Design, synthesis and characterisation of a novel anticancer prodrug having antiproliferative activity against prostrate tumour

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A curcumin glycine conjugate viz. 1,7-bis (4-O-glycinoyl-3-methoxyphenyl)-1,6-heptadiene- 3, 5 dione 1 previously shown by this lab to be having antioxidant, antibacterial and antifungal activity is synthesized and after attachment of a linker (-CH₂-CH₂-OH) 2 is phosphitylated. The phosphitylated unit 3 was covalently linked to 5'-GT TAG GGT TAG-3' 4, a complementary sequence of 5'-CU AAC CCU AAC-3', which is the repeat sequence of telomerase RNA template. The comparative melting temperatures (Tm) of modified and normal duplexes have indicated more thermal stability of duplex with bioconjugate. The biodegradable nature of the covalent bond of the prodrug has now been proved by carrying out enzymatic digestion studies. The prostrate tumour derived DU145 cells are transfected with 5'-O-curcumin DNA and study of antiproliferative activity indicated 80% reduction in cell proliferation by the end of 75 days.

Keywords: Telomerase, antiproliferative, pro-drug , curcumin-glycine-deoxyoligonucleotide conjugate

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Human telomeres are composed of simple double-stranded DNA sequence TTAGGG1, repeated over thousands of base pairs and are shortened by 50-200 bps with each cell division due to end-replication problem,2 leading to Phase III or MI stage3. Telomerase is a multi subunit ribo-nucleoprotein complex which includes a 445-base long human telomerase RNA component hTR and a reverse transcriptase subunit hTERT 4,5. The RNA component hTR contains a domain that is complementary to one hexameric unit of the DNA telomeric repeat sequence TTAGGG. The protein moiety contains the cloned catalytic subunit (human telomerase reverse transcriptase, hTERT in humans) that is homologous to reverse transcriptases.

Thus, this enzyme complex comprises both template and polymerase activity. Telomerase binds to the 3'-ends of DNA strands and extends them by copying its own RNA template in multiples of the hexamer repeat sequence6. Telomerase activity is expressed in more than 85% cancer cells but not in most normal and germline cells, suggesting that telomerase may be an important target for chemotherapy7-9. Recent literature shows use of oligonucleotides especially antisense oligos10,11 as telomerase inhibitors, mainly directed towards the template region of hTR. The RNA template contains 11-mer oligonucleotide (CUAACCCUAAC) that complements the 3’-5’ human telomere sequence. The current communication is an attempt to use antisense methodology12-14 along with, prodrug concept15 to target telomerase. Inhibition of telomerase by phosphorothioate-modified DNA16-18, however, has poor sequence selectivity, presumably because of interactions between the phosphorothioate backbone and the telomerase reverse transcriptase domain. Therefore, in the present communication, it is preferred to use unmodified backbone and in order to improve its cellular uptake bioconjugate of curcumin with glycine has been prepared so that the lipophilicity of the oligo may be enhanced and the prodrug could be a better target (targeted delivery). The present attempt is to develop an inhibitor, which can select the infected (hypoxic) cells among the normal somatic cells, so that its toxicity is minimized and it could be used as specific armour against cancerous cells. The targeting of telomerase with a prodrug molecule resulting in antiproliferation, has been tested in vivo during the present investigation.
Curcumin, the yellow pigment from rhizome of *Curcuma longa* Linn, has a wide spectrum of therapeutic applications as antibacterial, antifungal, antioxidant, anti-proliferative capability, etc. and is thus identified as a potent tool in cancer therapy\textsuperscript{19-27}. Curcumin has been shown to inhibit the proliferation of a wide variety of tumour cells, including B cell and T cell leukemia\textsuperscript{28-31}, colon carcinoma\textsuperscript{32} and epidermoid carcinoma cells\textsuperscript{33}. It has also been shown to suppress the proliferation of various breast carcinoma cell lines in culture\textsuperscript{34-36}. Zheng \textit{et al.} explored the apoptosis-inducing effects of curcumin in human ovarian tumour A2780 cells\textsuperscript{37}. We have noticed that with increased cellular uptake of its conjugate obtained by attaching glycine to its two phenolic groups, these properties are enhanced multifold\textsuperscript{38}. Thus, the present effort is to develop an anticancer drug based on the two major therapeutic approaches i.e. antisense and prodrug concept.

**Results and Discussion**

As shown in Scheme 1, 1,7-bis (4-O-glycinoyl-3-methoxyphenyl)-1,6-heptadiene-3, 5-dione 1 was prepared by esterification of curcumin with two moles of glycine followed by reaction with DMAP/Pyridine and EtOAc.

![Scheme 1](image-url)
of N-protected glycinoyl chloride. The compound \(1\) was treated with unimolar proportion of ethylene chlorohydrin to get \(1\)-(4-\(\text{O}-\text{glycinoyl-N-}\beta\text{-hydroxyethyl-3-methoxyphenyl})-7(4\text{-O-glycinoyl-3-methoxyphenyl})-1, 6\)-heptadiene-3, 5-dione \(2\). The phosphoramidite of \(2\) was prepared by using 2-cyanoethyl-\(N\), \(N\), \(N\)', \(N\)`-tetraisopropyl phosphoramidite\(^{39}\) in presence of pyridinium trifluoroacetate as an activator. These reagents have replaced the conventional reagents i.e. 2-cyanoethyl-\(N\), \(N\)-diiisopropyl-chlorophosphoramidite and 1H-tetrazole, respectively due to their cost effectiveness and relatively more stability and lesser toxicity.

This amidite \(3\) was coupled to 5` OH of 11-mer sequence 5´- GT TAG GGT TAG-3´ on solid support using the usual coupling procedure employed in the amidite approach\(^{40}\) (Scheme I). The hybridisation of \(4\) with the repeat sequence of the telomerase RNA template 5´-CU AAC CCU AAC-3´ was studied by \(Tm\) vis-à-vis the unmodified duplex of the two normal 11-mer sequences.

The curcumin - glycine conjugate is designed to act as a specific prodrug and gets activated only under hypoxic condition, a specific characteristc of the tumour cells (Scheme II). The normal tissues typically have median \(O_2\) concentration in range of 40-60 mm Hg whereas half of all solid tumours have median oxygen value less than 10 mm Hg with fewer than 10% in the normal range\(^{41}\). The curcumin conjugate has been designed keeping this rational in mind that it will be preferentially active only inside the cells/ tissues having lower level of \(O_2\) and inactive inside the cell having the normal range of \(O_2\). The curcumin conjugate released from curcumin oligonucleotide conjugate becomes an excellent substrate for various intracellular reductase enzymes, which can add single electron to the molecule thereby producing a free radical intermediate. In presence of oxygen i.e. in normal or diseased cells, this free radical is rapidly oxidized back to the parent molecule with the formation of super oxide radical. However, in absence of oxygen the situation prevailing in hypoxic cells, this does not occur and the highly reactive curcumin- conjugate radical will abstract hydrogen atom from nearby macromolecules causing their structural mutations. If this nearby molecule is DNA, then the curcumin-conjugate radical may break both single and double strands, leading to chromosomal aberrations and cell death\(^{42}\).

On the other hand, the oligonucleotide sequence released at the site due to enzymatic cleavage can hybridize with the complementary telomerase repeat inhibiting their reassembly to telomere, which is responsible for fast proliferation of the cancerous cells. The size of this prodrug molecules as compared to liposomes (> 1-2 \(\mu\)m) is small enough to get a thorough passage in pulmonary capillaries and the approach for improving the uptake involves such a design which would undergo enzyme mediated transformation within the targeted organ. The conjugate bonds reported herein, in case of amino acid curcumin bioconjugates are specifically enzyme sensitive to cause a positive systemic delivery as shown by hydrolysis with \(\alpha\)-chymotrypsin.

The present prodrug thus meets all the necessary conditions of an effective prototypical inhibitor. It can get activated only at the site of cancerous cells (hypoxic condition), is effective at nanomole level, is a relatively small molecule, can penetrate deeper in the tumor to reach cancerous cells, also is non toxic to normal cells and somatic cells and can be cleaved by the reductase only under hypoxia. Its higher \(Tm\) value (32°C) with complementary RNA sequence as compared to normal duplex (30°C) suggests its stronger and stable binding to the target.

This increased thermodynamic stability of the duplex might be responsible for the molecular activity \textit{in vivo} system vis-à-vis the unmodified strand. The normal duplex probably gets destabilized and is ineffective in modulating the expression of the cell.

To determine whether inhibition of telomerase would ultimately limit proliferation, the DU145 cells derived from the prostate tumor were used. The cells were transected with 5´-\(\text{O-glycinoyl curcumin-DNA}\) and unmodified DNA oligomers at 3 to 4 days intervals for 75 days. Addition of 5´-\(\text{O-Cur-DNA}\) to DU145 cells caused proliferation of cells to begin to slow after 30 days. By the end of the experiment (after 75days) only 20% of the cell population is found to be in the proliferation stage (Figure 1), while the cells treated with unmodified DNA remained unchanged.

The DU145 cells have a mean terminal restriction fragment (TRF) length of 3600 bp. TRF length corresponds qualitatively to telomere length and the length corresponds to the slow decrease in cell proliferation.

\textbf{Telomere shortening on addition of 5´-\(\text{O-Cur-}\)DNA and unmodified DNA into cells}

The mean TRF length of DU145 cells was examined after treating the cells for 75 days. Since
DU145 cells possess long telomere length, with the mean TRF length of cells treated with the 5'-O-Cur-DNA complementary to the hTR region, decreasing from 3,600 to 2,200 bp. There was no change in the cells treated with unmodified DNA (Figure 2).

TRF length corresponds qualitatively to telomere length and the length corresponds to the slow decrease in cell proliferation. Therefore, if a cell is having a comparatively longer TRF, it will take more time for treatment to effect significant loss of subtelomeric repeats than those cells, which are having smaller TRF.

Materials and Methods
All solvents used were purified and dried prior to use. 2-cyanoethyl-N, N', N'-tetraisopropyl
Phosphoramidite/bis-reagent and pyridinium trifluoroacetate (Py·TFA) were obtained from ISIS Pharmaceuticals, USA. Oligonucleotides were synthesized on the ABI DNA synthesizer on 0.2 µmole scale using standard phosphoramidite approach. Curcumin, 1-H tertrazole and trichloroacetic acid were purchased from Sigma Chemical Co. USA. UV absorption spectra were recorded on a Hitachi 220S UV-visible spectrophotometer. Hybridization studies were carried out on a Hitachi 220S, UV-Vis spectrophotometer attached with HAAKE DC-5 refrigerated circulatory-bath for temperature programming. Elemental data were recorded on a Schimadzu 34-408 analyser and ¹H NMR spectra were obtained using a Brookers DRX 300. γ³²P-ATP was purchased from Perkin-Elmer Scientific. T4 polynucleotide kinase and α-chymotrypsin were obtained from Invitrogen.

**Oligonucleotides used.** All the oligonucleotides were prepared from respective monomer phosphoramidites by solid phase synthesis at 0.2 µmole scale on 392 ABI DNA synthesizer. (5’-GT TAG GGT TAG-3’), an 11-mer sequence was modified at 5’-end by attaching curcumin-diglycinoyl monoethyl-O-phosphoramidite and was used for further studies, hereafter written as 5’-Cur-O-DNA. For control study, an unmodified normal sequence was also used. A complementary sequence (5’-CU AAC CCU AAC-3’) was synthesized for hybridization studies. 5´-³²P-TTA GGG TTA GGG TTA GGG-3’ was used as a telomeric probe for hybridization with chromosomal DNA.

**Cell lines.** The prostrate tumour derived DU145 cells were used in this study. The cells were maintained in DMEM (Dulbecco’s eagle medium) containing 10% (v/v) FBS (fetal bovine serum), 500 units/mL penicillin (Sigma) and 0.1 g/mL streptomycin (Sigma) and incubated at 5% CO₂ at 37°C.

**Experimental Section**

1. **7-Bis (4-O-glycinoyl-3-methoxy phenyl)-1,6-heptadiene-3, 5-dione 1.** Curcumin (1.104 g; 3 mmole) dissolved in dry pyridine and stirred with two moles of N-phthaloyl glycinoyl chloride (1.607 g; 7.2 mmole), the reaction completed after 6 hr as checked by TLC. The curcumin conjugate obtained was purified on column, yield 38% (732 mg); Rf 0.87 (MeOH-DCM; 0.5 : 9.5). The pure product was characterised by elemental data and ¹H NMR, Anal. Calcd for C₂₅H₂₆O₈N₂: C, 62.17; H, 5.39; N, 5.80. Found: C, 62.15; H, 5.48; N, 5.62 %. ¹H NMR (CDCl₃, δ) 3.85 (s, 6H, -OCH₃) 5.13 (s, 2H, C₄-H), 6.48 (d, 2H, C₂-H & C₆-H), 7.52 (d, 2H, C₁-H & C₇-H); 4.49-4.61(m, 4H, -CH₂-NH₂).

2. **1-(4-glycinoyl-N-β-hydroxyethyl-3-methoxyphenyl)-7-(4·glycinoyl-3’·methoxyphenyl)-1,6-heptadiene-3, 5-dione 2.** The compound 1 (723 mg, 1.5 mmole) was taken in dry pyridine (5 mL), to it dimethyl aminopyridine (36.6 mg; 0.2 equivalent)

**Figure 1** — DU145 cells transfected with 5’-O-Cur-DNA (■) to see the effect of long-term transfection. The cells treated with unmodified DNA (●) shows 100 % proliferating cells same as the untreated cells (♦).

**Figure 2** — Gel electrophoresis of digested DNA on 0.7% agarose; Nos 1, 2 and 3 are lanes and 1.0 to 6.0 is the length in cms. Reduction in Telomere restriction fragment length (TRF) in DU145 cells after treating with 5’-O-cur-DNA; lane 1 shows the chromosomal DNA from unmodified cells, lane 2 shows the fully complementary oligomers after the treatment, which is weak due to erosion of telomeres and very few telomeric repeats are left to hybridise with radiolabeled probe, lane 3 shows the chromosomal DNA from cells treated with unmodified DNA. Chromosomal DNA is loaded in equal amount in all the lanes. All the experiments were done in duplicate.
was added. To this reaction mixture cooled to 0°C, ethylene chlorohydrin (1.02 mL, 1.5 mmole) was added slowly during 10 min. The reaction mixture was stirred at room temperature for 6 hr. The completion of the reaction was assessed by TLC and pure product was isolated by silica gel column chromatography, yield 43% (339 mg). Anal. Calcd for C_{27}H_{31}O_{9}N_{2} Cl: C, 57.61; H, 5.57; N, 4.96. Found: C, 57.63; H, 5.63; N, 4.97.

The desired fractions were pooled, dried under reduced pressure to afford title compound as amorphous yellow powder, yield, 82% (357 mg), Rf 0.63 (CH_{2}Cl_{2}-MeOH; 9.5:0.5 v/v), 31P NMR (CD_{3}CN): 141.7 ppm.

Covalent attachment of phosphoramidite 3 to 5′-GT TAG GGT TAG-3′ (4). All the oligomers were synthesized at 0.2 μmole scale on an ABI 392 model DNA/RNA synthesizer. The phosphoramidite unit 3 was incorporated into the sequence 5′-GT TAG GGT TAG-3′ using standard phosphoramidite approach in the last coupling cycle. The last coupling cycle was increased by 10 min. All the oligomers were treated with 30% aqueous ammonia for 16 hr at 55°C to remove protecting groups from the bases and internucleotide phosphates and also to cleave oligomer from the solid support. The ammoniacal solution was concentrated under vacuum in a speed vac, and the residue was subjected to desalting on a reverse phase silica gel column. The oligomers were purified on anion exchange FPLC and then analyzed on reverse phase HPLC with a Merck lichrosphere RP-18 column using 0.1 M ammonium acetate buffer at pH 7.4 and acetonitrile solvent at a flow rate of 1mL/min, while monitoring the UV signal of DNA at 260 nm. However, the desired collected portion was reinjected in 25μL to get the 90-95% purified oligonucleotides.

Hybridisation studies. The hybridization studies of modified 11-mer with normal complementary strand was done. The study was carried out by mixing the modified sequence (5′- curcumin-glycine– O-P-O- GT TAG GGT TAG-3′) with the target complementary sequence (5′-CU AAC CCU AAC-3′) at total oligonucleotide concentration of 1.0 OD in buffer containing 0.1 mole sodium chloride, 0.01 mole potassium dihydrogen phosphate, and 0.01 mole sodium hydrogen phosphate (pH 7.0). The melting curves were studied by recording the change in OD concentration at 260 nm wavelength. The variation in temperature has been done at the rate of 0.5°C/min. The same experiment was repeated with unmodified 11-mer duplex. The duplex of the complementary sequence with modified 11-mer shows higher melting temperature, Tm (32°C) than the unmodified duplex, Tm (30°C).

Enzymatic digestion studies. The substrate 4 (1.0 A_{260} unit) and 50 mg of α-chymotrypsin were taken in 100 μL of Tris buffer (pH, 7.8, 25 mmole) and incubated at 37°C. Aliquots of 10 μL were taken out at 5 min. interval and were diluted to 150 μL with the same buffer and analyzed on reverse-phase HPLC. Free oligos were recovered after 12.5 min of this treatment.

Uptake of 5′-O-cur-DNA and unmodified DNA into cells. DU145 cells were plated at 30,000 cells per well in a 24-well plate (FALCON Multiwell, 24 well, marketed by Becton Dickinson) in DMEM supplemented with 10% (v/v) FBS. After allowing the cells to adhere, they were transfected with 2.0 μL of lipofectamine and 0.5 μL of 5′-O-cur-DNA in 200 μL of total opti-mem (Life Technologies) according to manufacturer's protocol. For control experiment a similar set of experiment was done with unmodified DNA. After 6 hr at 37°C, the transfecting mixture was removed and medium without antibiotics and with 10% (v/v) serum was added. Cells were then harvested with PBS buffer and treatment after 3 days, counted, replated and assayed for telomerase activity. The experiment was carried out for 75 days.

Estimation of telomere length after treatment with 5′-O-cur-DNA. Genomic DNA was isolated from cells and digested twice with six-fold excess of
enzyme HinFI (New England Biolabs), the digested DNA was electrophoresed on 0.7% agarose gel and transferred to a nitrocellulose membrane. Telomeric DNA was detected by hybridization with 5’-γ³²P-TTA GGG TTA GGG TTA GGG-3’ and the gel was scanned using phosphoimager (Molecular Dynamics).

**Conclusion**

Telomerase is the critical enzyme in overcoming growth limitations due to telomere dysfunction. Killing tumour cells in cancer patients with telomerase based strategies have never before looked so promising. The present communication is an effort to search for cost-effective therapeutic approaches which holds promise to be used as telomerase inhibitors as well as killing of the cancerous cells specifically and simultaneously.

The present attempt is to develop an antitumour prodrug, which meets all necessary conditions of an effective prototypical inhibitor. It can get activated only at the site of cancerous cells (hypoxic condition), is non toxic to normal and somatic cells and gets cleaved at the desired site by reductase, is thermodynamically stable and binds strongly to the target, is effective at nanomole level, is a small lipophilic molecule, can penetrate cells and can cause apoptosis of desired cells. In vivo it has reduced the proliferating cells population of DU145 cells from prostrate tumour by 80% at the end of 75 days. Further clinical studies are required to prove it as a successful antitumour prodrug.

**References**