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Curcuminoid derivatives enhance telomerase activity in an in vitro TRAP assay

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ABSTRACT

The length of telomeres controls the life span of eukaryotic cells. Telomerase maintains the length of telomeres in certain eukaryotic cells, such as germline cells and stem cells, and allows these cells to evade replicative senescence. Here, we report for the first time a number of curcuminoid derivatives that enhance telomerase activity in an in vitro TRAP assay. A preliminary analysis of structure–activity relationships found that the minimal requirement for this enhanced telomerase activity is a curcuminoid core with at least one *n*-pentylpyridine side chain, while curcuminoids with two such side chains exhibit even greater activity. The finding here might lead to a new class of telomerase activators that act directly or indirectly on telomerase, rather than through the reactivation of the telomerase reverse transcriptase (TERT) gene associated with other telomerase activators found in the literature.

Telomerase expression is associated with cell immortalization and tumorigenesis.[1] Most human somatic cells do not express telomerase; and therefore, their telomeres are gradually shortened with each cell division due to the 'end-replication' problem.[2] Once a few telomeres are shortened to a critical length, they signal the cell to enter growth arrest known as replicative senescence.[3] Cancer cells evade replicative senescence by maintaining their telomeres, mostly by reactivating telomerase reverse transcriptase (TERT) expression.[4] Inhibition of telomerase is thus a selective cancer therapy with the capacity to render cancer cells to replicative senescence.[5]

Telomerase is a multi-subunit ribonucleoprotein enzyme comprised of the telomerase reverse transcriptase (TERT), the telomerase RNA (TR), and species-specific accessory proteins.[6] TERT catalyzes the addition of a short repetitive telomeric sequence onto the 3′-end of telomeres using a section of TR as the template in a process known as repeat addition processivity.[7] This elongation mechanism requires a translocation step of the previous round of telomerase-extended product to the original position on the RNA template before the new round of telomeric repeat can be reverse transcribed onto the telomeric end.[7a] The activity and processivity of telomerase depends on several factors, including both TR and TERT, as well as some telomerase-associated proteins, notably POT1–TPP1 heterodimer, which enhances telomerase processivity by slowing primer dissociation and aiding translocation.[8]

Many small molecules have been shown to inhibit telomerase activity.[9] Some G-quadruplex ligands were also found to inhibit telomerase processivity.[10] Until now, however, there have been no reports of small molecules that directly enhance telomerase activity in a cell-free system. Here, we report for the first time a series of curcuminoid derivatives that enhance telomerase activity in an in vitro telomerase assay. The structures of these compounds are shown in Figure 1. The strategies used to synthesize these compounds followed the general procedures outlined in two previous publications and are described in detail in Supplementary data S1.[11]

In our search for telomerase inhibitors, we employed a modified telomerase repeat amplification protocol (TRAP) assay introduced by Szatmari and Aradi as our standard protocol because this method can retain the original length of telomerase products, and thus the processivity of telomerase can be analyzed.[12] To detect the amplified telomerase products, we modified the method by using a fluorescent-tagged primer instead of using radioactive nucleotides. The crude cell lysate from HEK293T cells transfected with hTERT and hTR plasmids was used as the source of telomerase

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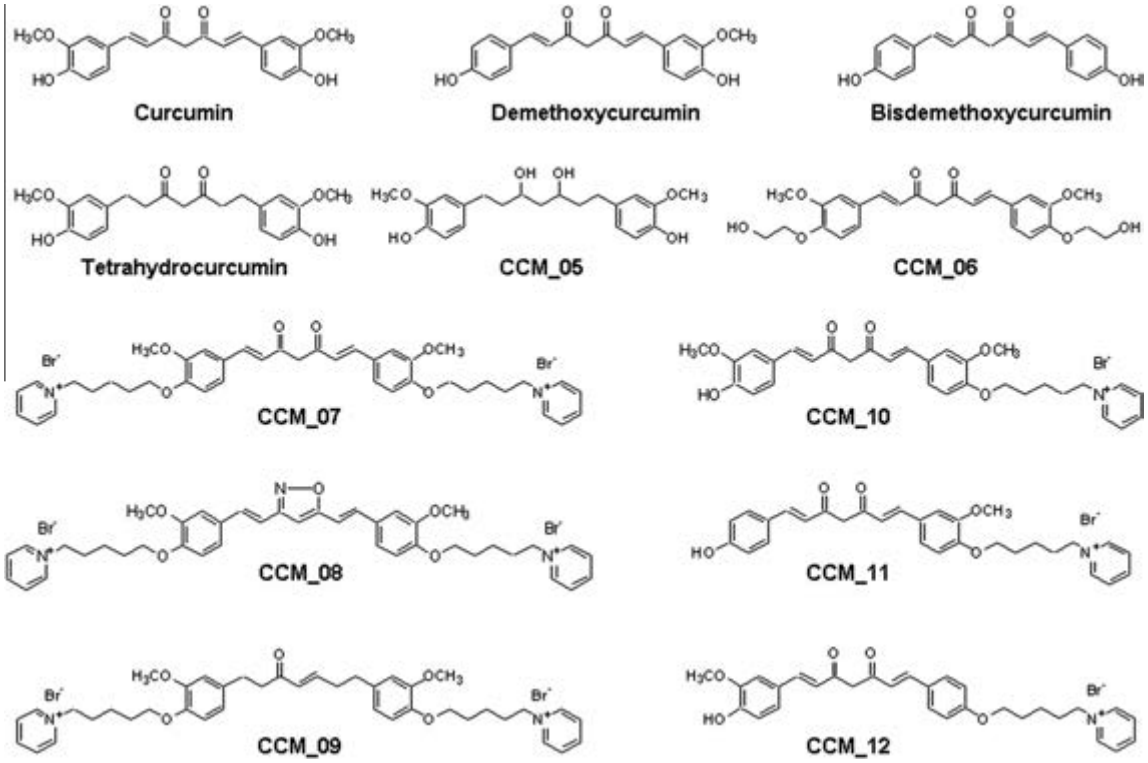

Figure 1. Structures of curcumin and curcuminoid derivatives evaluated in the present study.

according to a published protocol.[13] In all assays, the test compound was removed from telomerase products by phenol–chloroform extraction and ethanol precipitation before the amplification step to prevent any false negative result due to the inhibition of *Taq* DNA polymerase. The details of each TRAP assay are described in the figure captions. The DNA sequences of oligonucleotides used in the TRAP assay are listed in Table 1.

We first tested the first seven curcuminoid derivatives shown in Figure 1 (curcumin to CCM_07) using our modified TRAP assay. We found that most curcuminoids had no effect on telomerase activity (see Supplementary data Fig. S1), except for CCM_07, which was found to enhance telomerase activity in a concentration-dependent manner. As shown in Figure 2A, the telomerase products increase in intensity and length with increasing amounts of CCM_07, while the intensity of the internal control (IC) remains unchanged. Note that our TRAP system does not generate DNA ladders from a single telomeric DNA template as often seen with conventional TRAP assay.[12a] As shown in lanes 0.25R6 and 0.125R6, where the artificial telomerase product with 6 repeats (MTS-R6 at 0.25 and 0.125 amol) was used as the template for the amplification step, only one single amplified product was observed. The intensity of the amplified MTS-R6 correlates well with the input template, which implies that the PCR cycle used in our TRAP assay does not reach the plateau stage; and therefore, the amplified products reflect the amount of telomerase products in the reaction. We repeated this experiment three times, and the intensity of the telomerase products was quantified using Image J software. The average accumulated intensity was then plotted against the concentration and shown as a bar graph in Figure 2B. The graph demonstrates that CCM_07 enhances telomerase activity in the concentration-dependent manner, up to 5 fold at 60 μM of CCM_07.

The ability of CCM_07 to enhance telomerase activity is also evident from the time-course experiment, where we examined telomerase activity in the absence and presence of 40 μM CCM_07 at various incubation times. Figure 2C shows the phosphorimage of the time-course experiment, and Figure 2D shows the line graph plotted between the accumulated intensity of telomerase products and time. As shown in Figure 2C, the telomerase products, both length and intensity, increase with the incubation time in both the experimental and the control sets. However, as shown in Figure 2D, when compared with the control set, the intensity and length of telomerase products appear to increase in the presence of 40 μM CCM_07 at every time interval.

As mentioned above, the test compound was removed from telomerase products by phenol–chloroform extraction and ethanol precipitation before the amplification step in our TRAP protocol; therefore, the increase of amplified telomerase products by CCM_07 shown in Figure 2 does not occur in the amplification step. In fact, without compound removal, the gel data appear as if CCM_07 were a telomerase inhibitor (see Supplementary data Fig. S2A). We found out later, in a separate experiment using ordinary PCR, that CCM_07 strongly inhibited *Taq* DNA polymerase at concentrations as low as 5 μM (see Supplementary data Fig. S2B). Therefore, the increase in intensity of telomerase products observed in Figure 2 likely arises solely from an increase in telomerase activity. The recovery control (RC), which was added to each

Table 1
Oligonucleotides employed in our modified TRAP assay

Name	Sequence
MTS	AGCATCCGTCGAGCAGAGTT
RPc3g	TAGAGCACAGCCTGTCCGTG$(CTAACC)_3$GG
RP*	TAGAGCACAGCCTGTCCGTG
IC	TAGAGCACAGCCTGTCCGTGAAAAGGCCGAGAAGCGATCG
NT	CGATCGCTTCTCGGCCTTTT
RC(+)*	AAGCTTTAATACGACTCACTATACGGACGTCC
RC(−)	GGACGTCCGTATAGTGAGTCGTATTAAAGCTT
MTS-R6	AGCATCCGTCGAGCAGAGTTAG$(GGTTAG)_6$

* FAM-tagged strand.

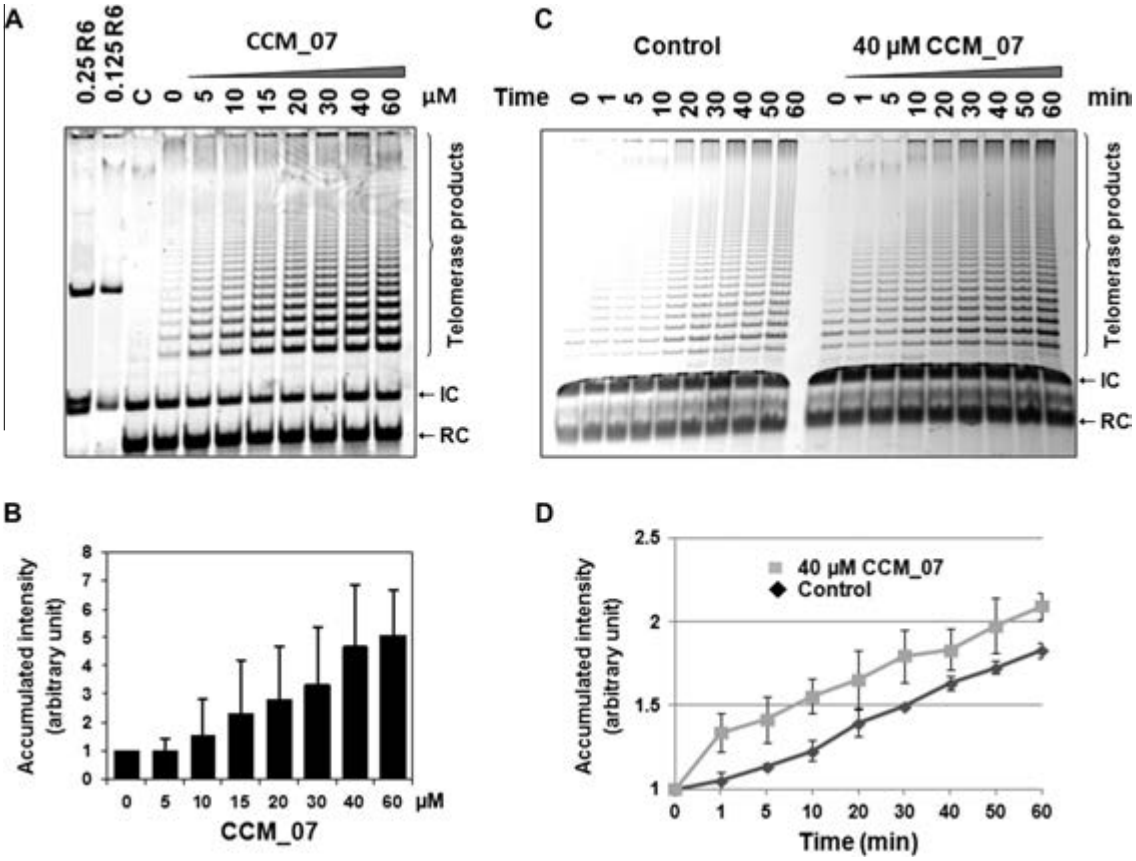

Figure 2. CCM_07 enhances telomerase activity in a concentration-dependent and time-dependent manner. (A) Phosphorimage from the concentration-dependent experiment. The MTS primer and the indicated concentration of CCM_07 were incubated in the 100 μl telomerase reaction buffer at 30 °C for 30 min. The compound was then extracted by two rounds of phenol–chloroform extraction, and the telomerase products were then ethanol precipitated and amplified by PCR. The amplification products were electrophoretically separated in 8% non-denaturing acrylamide gel and visualized with a phosphorimaging system (Typhoon; Molecular Dynamics). The lanes labeled as 0.25R6 and 0.125R6 are assay controls, when 0.25 amol and 0.125 amol of MTS-R6 (the artificial telomerase product with 6 repeats) were used as template in our TRAP assay. Notice that MTS-R6 only generated one amplified product, and not DNA ladders associated with conventional TRAP assay. (B) Phosphorimage from the time-course experiment. First, a 2-ml master mix of telomerase reaction mixture was prepared on ice to prevent any telomerase activity. Then, 990 μl of this master mix was dispensed into a tube containing 110 μl of 400 μM CCM_07 in 25% DMSO, and a tube containing 110 μl of 25% DMSO (the control set). The reaction mixture was mixed and incubated in a water bath at 30 °C. At the indicated times, 100 μl from this reaction mixture was drawn from both sets, and the enzyme activity was terminated by heating at 95 °C for 10 min. DNA purification, PCR amplification, and electrophoresis of the telomerase products were performed in the same manner as described above. IC represents the internal control, and RC represents the recovery control. (C and D) Graphs represent the accumulated intensity of telomerase products from three independent experiments of (A) and (B), respectively. The telomerase products were quantified using ImageJ software.

sample before the phenol–chloroform extraction and ethanol precipitation, also ensured that all the primer-extended products were fully recovered during the procedure. The internal control (IC) was used in the reaction to ensure that there was no inhibition or enhancement in the amplification step.

Since the existence of a telomerase activator of this kind had, to our knowledge, never been reported, we questioned whether the enhanced telomerase activity was specific to CCM_07, or would structurally related derivatives yield similar results. To this end, we synthesized five new *n*-pentylpyridine curcuminoid derivatives, CCM_08–CCM_12 (Fig. 1). In CCM_08, the double carbonyl groups in the curcumin core of CCM_07 are replaced with an isoxazole ring. CCM 09 possesses only one carbonyl group and one double bond in the center of the curcumin core. CCM_10 possesses only one *n*-pentylpyridine side chain on the curcumin core. CCM_11 and CCM_12 are isomers of the demethoxycurcumin core with one *n*-pentylpyridine side chain located in two distinct positions. We then tested these compounds for their effect on telomerase activity using the same TRAP assay. Figure 3A shows the gel data, and Figure 3B shows graphs representing the accumulated intensity of telomerase products enhanced by each curcuminoid derivative at various concentrations, averaged from three independent experiments. As shown in Figure 3B, CCM_08 and CCM_10, CCM_11, and CCM_12 enhance telomerase activity roughly threefold at 60 μM, compared to roughly fivefold for CCM_07. In contrast, CCM_09 had little or no effect on telomerase activity within the concentration range tested.

Based on the results obtained from the 12 compounds studies here, we can conclude that the minimal requirement for enhanced telomerase activity is a curcuminoid core having at least one *n*-pentylpyridine side chain, but the activity is greater when there are two such side chains. The curcuminoid core can be curcumin or demethoxycurcumin, and the existence or position of the methoxy group on the benzene ring is seemingly unimportant. The double carbonyl group in the heptyl-linker chain of the curcuminoid core can be replaced by an isoxazole ring, but the mono-oxygenated analog loses activity. It is likely that the conjugated double bonds, which keep the molecules in an extended planar configuration, are important for this activity. It should be noted, however, that these conclusions are based on a limited set of compounds; a larger library of compounds is needed for a full assessment.

Furthermore, we have observed this enhanced telomerase activity only in an in vitro TRAP assay. We do not know whether these compounds can enhance telomerase activity inside cells. To date, there have been few reports of telomerase activators; namely, an extract from *Astragalus membranaceus* root (TA-65) or from its

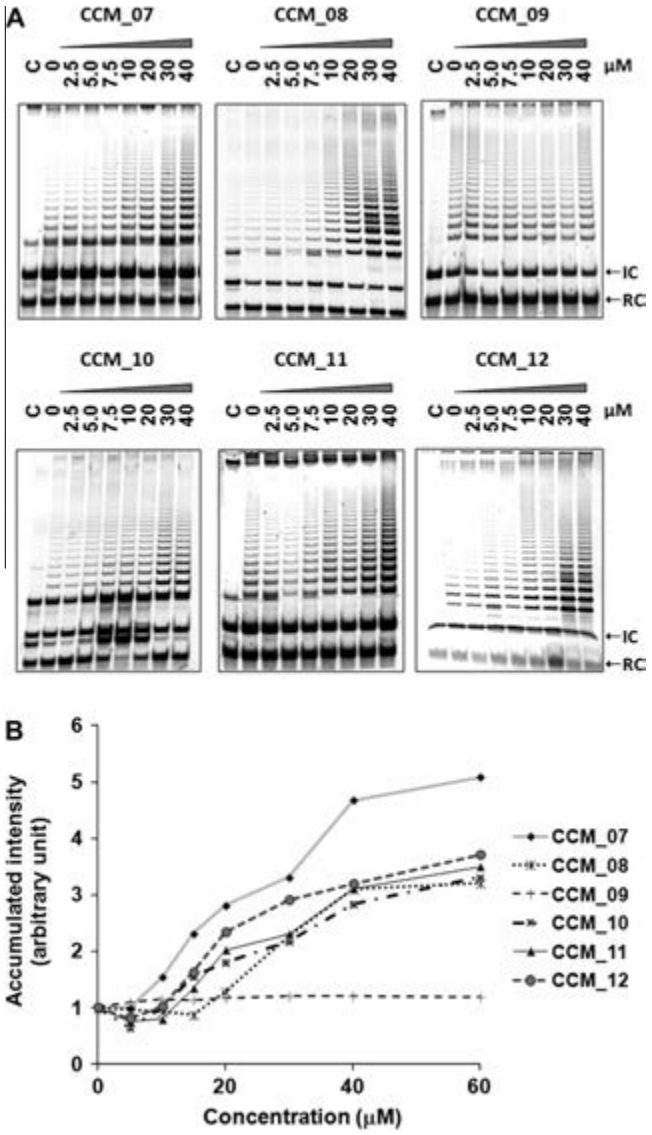

Figure 3. The effect of CCM_07–CCM_12 on telomerase activity. (A) Phosphor-images from the concentration-dependent experiment. The experiments were performed in the same manner as described in Figure 2. IC represents the internal control, and RC represents the recovery control. Lane C represents the negative control experiment in which telomerase was heat-denatured. (B) Graphs represent the accumulated intensity of telomerase products enhanced by each curcuminoid derivative at various concentrations, averaged from three independent experiments. The standard deviations are omitted from this figure for clarity of the data. Bar graphs with standard deviation are available in Supplemental data Figure S10.

active compound cycloastragenol (GRN665 or TAT2) and its derivative (GRN510).[14] However, the plant extract or its active compounds exert their activity through the enhancement of TERT expression, leading to an increase in telomerase activity and telomere lengthening inside the cells or in in vivo murine models.[14b–d] In contrast, our curcuminoid derivatives exert their telomerase enhancement via a completely different mechanism that involves no cellular activity. We believe that these agents might augment the binding of the telomeric end to the enzyme since the activity seems to increase immediately after the compound is added to the reaction mixture (Fig. 2C). However, further investigation into the mechanism of action is needed. Also, it would be interesting to investigate these compounds further at the cellular level or in in vivo models.

Most human somatic cells lack sufficient telomerase to maintain their telomeres, which leads to their limited life span.[2,3] Reactivation of telomerase by transfecting hTERT, the catalytic subunit of telomerase, into various human cell types has been shown to increase the cellular life span.[15] Moreover, restoration of telomerase in mice models has been shown to rescue critically short telomeres, to prevent or reverse tissue defects associated with cellular aging, and to increase life span.[16] Recent studies of telomerase activators TA-65 and GRN510 in mice have shown that small molecules can increase cellular telomerase activity in some tissues and rescue critically short telomeres, which leads to a longer life span, or in the case of GRN510, suppression of lung damage from bleomycin-induced fibrosis.[14b,c] The reports from humans taking TA-65 in a commercial health maintenance program, PattonProtocol-1, show an improvement in markers of immune, metabolic, bone, and cardiovascular health.[14a,d] These findings support the notion that activating telomerase might limit premature aging and increase organismal life span. Further, the findings in this Letter might lead to development of new telomerase activators that act directly on telomerase.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.09.059. These data include MOL files and InChiKeys of the most important compounds described in this article.

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