human tissues. Tissues found to naturally express active telomerase include germ line stem cells, [3,4] spermatocytes and somatic adult progenitor cells [5]. Even though human telomerase has not been crystallized, high- resolution crystal structures of model organisms such as Tetrahymena thermophila, provide much-needed insight into the intricate details of this unique enzyme [6,7]. The enzyme telomerase is made up of an RNA subunit, telomerase RNA, the telomerase reverse transcriptase (*TERT*), along with other accessory proteins [6,7]. Telomerase is responsible for adding the repeated 'TTAGGG' segments onto the ends of chromosomes, protecting the chromosomal coding DNA of the chromosome from becoming degraded during cellular division. Evidence from previous research studies has revealed that the *TERT* subunit is the catalytic subunit of the telomerase enzyme [7]. The *TERT* domain is organized into three structural components: a short C-terminal portion, a catalytic RT domain, and a long N-terminal extension that houses the nucleic acid binding domains. Perhaps one of the more notable characteristics of the structure of telomerase is the fact that it can be construed to resemble a human hand, containing a 'palm', 'thumb', and 'fingers'. These anatomical terms can be used to

refer to the three main structural elements that have been found to make up the telomerase enzyme. Recent evidence indicates that an increase in telomerase activity is due to up-regulation of the normally silent human *TERT* gene (*hTERT*) in cancer cells [8]. Inhibition of telomerase in a wide range of cell lines has resulted in the shortening of telomeres causing subsequent growth arrest, and senescence [9,10]. Telomerase inhibition, therefore, provides a promising novel approach to cancer treatment.

Multiple approaches in cancer therapeutics targeting telomerase and its mechanisms including vaccines, small molecule inhibitors, oligonucleotides and G-quadruplex approaches are being tested currently. A variety of small inhibitor molecules against telomerase have been tested with different success, amongst which BIBR-1532 emerged to be more effective [3]. BIBR-1532, also known as (2-{[(2E)-3-(2-Naphthyl)-2-butenoyl]amino}benzoic acid) (Figure 1) is a small anthranilic acid inhibitor molecule and the first effective telomerase inhibitor, shown in pre-clinical trials. As a mixed type, non-competitive inhibitor of *TERT* and hTR [3], BIBR-1532 acts independently of the telomerase active site, preventing DNA from binding to telomerase and preventing the telomere strand from elongating past the 5' end of a strand of DNA [10]. If the strand of the DNA is not elongated, the telomeres remain the same size and are degraded normally. Although positive results have been observed with BIBR-1532 in preclinical studies on breast, prostate and fibrosarcoma cancer cell lines, no further progress or entrance in clinical trials has been shown with BIBR-1532 [11].



BIBR1532

Figure 1: Patented structure of the telomerase inhibitor, BIBR-1532.

The critical structures important for telomerase activity are labelled. Without the carboxylic acid, aromatic ring, and conjugated amine group, the compound will not demonstrate anti telomerase activity.

Research on telomerase inhibitors such as BIBR-1532 has shown that no matter the overall structure of the compound, there are three substructures that must be present in order for it to act as a telomerase inhibitor: A carboxylic acid, connected to an aromatic ring, and a conjugated amine group. Based on this information, it was imperative for the synthesized analogues to mirror the structures in order for it to inhibit telomerase activity. This research aimed to synthesize novel telomerase inhibitors that are derivatives of cinnamic acids. Cinnamic acids have a long history of cancer treatment, being effective constituents of ginseng, a compound valued by traditional Chinese medicine for promoting health and preventing disease [12]. Cinnamic acids are also found in other compounds that have been found to have anti-cancer and anti-inflammatory properties, such as green tea, coffee, and citrus fruits [13]. Cinnamic acids have been shown to be less toxic than other synthetic pharmaceuticals currently on the market. In the treatment of melanoma, cinnamic acid is believed to induce apoptosis, or controlled cell death. Cinnamic acids also have been shown to inhibit certain enzymes, including telomerase [13]. It was because of the anti-telomerase enzymatic properties that cinnamic acid was chosen as the base compound for the synthesized telomerase inhibitors. The natural origins of cinnamic acid led to the hypothesis that the novel inhibitors would demonstrate less cytotoxic effects on healthy tissues compared to BIBR1532 alone. However, only clinical trials will be able to confirm this, which is beyond the scope of this particular research. A series of cinnamic acid analogues were synthesized [14] to mirror the anthranilic acid derivative structure of BIBR-1532 as shown in Figure 2.

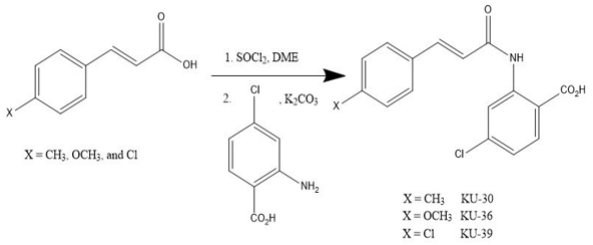


Figure 2: Synthesis of cinnamic-anthranilic derivatives, the portion marked X was replaced by a methyl- (KU-30), chloro- (KU-39), or methoxy- (KU-36) group via organic synthesis.

It was hypothesized that a change in the electronegativity of the substitution on the cinnamic acid would lead to an increase in the anti-telomeric activity demonstrated by BIBR-1532. A total of three cinnamic acid analogues (KU-30, KU-36 and KU-39) were synthesized, purified, and then tested for anti-cancer and anti-telomeric properties.

Prostate, breast, bladder, lung and colorectal cancers have all been shown to have abnormally high levels of telomerase than other forms of cancer [4]. These are also among the most common kinds of cancer to occur in the American population, which makes this research extremely timely and relevant. Prostate cancer is the second leading cause of cancer death among men in the US and high telomerase activity has been observed in 85% of prostate cancers [4]. PC3 metastatic prostate cells were chosen to test these compounds in our lab as these cell lines have been shown to exhibit high telomerase activity [15]. The newly synthesized compounds along with BIBR-1532 were tested for their efficacy in metastatic PC3 prostate cell line for anti-proliferative and further for anti-telomerase activity.

Materials and Methods

Synthesis of cinnamic acid derivatives

4-chloroanthranilic acid, 4-chlorocinnanmic acid, 4-methyl cinnamic acid, and 4methoxycinnamic acid were purchased from the Sigma-Aldrich and used with further purification. Nuclear Magnetic Resonance Spectra were obtained with a Varian Unity Inova 400 MHz spectrometer.

Synthesis of N-(4-chlorocinnamoyl) -4-chloroanthranilic acid (KU-39): To a mixture of 4-chlorocinnanmic acid (0.01M) and thionyl

chloride (5 mL) were added 2 drops of dimethylformamide. A vigorous reaction ensued, and the acid dissolved. After one hour of stirring, the excess thionyl chloride was moved under pressure on a Rotovape resulting acid chloride was dissolved in 1,2-dimethoxyethane (15 mL) and the resulting solution was added drop wise, over a 5 minute period, to a well-stirred mixture of 4chloroanthranilic acid (0.01M), 1,2 dimethoxyethane (20 mL), and 25% potassium carbonate solution (25 mL) at 5°C. The mixture was allowed to warm to room temperature and stirred for an additional 2 hours. Addition of water (30 mL) and excess 6N HCl gave a precipitate of the product N-(4-chlorocinnamoyl) -4-chloroanthranilic acid which was filtered off, washed with water, and recrystallized with aqueous isopropanol.

Yield 78%, Melting Point 260-261°C, 1HNMR (400 MHz, DMSO-d6): 11.5 (s, 1 H), 8.65 (d, J=2.15 Hz, 1 H), 7.95 (d, J=8.6 Hz, 1 H), 7.7 (d, J=8.6 Hz, 2 H), 7.5 (d, 15.8 Hz, 1 H), 7.4 (d, J=8.6 Hz, 2 H), 7.2 (dd, J=2.15 & 8.6 Hz, 1 H), 6.8 (d, J=15.6 Hz, 1 H), and 2.3 (s, 1H).

Synthesis of N-(4-methylcinnamoyl) -4-chloroanthranilic acid (KU-30): N-(4-methylcinnamoyl)-4-chloroanthranilic acid as prepared in the same fashion as N-(4chlorocinnamoyl)-4-chloroanthranilic acid using 4-methylcinnanmic acid (0.01M) and 4chloroanthranilic acid (0.01M).

Yield 80%, Melting Point 226-227°C, 1HNMR (400 MHz, DMSO-d6): 11.4 (s, 1 H), 8.67 (d, J=2.15 Hz, 1 H), 7.95 (d, J=8.6 Hz, 1 H), 7.55 (d, J=8.6 Hz, 2 H), 7.5 (d, 15.8 Hz, 1 H), 7.2 (d, J=8.6 Hz, 2 H), 7.19 (dd, J=2.15 & 8.6 Hz, 1 H), 6.7 (d, J=15.8 Hz, 1 H), 2.4 (s, 1 H) and 2.2 (s, 3H).

Synthesis of N-(4-methoxycinnamoyl)-4-chloroanthranilic acid (KU-36): N-(4-methoxycinnamoyl)-4-chloroanthranilic acid as prepared in the same fashion as N-(4chlorocinnamoyl)-4-chloroanthranilic acid using 4-methoxycinnanmic acid (0.01M) and 4chloroanthranilic acid (0.01 M).

Yield 73%, Melting Point 217-219°C, 1 HNMR (400 MHz, DMSO-d6): 11.4 (s, 1 H), 8.7 (d, J=2.15 Hz, 1H), 7.9 (d, J=8.6 Hz, 1H), 7.6 (d, J=8.6 Hz, 2H),7.5 (d, 16.4 Hz, 1 H), 7.2 (dd, J=2.15 & 8.6Hz, 1H), 6.9 (d, J=8.6 Hz, 2H), 6.6 (d, J=16.4 Hz, 1H), 3.8 (s, 3H) and 2.3 (s, 1H).

All compounds were dissolved in dimethyl sulfoxide (DMSO) and a stock solution was made at 1 mM concentration and was further used in the cell proliferation assay.

Cell proliferation assay

PC3 cells obtained from ATCC (American Type Culture Collection, ATCC) were grown in F12K culture media, supplemented with 10% FBS (Invitrogen) and grown in an incubator at 370C with 5% CO_2 and unused cultures were frozen and stored at -80°C in liquid nitrogen until further use. PC3 cells were maintained in culture on plates with F12K culture media (supplemented with 10% FBS) at 37°C with 5% CO_2 until they reached confluency. The cells were tested from time to time for mycoplasma (mycoplasma detection kit, Thermo Fisher Scientific) while in culture. Right before the assay, the cells were prepped for the assay as follows: the media in the plate was removed, washed twice with phosphate buffered saline solution (PBS, pH7.4) and trypsinized with 0.05 M trypsin/EDTA for 2 minutes in the incubator. Once the cells were non-adherent and floating, the cells were pooled in a centrifuge tube and subject to centrifugation at 1000 rpm for 5 minutes. The cells were then resuspended in the F12K medium and counted using a Beckman Coulter™ SZ Particle Counter and/or a cell counter in order to estimate the number of cells/ml. Once the cell number was determined, approximately 25,000 cells were added to each well of a sterile six-well plate. The plate was incubated for 24 hours in the incubator for the cells to adhere. After 24 hours the drugs were added to the plates in 50 μM, 75 μM, and 100 μM concentrations, respectively. A control well with the same volume of DMSO (dimethyl sulfoxide) was maintained as a negative control. The cells were incubated with DMSO, BIBR1532 and the cinnamic-derivatives (KU-30, KU-36 and KU-39) for 48 hours at 37°C (with 5% CO_2) after which time the cells from each sample were pooled individually, and the number of live cells was counted using the following protocol. The cells treated with the drugs were removed from the incubator after 48 hours, trypsinized and prepared for counting to the point of centrifugation, as described above. Soon after the centrifugation, the supernatant was removed via aspiration and the cell precipitate re-suspended in 1ml media and mixed thoroughly. A small volume of Trypan Blue (1/100 dilution) was then mixed with the cell suspension. The number of live cells (without trypan blue) from each sample was counted in triplicates using a hemocytometer and/ or using a Beckman Coulter™ SZ particle cell counter (without the trypan blue dilution). The cell numbers, both for the negative control (DMSO) and the positive control (BIBR-1532) and the cinnamic derivatives (KU-30, KU-36 & KU39), were counted in order to determine the quantitative effect each drug had on the PC3 prostate cell proliferation. Once the optimum concentration of the drug was determined (75 μM), the drugs assays were repeated with BIBR-1532 and the cinnamic derivatives (KU30, KU-36, & KU-39) at 75 μM concentration, along with the DMSO control.

To compare the anti-cancer activity of the cinnamic derivatives against BIBR-1532 for significance, statistical tests were done using JMP Statistical Analysis Software (SAS). Data from a total of seven trials with 75 μM concentration of all the compounds against the control (DMSO) were compared first and then data from BIBR1532 was compared against the cinnamic derivatives for their significance. Since the cell count data distribution was skewed, we used Wilcoxon nonparametric ANOVA tests to compare the efficacy of the control to drug treatments and then BIBR1532 (parent compound) to cinnamic derivatives (new drugs), using the JMP software.

Telomerase repeat amplification protocol (TRAP) assay

In order to test for evidence of telomerase inhibition, a TRAP assay kit i.e., TRAPeze® Telomerase Detection Kit from EMD Millipore was used. The assay was carried out according to the kit's protocol, using lysates from cells collected from the drug assays. One set of the sample was used for the assay without any treatment while a second set of the sample was heat treated at temp 85°C for approximately 30 minutes to inhibit the telomerase activity. Both sets of samples were subject to PCR amplification with the parameters described in the TRAP assay kit. The resulting sample was then run through a 12.5% denaturing SDS gel. The gel was further stained with ethidium bromide and visualized under a UV light source using a UVP EpiChemi3 Darkroom UV –transilluminator and captured via a Hamamatsu camera attached to it.

Results quantitative analysis of anti-cancer properties of the BIBR derivatives

The data on the effect of BIBR-1532 and the cinnamic derivatives (KU-30, KU-36, KU-39) was collected by counting the number of live cells left after the cells had been exposed to the compounds for a full 48 hours of incubation at 37°C with 5% CO_2. The initial set of drug assays

were done to determine the optimum concentration of the drug. Once the optimum concentration was determined, the second set of drug assays was performed to observe proliferative activity. The optimum concentration/dosage of the drugs for the proliferation assay was accomplished by comparing the live cells remaining after drug treatment dosages of 50μM, 75μM, and 100μM respectively. The drug concentration range was determined based on BIBR1532 concentrations, previously described in several BIBR-1532 based drug studies [10,11,16]. PC3-parental cells were plated with the above 3 concentrations of the drug yielded the results as shown in Figure 3. In Figure 3, at 50 μM drug concentration all compounds showed a decrease in proliferative activity, with the number of live cells with DMSO control being 50,000, compared to 20,000 for BIBR1532, 42,500 for KU-30, 12,500 for KU-36 and 7,500 for KU-36 treatment respectively. The 100 μM concentration treatment on the other hand led to treated cells displaying apoptotic qualities.

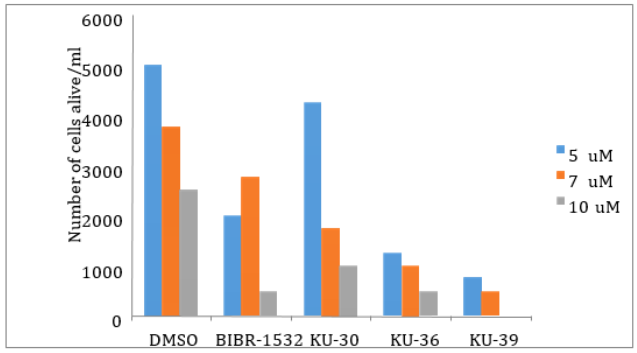


Figure 3: A sample data of the drug assay with different telomerase inhibitors with three different drug concentrations.

BIBR-1532 and the anthranilic derivatives along with a DMSO control was used in the drug cell assay. Three different concentrations of the drug (50 μM,75 μM and100 μM) were used for the drug assay. After 48 hrs. of treatment, the live cells remaining were counted in triplicates and the average count was plotted against the respective drug treatment.

Even though concentration of 100μM was found to be extremely effective in its anti-cancer activity, there were not enough viable cells left behind for a telomerase assay to be carried out (Figure 3) i.e., the live cell counts were 25,000 for DMSO control compared to 5000 for BIBR, 10,000 for KU-30, 5000 for KU-36 and with no live cells left upon KU-39 treatment. Since the treatment with 50 μM was not powerful enough but at the same time 100 μM mostly destroyed all the cells, a 75 μM concentration was used in the subsequent drug assays (Figure 4) i.e., a concentration that was effective in arresting cell proliferation but would allow at the same time a TRAP assay to be carried out on the treated cells. At 75 μM treatment, BIBR-1532 left 27,500 live cells, KU-30 with 17,500 KU-36 with 10,000 and KU-39 with 5000 live cells respectively (Figure 3), compared to the control DMSO treatment with 37,500 live cells.

DMSO treated cells served as a negative control, demonstrating the number of cells that would have survived the incubation period had the compounds not been added to the media. Compared to the control, BIBR-1532 and all three experimental compounds demonstrated anti-cancer properties, as seen by the decrease in the percentage of live cells remaining (Figure 4) i.e., Using BIBR-1532 as a positive control, it is evident that all the experimental compounds show anti-cancer properties that were more significant or equivalent to BIBR1532. At a concentration of 75 μM, KU-36 and KU-39 were more effective at terminating PC3-parental cells than BIBR-1532. BIBR-1532 had an average of a 46.7% decrease in the total live cell population, while KU-30 demonstrated a 53.3% decrease, KU-36 a 67.8% decrease, and KU-39 marginally better, showing a 70.4% decrease in respectively after the 48-hour incubation period compared to the DMSO treated control (Figure 5).

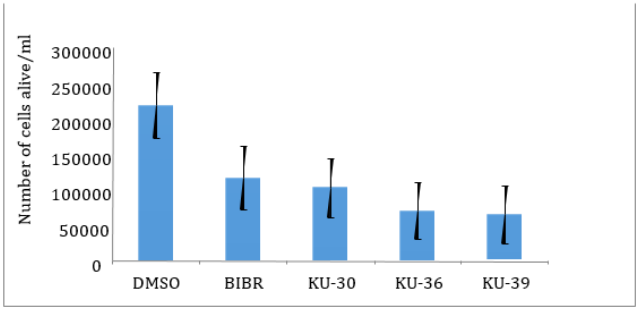


Figure 4: A comparative drug assay of different telomerase inhibitors using the optimum drug concentration (75 μM).

The data expressed here is a compilation of data from seven drug trials with BIBR-1532 and the cinnamic acid derivatives (KU-30, KU-36 and KU-39) with a control DMSO. Live cells remaining after 48 hours of drug treatment were counted in triplicates after each drug trial and the average cell counts from these drug trials with the standard deviation values are plotted against their respective treatments.

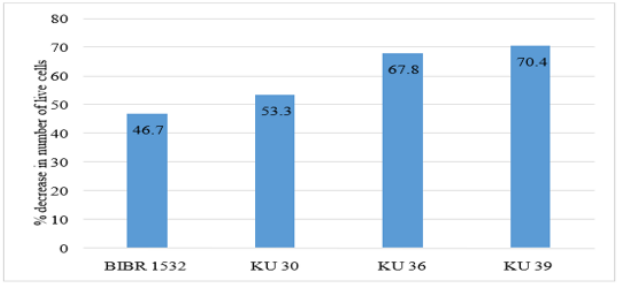


Figure 5: Compiled percentage decrease in the number of live cells obtained from the drug study.

The percentage decrease in the average number of live cells from the different drug treatments compared to control DMSO from Figure 4 were compiled and presented in this figure.

Overall, the cinnamic derivatives had less live cells compared to BIBR-1532 and performed better than the parent compound BIBR-1532. The data was subject to statistical analysis to determine if the anti-proliferative activities of BIBR-1532 and the cinnamic derivatives were statistically significant than the control sample and if they indeed performed better than BIBR1532. It should be noted however that the presence of anti-proliferative activity is not an

indicator of telomerase inhibition. Further experimentation (TRAP assay) was done in order to confirm that the results seen in the drug assay were due to telomerase inhibition, and not by a different mechanism. Statistical analysis to evaluate the anti-proliferative activity of BIBR1532, KU-30, KU36, and KU-39 Statistical analysis was performed comparing live cell count from control treatment with all the inhibitors (including BIBR1532) and then comparisons were made on the live cell count between BIBR1532 against KU-30, KU-36, and KU-39. The results are summarized in Figure 6. The live cell count for the experimental compounds overall was significantly lower than those for the control [Figure 6a; Wilcoxon: $\chi 2=20.66$; $p=0.0004$]. Moreover, cell counts for the novel compounds KU-30, KU-36 and KU-39 were significantly lower than for BIBR-1532 (Figure 6b; Wilcoxon: $\chi^2=4.87$; $p=0.027$). When analyzed all together, cell counts for all compounds, BIBR-1532, KU-30, KU-36, and KU-39 overall were significantly lower than for the control (Figure 6c; Wilcoxon: $\chi^2=14.59$; $p<0.0001$).

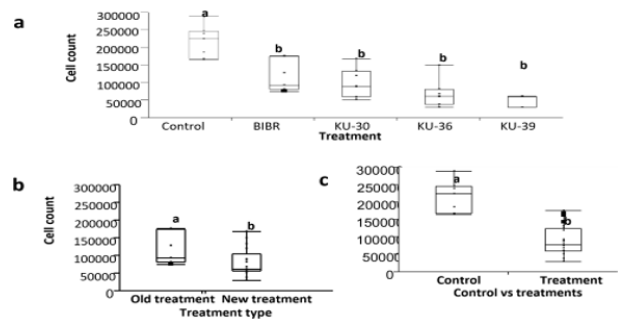


Figure 6: Statistical analysis where live cell counts is compared by treatments.

(a) Control (DMSO-a) treatment compared to all drug treatments (BIBR-1532, KU-30, KU-36, KU-39 -b) (Figure 6a; Wilcoxon: $\chi^2=20.66$; $p=0.0004$) (b) Comparison of BIBR-1532 (old treatment- a) with the new anthranilic derivatives (new treatment -b) combined (Figure 6b; Wilcoxon: $\chi^2=4.87$; $p=0.027$) (c) Combined treatments of the telomerase inhibitors (BIBR-1532, KU-30, KU-36 & KU-39-treatments-b) were compared to the control DMSO (a) (Figure 6c; Wilcoxon: $\chi^2=14.59$; $p<0.0001$).

Analysis of telomerase inhibition

Once the presence of anti-proliferative property was confirmed with the compounds, the compounds were then further tested for their ability to inhibit telomerase. A TRAPeze® Telomerase Detection Kit was used to analyze the drug-treated cell lysates for telomerase activity. The TRAPeze® Telomerase Detection Kit contains a set of primers that are specific for telomeres and a PCR assay was run with the drug-treated lysates along with the control (DMSO treated) sample to determine if telomerase activity is still present in these drugs treated cell lysates. Using the appropriate buffers, the telomeric DNA generated from the PCR run was isolated and separated using a 12.5% denaturing SDS gel. The result from the telomerase assay is summarized in the gel picture in Figure 7.

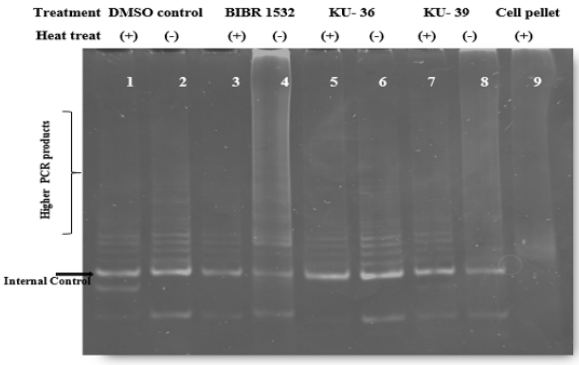


Figure 7: Evidence of telomerase inhibition demonstrated by the novel compounds.

PC3 prostate tumor cells subject to 48 hours of drug treatment were used for the TRAP assay. Trap assay was performed on the cells with and without heat treatment. The RT-PCR products formed were then run on a 12.5% SDS gel and picture taken after treatment of the gel with ethidium bromide. The samples were loaded on the gel as follows Lane 1: DMSO control, heat treated (+), Lane 2: DMSO control, no heat (-) Lane 3: BIBR-1532, heat treated (+), Lane 4: BIBR-1532, no heat (-), Lane 5: KU-36 heat treated (+), Lane 6: KU-36, no heat (-), Lane 7: KU-39 heat treated (+), Lane 8: KU-39, no heat (-), Lane 9: control cell pellet.

Each sample was run with the corresponding heat-treated samples in the lane before and used as negative controls against the normal cell pellets collected from the drug assay. Lane 1 & 2 contained control samples (DMSO) with no drug treatment, while Lane 3 & 4 were BIBR-1532 treated samples, followed by PC3 cell pellets treated with KU-36 (Lanes 5 & 6), and cell pellets treated with KU-39 (Lanes 7 & 8). Lane 9 serves as a positive control, with a slight band. Review of other articles that have cited this particular TRAPeze kit has also noted that the positive control band rarely presents itself as the clearly defined band that is typically desired. KU-30 treated cells were not included in the TRAP assays as it did not perform substantially better than BIBR1532 in our proliferative assays. All samples show a strong band corresponding to the size of the control cell pellet (Figure 7, internal control band with arrows) demonstrating that the PCR was carried out to completion. Lane 3 contains the results for BIBR-1532, which serves as a positive control. When compared to the negative controls (lane 1 & 2), each experimental sample, including BIBR-1532, showed evidence of telomeric DNA that is shorter than the typical, unmodified telomeres. This indicates that the novel anthranilic derivative compounds (KU-36, and KU-39) are indeed functioning as telomerase inhibitors, like that of BIBR-1532.

Discussion

Cancer is the second leading cause of death after heart disease in the United States. Several different mechanisms are involved in the transformation of a normal cell to cancer cell. Among all these mechanisms the predominant one that imparts immortality to cancer cells is the activation of the enzyme telomerase. Increased activity of telomerase has been identified in more than 80-95% of the cancers and is high predominantly in prostate, breast, lung and colorectal cancers.

Prostate cancer has been proven to demonstrate high levels of telomerase activity; in particular, the tumor-initiating cells found in prostate cancer are ideal targets for novel telomerase inhibitors [16]. These inhibitors are designed to interfere with telomerase activity and with telomerase activity halted, cancer cells are no longer immortal and are limited in the number of cell divisions before apoptosis endures.

BIBR-1532 has been shown to be an active telomerase inhibitor, preventing the enzyme from functioning via non-competitive inhibition and has undergone preclinical trials, but to enter clinical trials. Since it has not yet entered clinical trials due to issues with bioavailability [17,18], there is an increased need to develop new anti-telomerase compounds for cancer therapy. It was therefore the goal of this experiment to design novel compounds that are telomerase inhibitors, and are better than BIBR1532, the parent compound. The compounds synthesized in our lab (KU-30, KU-36 and KU-39) are derivatives of cinnamic acid that occurs naturally. Cinnamic acid is found commonly in green tea, coffee, citrus fruits and ginseng and is known for its anti-cancer and anti-inflammatory properties, along with anti-telomerase activity. Our compounds when tested on prostate metastatic cell lines with high telomerase activity (PC3), exhibited extensive anti-proliferative properties. The anti-proliferative properties exhibited by these compounds were similar (KU-30) or significantly even more effective (KU-36, KU-39) than the parent compound BIBR-1532. i.e., BIBR-1532 had an average of a 46.7% decrease in the total live cell population, while KU-30 demonstrated a 53.3% decrease, KU-36 a 67.8% decrease, and KU-39 marginally better, showing a 70.4% decrease in respectively after the 48-hour incubation period compared to the DMSO treated control (Figure 5). TRAP assays further confirmed that our compounds (KU-36, KU-39) exhibited anti-telomerase activity similar to BIBR1532. Even though our compounds exhibited anti-proliferative and anti-telomerase activities similar to BIBR-1532 in prostate cancer cells, they have not been tested in other cancer cells that exhibit high telomerase activity. In addition, it is not clear exactly where these compounds bind in the telomerase molecule. Since they are anthranilic derivatives and contain all three sub-structures similar to BIBR-1532, needed for telomerase inhibition we can assume that our compounds are also binding in a similar fashion as BIBR-1532. We predict our novel compounds also to be non-competitive inhibitors of *TERT* and hTR, like BIBR-1532 and may act independently of the telomerase active site, preventing DNA from binding to telomerase thus preventing telomere strand elongation. All these possibilities and the exact binding regions where these compounds may possibly bind are currently being explored in our lab through bioinformatics techniques.

Conclusion

The three novel cinnamic acid derivatives we synthesized have proven anti proliferative and anti-telomerase activity and have the potential to become therapeutic agents to treat cancer. The effects demonstrated by these drugs on proliferation and telomerase inhibition is similar or even better than BIBR-1532, a known telomerase inhibitor. The natural origins of cinnamic acid leads us to believe that the novel inhibitors we generated would demonstrate less cytotoxic effects on healthy tissues compared to BIBR1532 and may offer a more organic and a better approach to BIBR1532 in telomerase inhibition and cancer treatment.

Acknowledgment

We would like to thank Dr. Deby Cassill, professor from University of South Florida, St. Petersburg for her expertise and help in the statistical analysis of our data.

Availability of Data and Materials

The datasets generated during and/or analysed during the study are available from the corresponding author on request.

Conflict of Interests

The authors declare no competing interests

References

1. Rahib L, Smith BD, Aizenberg R, Rosenweig A, Fleshman JM, et al. (2014) Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Research 74(11): 29132924.
2. Edwards BK, Noone AM, Mariotto AB, Simard EP, Boscoe FP, et al. (2014) Annual Report to the Nation on the status of cancer, 19752010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast, or prostate cancer. Cancer 20: 1290-1314.
3. Gomez DL, Armando RG, Cerrudos CS, Ghiringhelli PD, Gomez DE (2016) Telomerase as a cancer target- development of new molecules. Current topics in medicinal chemistry 16: 2432-2440.
4. Cassandra DB, Thomas RY, Patricia ML, Catherine AR (1997) Telomerase activity: A marker of cell proliferation non-malignant transformation. PNAS 94: 1367713682.
5. Shay JW, Wright WE (2010) Telomeres and telomerase in normal and cancer stem cells. FEBS Letters 584: 3819-3825.
6. Sandin S, Rhodes D (2014) Telomerase structure. Current Opinion in Structural Biology 25: 104-110.
7. Wyatt HDM, West, SC, Beattie TL (2010) InTERTpreting telomerase structure and function. Nucleic Acids Research 38: 5609-5622.
8. Jafri MA, Ansari SA, Alqahtani MH, Shay JW (2016) Role of telomeres and telomerase in cancer and advances in cancer-related therapies. Genome Medicine 8: 69-87.
9. Puri N and Girard J (2013) Novel therapeutics targeting telomerase and telomeres. J Cancer Sci Ther 5: 1-3.
10. Emanuelle P, Wenz C, Linger J, Hauel N, Priepke H, et al. (2002) Mechanism of Human Telomerase Inhibition by BIBR-1532, a Synthetic, Non-nucleosidic Drug Candidate. Journal of Biological Chemistry 277: 15566-15572.
11. Helder MN, Wisman GB, Van der Zee GJ (2002) Telomerase and telomeres: From basic biology to cancer treatment. Cancer Invest 20: 82-101.
12. De P, Baltas M, Bedos-Belval F (2011) Cinnamic acid derivatives as anticancer agents-A review. Current Medicinal Chemistry18: 1672-1703.
13. Evandro N, Machado-Santelli G (2013) Cinnamic acid induces apoptotic cell death and cytoskeleton disruption in human melanoma cells. Journal of Experimental and Clinical Cancer Research 32: 1-14.
14. Stabile RG, Dicks AP (2004) Two-step semi-microscale preparation of a cinnamate ester sunscreen analog. Journal of Chemical Education 18: 1488-1491.
15. Nayaka JS, Kakara R, Chaudharia UK, Joshib D, Vundintib BR, et al. (2016) A triad of telomerase, androgen receptor and early growth response in prostate cancer cells. Cancer biology and therapy 17: 439-448.
16. Marian CO, Wright WE, Shay JW (2010) The effects of telomerase inhibition on prostate tumor-initiating cells. International Journal of Cancer 127: 321-331.

17. El-Daly H, Kull M, Zimmermann S, Pantic M, Waller CF, et al. (2005) Selective cytotoxicity and telomere damage in leukemia cells using the telomerase inhibitor BIBR-1532. Blood 105: 1742-1749.

18. Wong HL, Unciti- Broceta A, Spitzer M, White, A, Tyers M, et al. (2013) Yeast chemical genetic screen identifies inhibitors of human telomerase. Chemistry and Biology 20: 333-340.