Ginger Extract Promotes Telomere Shortening and Cellular Senescence in A549 Lung Cancer Cells

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Supporting Information

ABSTRACT: Replicative senescence, which is caused by telomere shortening from the end replication problem, is considered one of the tumor-suppressor mechanisms in eukaryotes. However, most cancers escape this replicative senescence by reactivating telomerase, an enzyme that extends the 3′-ends of the telomeres. Previously, we reported the telomerase inhibitory effect of a crude Zingiber officinale extract (ZOE), which suppressed hTERT expression, leading to a reduction in hTERT protein and telomerase activity in A549 lung cancer cells. In the present study, we found that ZOE-induced telomere shortening and cellular senescence during the period of 60 days when these A549 cells were treated with subcytotoxic doses of ZOE. Using assay-guided fractionation and gas chromatography/mass spectrometry analysis, we found that the major compounds in the active subfractions were paradols and shogaols of various chain lengths. The results from studies of pure 6-paradol and 6-shogaol confirmed that these two compounds could suppress hTERT expression as well as telomerase activity in A549 cells. These results suggest that these paradols and shogaols are likely the active compounds in ZOE that suppress hTERT expression and telomerase activity in these cells. Furthermore, ZOE was found to be nontoxic and had an anticlastogenic effect against diethylnitrosamine-induced liver micronucleus formation in rats. These findings suggest that ginger extract can potentially be useful in dietary cancer prevention.

INTRODUCTION

Telomeres are specialized nucleoprotein structures found at the ends of all eukaryotic chromosomes. Telomeres maintain chromosome stability by preventing nucleolytic degradation and end-to-end fusion1 as well as facilitating chromosome segregation during meiosis.2 The long repetitive telomeric DNA sequence and the specific telomeric protein complex called shelterin allow the telomere to form a loop structure called T-loop, which differentiates telomeric DNA from other double-stranded DNA breaks.3 Telomeric DNA is shortened 50–200 base pairs during each round of DNA replication because of the end replication problem.4 The long telomeric DNA allows somatic cells to replicate for a number of cell divisions until one of the cell’s telomeric DNAs is shortened to a critical length when it triggers the irreversible cell cycle arrest called “replicative senescence”.5,6

Replicative senescence is generally considered a tumor-suppressor mechanism. To escape replicative senescence, the majority of human cancers maintain their telomere length by reactivating telomerase, an enzyme that normally adds telomeric DNA to the 3′-ends of chromosomes in germline cells.7,8 The isolated active human telomerase consists of two sets of human telomerase reverse transcriptase (hTERT), human telomeric RNA (hTR), and dyskerin.9,10 Although both hTERT and hTR are necessary for telomerase activity, the transcriptional regulation of hTERT expression is the principal mechanism for controlling the telomerase activity.9,11

Dietary phytochemicals have attracted considerable interest for cancer prevention because of at least three factors: (1)
potential therapeutic effects, (2) low cost, and (3) good bioavailability.\textsuperscript{12,13} However, applying plant chemicals for cancer prevention requires an in-depth knowledge of their mechanisms of action and their biosafety. One of the attractive targets for cancer prevention is telomerase because telomerase-specific inhibition causes cancer cells and cancer-initiating cells to enter replicative senescence and apoptosis without any significant effect on normal somatic cells.\textsuperscript{14,15} A literature search for plant-derived telomerase inhibitors found a few natural phytochemicals that inhibited telomerase in cancer cells; these include curcumin, epigallocatechin-3-gallate (EGCG), resveratrol, genistin, sulforaphane, silibinin, and pristimerin, among others.\textsuperscript{16} However, only a few reports show the long-term effects of these phytochemicals on telomere shortening and cellular senescence because the cancer cells must grow and normally divide for several generations in a nontoxic dose in order to attain a discernible telomere shortening. This aspect is crucially important because the viability of using dietary phytochemicals for cancer prevention depends on these agents being effective at nontoxic doses.

For millennia, the ginger (\textit{Zingiber officinale Roscoe}) rhizome has been traditionally used for various ailments, including many gastrointestinal disorders such as nausea, vomiting, and abdominal spasm, as well as rheumatic disorders, arthritis, and muscular discomfort.\textsuperscript{17,18} In recent years, scientists have revealed that various chemicals found in ginger rhizome—most notably gingerols, paradols, and shogaols—possess anticancer properties as shown in many experimental models.\textsuperscript{19–22} Previously, we reported the telomerase inhibitory effect of the crude ethyl acetate fraction of \textit{Z. officinale} extract (ZOE), which suppressed \textit{hTERT} expression, leading to a reduction in the \textit{hTERT} protein and telomerase activity in A549 lung cancer cells.\textsuperscript{23} However, there were two remaining important questions that we wanted to address; these are as follows: (1) would telomerase suppression by the ginger extract lead to telomere shortening and cellular senescence at subcytotoxic doses? (2) What are the active compounds in the ginger extract that suppress \textit{hTERT} expression? In this report, we demonstrated that ZOE induced telomere shortening and cellular senescence during long-term treatment when these A549 cells were treated with subcytotoxic doses of ZOE. We then identified the telomerase suppressors in the crude ginger extract using assay-guided fractionation and gas chromatography/mass spectrometry (GC/MS) analysis. Furthermore, we evaluated the safety, clastogenicity, and anticlastogenicity of this extract in rats.

\section*{RESULTS}

We previously reported that ZOE suppressed \textit{hTERT} expression in A549 lung cancer cells, leading to the decrement of the \textit{hTERT} protein and telomerase activity.\textsuperscript{23} In the present study, ZOE was extracted in the same manner, and the thin-layer chromatography (TLC) fingerprint of ZOE was similar to what we previously reported (Supporting Information, Figure S1). The IC\textsubscript{50} growth inhibitory effect of ZOE in A549 cells was 58 $\pm$ 2 $\mu$g/mL, compared to 50 $\pm$ 4 $\mu$g/mL as previously reported.\textsuperscript{23}

\textbf{ZOE Suppressed \textit{hTERT} Expression and Telomerase Activity in A549 Lung Cancer Cells.} We retested the new batch of ZOE for the suppression of \textit{hTERT} expression and telomerase activity in A549 lung cancer cells. For the gene expression assay, we incubated the A549 cells with the indicated concentrations of ZOE for 24 h, before their RNAs were extracted and assayed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. For each gene expression assay, we carefully chose the PCR cycle so that the detected amplified product could represent the initial amount of each cDNA in the reaction (Supporting Information, Figure S2). As shown in Figure 1A, the \textit{hTERT} and \textit{c-Myc} mRNA expressions were suppressed after the A549 cells were treated with ZOE for 24 h in a concentration-dependent manner. On the other hand, the housekeeping gene \textit{GAPDH} and other telomerase-related genes (\textit{hTR}, \textit{TRF1}, \textit{TRF2}, and \textit{hTEP1}) were not affected. For the telomerase activity assay, we incubated the A549 cells with the indicated concentrations of ZOE for 48 h. The crude protein was then extracted and used as the telomerase source in a modified TRAP assay. Figure 1B demonstrates that the telomerase activity of the A549 cells treated with ZOE for 48 h was also suppressed in a concentration-dependent manner. These results confirmed our initial contention that our new batch of ZOE could suppress \textit{hTERT} expression and telomerase activity in A549 cells.

\textbf{Long-Term Treatment with ZOE Led to Telomere Shortening in A549 Cells.} Most cancers treated with a telomerase suppressor should exhibit telomere shortening after successive rounds of cell replication, just like normal somatic cells. In order for this to happen and be observed, the dose of the agent must allow the cancer cells to proliferate normally for several passages. In this long-term treatment study, we treated the A549 cells with two subcytotoxic doses of ZOE (5 and 10 $\mu$g/mL) added in the culture media, with the changing of fresh media every 3 days and subculturing every 6 days for up to 60 days. Cells were collected and counted with each 6 day passage. We then plotted a graph between the cumulative number of population doublings and time (Figure 2A), which shows that the A549 cells in all three sets were steadily proliferating during the course of the 60 day experiment, although the population doublings in the experimental sets

\begin{figure}[h]
\begin{center}
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\caption{Short-term treatment of ZOE inhibits \textit{hTERT} expression (A) and reduces telomerase activity in A549 lung cancer cells (B). (A) To assay for gene expression, the A549 cells were incubated with the indicated concentrations of ZOE for 24 h before their RNAs were extracted and assayed by semiquantitative RT-PCR analysis. (B) To assay the effect on telomerase activity, the A549 cells were incubated with the indicated concentrations of ZOE for 48 h before the crude protein extract was used as the source of telomerase in a modified TRAP assay. Lane N represents the negative control experiment when telomerase was heat-denatured. IC and RC represent the internal control and recovery control.}
\end{figure}
doses of ZOE led to telomere shortening. On the basis of this finding, we further investigated whether this telomere shortening would accompany with the manifestation of cellular senescence. We conducted a senescence-associated β-galactosidase activity assay on the A549 cells collected from the long-term treatment study mentioned above. The A549 cells collected on days 30 and 60 were recultured in a six-well plate and allowed to grow for 24 h, before they were fixed, stained with X-gal solution, and photographed under a phase-contrast microscope. The blue-stained cells indicated the β-galactosidase positive cells, which are often accompanied with morphological changes. As shown in Figure 3A, there are more blue-stained cells in the experimental sets in which A549 cells were treated with ZOE than those found in the control set. The cells in each set were counted, and a graph between the percentage of the β-galactosidase positive cells (blue-stained cells) and time was plotted (Figure 3B). In the control set, the percentage of these cells (B) after long-term treatment with subcytotoxic doses of ZOE. The A549 cells from the long-term treatment study mentioned above. The A549 cells collected on days 30 and 60. However, after the A549 cells were treated with 5 μg/mL ZOE, the percentage of the β-galactosidase positive cells increased to 35% on day 30 and then to 55% on day 60, respectively. The percentage of the β-galactosidase positive cells was more profound after the A549 cells were treated with 10 μg/mL ZOE, with the percentage of β-galactosidase positive cells was more profound after the A549 cells were treated with 10 μg/mL ZOE, compared to the control group.

Figure 2. Population doublings (A) and telomere shortening (B) in long-term treatment of A549 cells with subcytotoxic doses of ZOE. A549 cells were incubated with or without (control) the indicated concentration of ZOE supplemented in the culture media, with a change of fresh media every 3 days and subculturing of the cells every 6 days, up to 60 days. (A) Cells from each passage were counted, and the growth curves between the cumulative numbers of population doublings were plotted against time. (B) A549 cells collected on days 6, 30, and 60 were subjected to the telomere length assay. The genomic DNA was extracted, and the mean TRFs were analyzed using the TeloTAGGG Telomere Length Assay kit. M represents a molecular weight marker.

Figure 3. Senescence-associated β-galactosidase positive cells (A) and percentage of these cells (B) after long-term treatment with subcytotoxic doses of ZOE. The A549 cells from the long-term treatment with the subcytotoxic dose experiment, collected on the indicated days, were subjected to the senescence-associated β-galactosidase activity assay. The A549 cells (1 × 10⁵ cells) were recultured in a six-well plate and allowed to grow for 24 h. The cells were fixed, stained with X-gal solution, and photographed under a phase-contrast microscope. (A) Morphological changes and β-galactosidase positive cells (blue-stained cells) are indicators of cell senescence. (B) Cells in each set were counted, and the percentage of the β-galactosidase positive cells was plotted against time. Differences are considered statistically significant when *p < 0.05 or **p < 0.01, compared to the control group.
the β-galactosidase positive cells rising to 43% on day 30 and then to 69% on day 60.

Effect on Clonogenicity after Long-Term Treatment with ZOE. Cellular senescence is the state by which mitotic cells irreversibly stop dividing. This inability to divide can be tested using a simple colony-forming assay in which individual cells are spread on a tissue culture plate and allowed to form colonies. To evaluate the effect on the clonogenicity of A549 cells after long-term treatment with ZOE, the A549 cells collected on days 30 and 60 from the long-term treatment study were seeded at a low density (2 × 10^4 cells) in a 10 cm tissue culture dish and allowed to form colonies for a period of 14 days. The colonies were then stained with crystal violet and digitally scanned. The number of colonies was determined using the ImageQuant TL software. Figure 4A shows the pictures of the colony formation of the A549 cells collected from the untreated control set and the treated experimental sets (with 5 and 10 μg/mL of ZOE) on days 30 and 60, respectively. The colonies from the untreated control set are densely populated, whereas the colonies from the treated experimental sets are less populous in a time- and concentration-dependent manner. The numbers of colonies were quantified, and the percentage of colony formations was compared to the control and plotted against time. The graph in Figure 4B shows that after the A549 cells were treated with 5 μg/mL ZOE, the percentage of colony formation decreased to 48% on day 30 and to 42% on day 60. After the A549 cells were treated with 10 μg/mL of ZOE, the percentage of colony formation decreased to 42% on day 30 and to 35% on day 60. From all of the experiments presented above, we conclude that ZOE suppresses hTERT expression and telomerase activity in A549 cells. The long-term treatment with subcytotoxic doses of ZOE in this cancer cell line leads to a gradual loss of telomere length, an induction of cellular senescence, and a reduction in clonogenicity.

Identification of Telomerase Suppressors in ZOE. We employed assay-guided fractionation and GC/MS analysis to identify the active compounds in ZOE that suppress telomerase expression and activity in A549 cells. ZOE was purified by column chromatography to obtain four fractions: E1–E4. The ZOE and its fractions were fingerprinted by TLC and high performance liquid chromatography (HPLC; Supporting Information, Figures S1, S3, and S4). The amount of 6-gingerol in these fractions was also quantified by HPLC. The E1 and E2 fractions contained an undetectable amount of 6-gingerol, whereas the E3 and E4 fractions contained about 30% and 40% of 6-gingerol, respectively. The semiquantitative RT-PCR analysis found that only the E2 fraction significantly suppressed hTERT expression in a dose-dependent manner, whereas the E3 and E4 fractions, which contained a large amount of 6-gingerol, were not found to suppress hTERT expression (Supporting Information, Figure S5A). These results confirm our previously published finding that 6-gingerol does not suppress hTERT expression.23

We further fractionated the E2 fraction by column chromatography into four more subfractions: E2.1–E2.4. The results from RT-PCR studies showed that all of these subfractions could suppress the expression of hTERT in A549 cells (Supporting Information, Figure S5B). These subfractions were then subjected to the same telomerase activity assay as previously described. All four subfractions were found to suppress the telomerase activity in a concentration-dependent manner (Supporting Information, Figure S6).

We then employed GC/MS to identify the compounds within these subfractions. The GC chromatograms, GC data, and selected GC/MS spectra are shown in the Supporting Information (Figures S7–S10). By using the fragment analysis of the ginger compounds reported by Tao et al.25 and Jolad et al.,26 we found that these active subfractions contained mostly paradols and shogaols of varying chain lengths (Table 1). Therefore, we conclude that paradols and shogaols are likely to be the active compounds in ZOE that are responsible for the suppression of hTERT expression and telomerase activity. Although there are concerns about the thermal degradation and dehydration of compounds containing a β-hydroxyketone group such as gingerols to aliphatic aldehyde, zingerone, and

![Figure 4. Clonogenicity of A549 cells (A) and percentage of colony formation (B) after long-term treatment with subcytotoxic doses of ZOE. The A549 cells collected on the indicated days were subjected to the colony formation assay. The low number of cells (2 × 10^4 cells) was recultured on a Petri dish. The cells were allowed to form colonies for 14 days and stained with crystal violet. Each plate was then scanned by a phosphoimager, and the colonies were counted. The number of colonies was determined using the ImageQuant TL software. Figure 4A shows the

Table 1. Major Compounds in the E2 Subfractions by GC/MS Analysis

| Compounds         | E2.1      | E2.2      | E2.3      | E2.4      |
|-------------------|-----------|-----------|-----------|-----------|
| 11-paradol (5%)   | 11-paradol (26%) | 7-paradol (42%) | 6-shogaol (68%) |
| 13-paradol (9%)   | 7-paradol (22%) | 10-shogaol (33%) | 8-shogaol (8%) |
| Bisabolene (7%)   | 6-paradol (22%) | 11-paradol (9%) | 10-shogaol (6%) |
| 9-paradol (16%)   | 6-paradel (6%)  | 7-paradel (5%)  |           |

* (% represents percentage of the peak area in GC chromatogram.
the corresponding shogaols under GC condition, we do not believe the paradols and shogaols we found in the E2.1–E2.4 subfractions were the products of thermal degradation or dehydration because gingerols were absent in the active E2 fraction and were found only in E3 and E4 fractions.

Although the data and results above suggest that paradols and shogaols are likely the active compounds in ZOE and responsible for the suppression of hTERT expression and telomerase activity, we cannot rule out the possibility that some minor ingredients in the extract are responsible for the observed effects. To verify whether the activities arose specifically from paradols and shogaols, we obtained pure 6-paradol and pure 6-shogaol as representative compounds and also pure 6-gingerol as a negative control. The same gene expression analysis by semiquantitative RT-PCR and telomerase activity assay were performed. The results are shown in the Supporting Information (Figure S11). The results showed that 6-paradol and 6-shogaol significantly suppressed hTERT expression in a dose-dependent manner, whereas 6-gingerol only slightly suppressed the hTERT expression at a higher concentration. Telomerase activity in the cells treated with 6-paradol and 6-shogaol, but not 6-gingerol, was also suppressed in a concentration-dependent manner. These results confirmed that the suppression of hTERT expression and telomerase activity found in ZOE arose from the paradols and shogaols rather than the gingerols.

The chemical structures of gingerols, shogaols, and paradols (Figure 5) are similar in that they all contain the 4-hydroxy-3-methoxyphenyl nucleus linked to different side chains of various lengths at position 1. Gingerols contain a β-hydroxy ketone side chain, whereas shogaols contain an α,β-unsaturated ketone side chain and paradols contain only one ketone group in their allyl side chain. In terms of chemistry, shogaols are the dehydrated form of gingerols and paradols are the hydrogenated form of shogaols. The loss of activity to suppress hTERT expression and telomerase activity in gingols might be due to the presence of the β-hydroxy group in the hydrocarbon side chain. The length of the side chain in paradols and shogaols might not affect the activity because all four subfractions (E2.1–E2.4), which have different compositions of paradols and shogaols (Table 1), had the capacity to suppress hTERT expression and telomerase activity.

**Influence of ZOE on Acute Toxicity and DEN-Induced Clastogenicity in Rats.** To evaluate the biosafety of our ZOE, the same ZOE used in the above experiments was assayed for acute toxicity, clastogenicity, and anticlastogenicity in rats. The results from the acute toxicity test showed that rats did not show any signs of toxicity or mortality after treating with the single maximum dose of 5000 mg/kg bw of ZOE for 14 days. Their body weight, food and water intake, and relative organ weight were also similar to those from the control group (Supporting Information, Tables S1 and S2). These results suggest that ZOE is safe in rats, which is in agreement with the ranking of ginger in the generally recognized as safe (GRAS) list by the Food and Drug Administration.

We then employed the liver micronucleus assay to investigate the clastogenicity and anticlastogenicity of ZOE in rats. The treatment scheme is illustrated in Figure 6. Male Wistar rats were divided into four groups: Group 1 is a negative control group, whereas group 3 is a positive control group. Group 2 is the experiment group to determine the clastogenic effect of ZOE, whereas group 4 is the experiment group to determine the anticlastogenic effect of ZOE. The liver micronucleus was used as the end-point marker.

![Figure 5. Chemical structures of gingerols, shogaols, and paradols.](image)

![Figure 6. Protocol for clastogenicity and anticlastogenicity determination of ZOE in rats.](image)
Table 2. Influence of ZOE on DEN-Induced Clastogenicity in Rats$^a$

| test group                        | rat body weight (g) | MNH (per 1000 HEP) | mitotic index |
|-----------------------------------|---------------------|--------------------|---------------|
|                                   | initial             | final              |               |
| negative control (5% Tween 80)    | 113 ± 3             | 289 ± 14           | 1.62 ± 0.85   | 3.36 ± 0.45   |
| ZOE (500 mg/kg bw)                | 112 ± 7             | 284 ± 5            | 1.79 ± 0.45   | 3.13 ± 0.36   |
| positive control (DEN + 5% Tween 80) | 110 ± 6          | 258 ± 11*          | 8.51 ± 1.82*  | 3.25 ± 0.33   |
| DEN + ZOE (DEN + 500 mg/kg bw)    | 110 ± 6             | 265 ± 4*           | 4.24 ± 1.02** | 3.39 ± 0.52   |

$^a$Values expressed as mean ± SD; MNH: micronucleated hepatocytes; HEP: hepatocytes; DEN: diethylnitrosamine; (*) indicates statistical significance at $p < 0.05$ compared to the negative control group; (**) indicates statistical significance at $p < 0.05$ compared to the positive control group.

**DISCUSSION**

Reproductive senescence is a basic feature of normal somatic cells and is widely considered as a cancer prevention mechanism. However, 85–90% of cancers escape this phenomenon by reactivating telomerase, which adds telomeric repeats to the 3’-end of telomeres. Telomerase-specific inhibition should, therefore, not affect normal somatic cells. It would render cancer cells entering replicative senescence naturally, with the manifestation of telomere erosion occurring with each round of cell division similar to that of normal somatic cells. With this safe mode of action, telomerase-specific inhibition is an attractive strategy for cancer chemoprevention.

Dietary phytochemicals have attracted considerable interest for cancer prevention for some time because of their potential therapeutic effects and safety. In search of telomerase inhibitors from plants, a few natural phytochemicals were found to inhibit telomerase in cancer cells; these include curcumin, EGCG, resveratrol, genistein, sulforaphane, and pristimerin, among others. However, it is worth noting that only a few reports show the long-term effect of these phytochemicals on telomere shortening and cellular senescence after successive rounds of cell divisions using a nontoxic dose. This is probably due to their broad mechanism of actions and cytotoxicity. Among these few reports, EGCG is the only example that was found to induce telomere shortening and cellular senescence (in U937 leukemia cells and HT29 colon adenocarcinoma cells) with its nontoxic dose up to 55–60 population doublings. Curcumin was also found to induce telomere shortening in several brain tumor cell lines after treating these cells with half the concentration of their IC$_{50}$ for 15 days. Similar to curcumin, thymoquinone (a compound from *Nigella sativa*) was found to induce telomere shortening in glioblastoma cell line M059K after treating these cells with approximately half the concentration of their IC$_{50}$ for 15 days. The *Inula viscosa* extracts, and its purified sesquiterpene lactone, tomentosin, were claimed to induce telomere shortening in cervical cancer cells. However, these two reports only measured the length of the 3’ G-rich telomeric overhang after the cells were treated with the test sample for 72 h.

In the present study, we add ZOE to this short list of plant-originated telomerase inhibitors that induce telomere shortening and cellular senescence in cancer cells using subcytotoxic doses. We first demonstrated that ZOE could suppress *hTERT* expression and telomerase activity in A549 lung cancer cells. The cells treated with subcytotoxic doses of ZOE proliferated normally but were soon found to manifest telomere shortening and cellular senescence. Using assay-guided fractionation and GC/MS analysis, we found several paradols and/or shogaols as the major compounds in the active subfractions. The results from pure 6-paradol and 6-shogaol showed that these two compounds could suppress *hTERT* expression and telomerase activity in the A549 cells, although 6-gingerol could not. We have concluded that the active compounds in ZOE that are responsible for the suppression of *hTERT* expression and telomerase activity in A549 lung cancer cells are paradols and shogaols. Last, we also found that ZOE did not induce acute toxicity in rats and showed the anticlastogenic effect against liver micronucleus formation in rats.

Nowadays, much attention has been focused on products derived from food sources that provide extra health benefits. Cancer chemoprevention by dietary phytochemicals is of considerable interest because of their potential therapeutic effects and safety. Many dietary phytochemicals were found to possess chemopreventive properties in various epidemiological and preclinical studies. Recently, clinical trials have added to the evidence supporting the efficacy of some selected compounds. Ginger (*Z. officinale Roscoe*), an already established nutraceutical product, has been reputed to have anticancer properties in various experimental models. Experiments in A549 lung cancer cells reveal that gingerol, shogaol, and zerumbone exhibit anticancer activity through various mechanisms. For example, gingerol was found to sensitize human lung cancer cells apoptosis. 6-Shogaol was found to induce autophagy through the AKT/mTOR pathway and inhibit cancer via microsomal prostaglandin E2 synthase 1 (mPGES-1), β-catenin, and glycogen synthase kinase 3β (GSK-3β) pathways. Moreover, 6-shogaol and its cysteine-conjugated metabolite induce lung cancer cell apoptosis through a p53 pathway in both in vitro and in vivo experiments. Zerumbone was also found to suppress cell invasion through inhibiting the FAK/AKT/ROCK pathway.

Our present study shows that paradols and shogaols in the ginger extract (ZOE) suppressed telomerase, which led to telomere shortening and cellular senescence, with a significant reduction in the clonogenicity of the A549 lung cancer, using only subcytotoxic doses. The extract was also found to be safe in rats, with an additional chemoprotective effect against DEN-induced liver micronucleus formation. These results lead us to believe that the ginger extract could potentially be a valuable tool in dietary cancer prevention against lung cancer.

**METHODS**

**Chemicals.** We purchased all materials from commercial suppliers. All oligonucleotides were supplied by Ward Medic (Thailand). Sulforhodamine B, 6-gingerol, and DEN were purchased from Sigma-Aldrich. Taq DNA polymerase was purchased from Vivantis. The collagenase type IV was purchased from Invitrogen (USA). 6-shogaol and 6-paradol were provided by Prof. Apichart Suksamrarn (details of which are provided in the next section).
of extraction, purification, and identification are shown in Supporting Information, S1).

**Plant Collection and Extraction.** Ginger rhizomes were collected in March 2012 from Lampang Province, Thailand, and was identified as Z. officinale Roscoe. A voucher specimen (BKF no. 118527) was deposited in the Forest Herbarium, Ministry of Natural Resources and Environment, Bangkok, Thailand. For the assay-guided fractionation study, ginger rhizomes (4.0 kg) were air-dried and finely pulverized. The ginger powder was then extracted into hexane, ethyl acetate, acetone, and methanol sequentially. After drying the solvents, we afforded 371.5, 235.8, 276.1, and 189.6 g of residues, respectively. The crude ethyl acetate extract (235.8 g) was purified by column chromatography using silica gel (Merck no. 7734, Mesh 70–230 ASTM) as the stationary phase. The first mobile phase was a gradient mixture of hexane (1 L) and ethyl acetate (1 L), followed by a gradient mixture of ethyl acetate (1 L) and methanol (1 L). The eluent was collected in a series of fractions, and the composition was visualized by TLC, before the fractions with similar composition were collected into four main fractions, E1–E4. After evaporation, dry powders of E1 (85.7 g), E2 (16.5 g), E3 (23.1 g), and E4 (46.2 g) were obtained. The E2 fraction was found to suppress hTERT mRNA expression and telomerase activity, and 10 g of the E2 fraction was further purified by the same chromatography. The first mobile phase was a gradient mixture of hexane (100 mL) and ethyl acetate (100 mL), followed by a gradient mixture of ethyl acetate (100 mL) and methanol (100 mL). After evaporation, dry powders of E2.1 (2.2 g), E2.2 (1.7 g), E2.3 (3.1 g), and E2.4 (2.8 g) were obtained. These fractions and subfractions were dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C. Prior to use, the stock solutions were diluted with distilled deionized water to working solutions with the same concentration of DMSO. The final concentration of DMSO was the same in all samples and was between 0.16 and 0.64% in the short-term cell culture experiments. For the long-term treatment of A549 cells, the crude ethyl acetate fraction of Z. officinale (ZOE) was obtained in a similar manner. The final concentration of DMSO in both experimental set and control set was 0.05%.

**TLC Analysis.** ZOE and its fractions and subfractions were TLC fingerprinted using silica gel GF254 (Fluka) as the stationary phase, and a mixture of hexane and ethyl acetate was used as the mobile phase. The TLC plate was then dipped in p-anisaldehyde/sulfuric acid reagent before color developing by heating at 100 °C.

**HPLC Analysis.** We performed the HPLC analyses using an HPLC system (Agilent 1200 Infinity series, Agilent, USA) and a C18 reverse phase column (4.6 mm × 150 mm, ZORBAX Eclipse Plus) as the stationary phase. A 50 μL sample of ZOE and its fractions (E1–E4) (10 mg/ml) were separated using a mixture of acetonitrile (A) and water (B) as the mobile phase at a flow rate of 2.0 mL/min. Within the total run time of 10 min, the solvent mixture profile of the mobile phase was as follows: 0–0.5 min, 35A:65B; 0.5–5 min, gradient mixture from 35A:65B to 95A:5B; and 5–10 min, 95A:5B. The compounds were detected using a UV–visible detector (Spec Monitor 3200) at 280 nm. A mixture of standard 6-gingerol (0.4 mg/mL), 6-shogaol (0.2 mg/mL), and 6-paradol (0.2 mg/mL) was run separately using the same conditions.

**Cell Culture.** The A549 human lung carcinoma cell line was obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 culture media with 10% fetal bovine serum and 1% antibiotics (50 μg/mL streptomycin, 50 units/mL penicillin) at 37 °C in a humidified incubator with 5% CO2.

**In Vitro Growth Inhibition Assays.** We determined the growth inhibition of A549 cells by the ginger extract and selected ginger compounds using the sulforhodamine B (SRB) assay according to a published protocol.44 The A549 cells (1.0 × 104 cells) were incubated with various concentrations of the indicated ginger fraction or the pure compound at 37 °C for 72 h in a humidified incubator with 5% CO2. The graphs between the concentration of the test sample and the percentage of cell viability, from three independent experiments, were plotted, and the 50% growth inhibitory concentrations (IC50) were determined. The IC50 growth inhibitory concentrations of A549 lung cancer cells by ZOE, its subfractions, and some pure compounds are summarized in the Supporting Information (Table S3).

**Semiquantitative RT-PCR Analysis.** We grew A549 cells (5.0 × 105 cells) on a six-well tissue culture plate for 24 h at 37 °C before they were treated with the indicated concentration of the test sample for another 24 h. The total RNA was collected, and the mRNA was converted into cDNA using RevertAid reverse transcriptase (Thermo Scientific). PCR amplification for each gene was carried out with the gene-specific primers. The primer sequences, annealing temperatures, and PCR cycles are summarized in the Supporting Information (Table S4).

**Modified TRAP Assay.** We performed the TRAP assay according to a published protocol and our previous publication.23,45 Briefly, the A549 cells (5.0 × 105 cells) were seeded on a six-well tissue culture plate for 24 h before the test sample at the indicated concentration was added to the culture media. The cells were incubated for another 48 h before they were lysed with 200 μL of CHAPS lysis buffer. The 10 μg of the crude cell extract was then used as the telomerase source in the telomerase reaction mixture, in which it extends a primer at 30 °C for 30 min. The DNA amplification mixture was added, and the telomerase-extended products were amplified by PCR. The amplified products were separated by nondenaturing acrylamide gel electrophoresis, and results were recorded using a phosphoimaging system (Typhoon; Molecular Dynamics). The oligonucleotides used in this assay are summarized in the Supporting Information (Table S5).

**Long-Term Proliferation Assay.** We compared three sets of A549 cell cultures for the long-term proliferation assay: the control group and the two experimental groups with the subcytotoxic doses (5 and 10 μg/mL) of ZOE added in the culture media. We subcultured A549 cells (1.5 × 105 cells) onto a 75 cm2 tissue culture flask, with or without the indicated concentration of ZOE, in RPMI 1640 medium supplemented with 10% fetal bovine serum. We changed the culture media after 3 days, and on day 6, we trypsinized, collected, and counted the cells. Then, the process was repeated up to 60 days. The equation: $n = \left(\log P_n - \log P_0\right)/\log 2$, where $P_n$ is the number of cells after n doublings and $P_0$ is the initial seeding density, was then used to calculate the number of population doublings. The graph between the cumulative number of population doublings and time was then plotted.

**Telomere Length Assay.** We performed a telomere length analysis using the TeloTAGGG Telomere Length Assay kit (Roche Applied Science) according to the manufacturer’s instruction and our published protocol.46 The formula $\sum(OD_1)/\sum(OD/L_1)$, where ODi indicates the signal at the
position i and \( L_i \) is the molecular weight marker at the same position, was used to calculate the mean TRF length.

Senescence-Associated β-Galactosidase Activity Assay. We performed a β-galactosidase activity assay according to our published protocol.\(^{46}\) We first grew the A549 cells (1 × 10⁵ cells) collected from the long-term proliferation study in a six-well plate for 24 h before they were fixed with 2% formaldehyde and 0.2% glutaraldehyde solution. After that, the cells were washed and incubated in the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution overnight at room temperature. Cells with β-galactosidase activity cleave X-gal and produce a blue stain. The β-galactosidase positive cells were monitored under a phase contrast microscope with a blue stain, usually accompanied by cell morphological changes. In each experiment, the blue-stained cells were counted in the field with more than 400 cells for at least 10 fields. The graph between the percentage of β-galactosidase positive cells and time was then plotted.

Colony-Forming Assays. We performed a colony-forming assay according to our published protocol.\(^{46}\) We first seeded the A549 cells collected from the long-term proliferation assay in a 10 cm dish at a low density (2 × 10⁵ cells) and allowed the colonies to form for 2 weeks, with the change of fresh growth media every 3 days. Crystal violet was then used to stain the colonies. The number of colonies was obtained using the ImageQuant TL software (Nonlinear Dynamics).

Gas Chromatography/Mass Spectrometry. GC/MS data were collected with a GC7890 instrument from Agilent Technologies, with a DB-5MS column (30 m × 0.25 μm film thickness). The temperature programming was as follows: 50 °C, 5 min; to 200 °C at 10 °C/min; to 250 °C at 5 °C/min; and 250 °C, 35 min, and the ionizing voltage was 70 eV, with 1 μL split injection (split ratio 25:1). The flow rate of helium gas was 1.5 mL/min. The identification of compounds was obtained using the Agilent Enhanced Chemstation MSD Data Analysis Tool with the W8N08 mass spectrum library (John Wiley & Sons, Inc., USA).

Animals. Wistar rats were fed with the CP082 diet (Perfect Companion Group) and tap water ad libitum under constant conditions of 12 h light/dark cycle and 50–60% humidity at 25 °C. The Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University, approved our experimental protocols.

Acute Toxicity Test. The acute toxicity of ZOE was evaluated according to OECD Guideline 425.\(^{47}\) The 6 weeks old (190–200 g) female Wistar rats were divided into two groups of five rats. The vehicle control group received 5 mL/kg of 5% Tween 80, whereas the experimental group received a single dose of 5000 mg/kg bw of ZOE by oral gavage. The body weight, behaviors, signs of toxicity, and mortality were observed and recorded every day for 14 days. The gross pathological observation of the tissues and organs was performed after the rats were sacrificed at the end of the study.

Liver Micronucleus Assay. We performed a liver micronucleus assay according to our published protocol.\(^{48}\) The 4 weeks old (110–120 g) male Wistar rats were divided into four groups of six rats. Group 1 was a negative control group in which the rats were supplemented with 5% Tween 80 for 28 days, whereas group 3 was a positive control group in which the rats were treated in the same way as group 1 but with an injection of 30 mg/kg bw of DEN on day 22 and day 25 to induce micronucleated hepatocytes. Group 2 was the experimental group to determine the clastogenic effects of ZOE, in which 500 mg/kg bw of ZOE was administered to rats by oral gavage for 28 days. Group 4 was the experimental group to determine the anticlastogenic effects of ZOE, in which 500 mg/kg bw of ZOE was administered to rats by oral gavage for 28 days, and rats were injected with 30 mg/kg bw of DEN on day 22 and day 25. On day 29, partial hepatectomy was performed, and single hepatocytes were isolated by the two-step collagenase perfusion method. The liver cell suspension was stained with the 4’,6-diamidino-2-phenylindole solution, and the incidence of micronucleated hepatocytes was analyzed under a fluorescent microscope on day 33, 4 days after partial hepatectomy. The initial body weight, final body weight, and mitotic index were also recorded.

Statistical Analysis. For assays in cell culture, data were taken from triplicate samples of three independent experiments. Statistical significance between treatments and controls was analyzed using Student’s t-test analysis. For assays in rats, data of each variable for each group are reported as means ± SD. The significance of differences between groups was analyzed using one-way analysis of variance with the least significant difference for post hoc tests. Values of \( p < 0.05 \) (*) were considered to be significant.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02853. TLC fingerprinting and HPLC chromatograms of the crude ethyl acetate extract of Z. officinale (ZOE) and its fractions; effects of some ZOE fractions on hTERT expression and telomerase activity; GC chromatogram and selected GC/MS spectra of ginger extract subfractions (E2.1–E2.4); effects of 6-gingerol, 6-paradol, and 6-shogaol on gene expression and telomerase activity in A549 lung cancer cells; effects of ZOE on body weight, food and water intake and relative organ weights from acute cytotoxicity tests in rats; extraction, purification, and identification of 6-paradol and 6-shogaol; IC₅₀ of ZOE, its subfractions, and selected pure compounds; primer sequences and conditions used in semiquantitative RT-PCR; and oligonucleotides used in the modified fluorescent TRAP assay (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Meena, J.; Rudolph, K. L.; Günes, C. Telomere dysfunction, chromosomal instability and cancer. Recent Results Cancer Res. 2015, 200, 61–79.

(2) Tomita, K.; Cooper, J. P. The telomere bouquet controls the meiotic spindle. Cell 2007, 130, 113–126.

(3) Martinez, P.; Blasco, M. A. Role of shelterin in cancer and aging. Aging Cell 2010, 9, 653–666.

(4) Shay, J. W.; Wright, W. E. Hayflick, his limit and cellular ageing. Nat. Rev. Mol. Cell Biol. 2000, 1, 72–76.

(5) Victorelli, S.; Passos, J. F. Telomeres and cell senescence - Size matters not. EBioMedicine 2017, 21, 14–20.

(6) Benarroch-Popivker, D.; Pisano, S.; Mendez-Bermudez, A.; Lototska, L.; Kaur, P.; Bauwens, S.; Djerbi, N.; Latrick, C. M.; Fraisher, V.; Pei, B.; Gay, A.; Jaune, E.; Foucher, K.; Chертиль-Винчи, J.; Aebly, E.; Miron, S.; Londoño-Vallejo, A.; Ye, J.; Le Du, M.; Wang, H.; Gilson, E.; Giraud-Panis, M.-J. TRF2-mediated control of telomere DNA topology as a mechanism for chromosome-end protection. Mol. Cell. 2016, 61, 274–286.

(7) Ohtani, N.; Mann, D. J.; Hara, E. Cellular senescence: Its role in tumor suppression and aging. Cancer Sci. 2009, 100, 792–797.

(8) Kim, N.; Patytszek, M.; Prowse, K.; Harley, C.; West, M.; Ho, P.; Coviello, G.; Wright, W.; Weinrich, S.; Shay, J. Specific association of human telomerase activity with immortal cells and cancer. Science 1994, 266, 2011–2015.

(9) Kumar, M.; Lechel, A.; Günes, Ç. Telomerase: The Devil Inside. Genes 2016, 7, No. E43.

(10) Cohen, S. B.; Graham, M. E.; Lovrecz, G. O.; Bache, N.; Robinson, P. J.; Reddel, R. R. Protein composition of catalytically active human telomerase from immortal cells. Science 2007, 315, 1850–1853.

(11) Ramlee, M. K.; Wang, J.; Toh, W. X.; Li, S. Transcription regulation of the human telomerase reverse transcriptase (hTERT) gene. Genes 2016, 7, No. E50.

(12) Scalbert, A.; Andres-Lacueva, C.; Arita, M.; Kroon, P.; Manach, C.; Urpi-Sarda, M.; Wishart, D. Databases on food phytochemicals and their health-promoting effects. J. Agric. Food Chem. 2009, 57, 653−666.

(13) Gullett, N. P.; Ruhul Amin, A. R. M.; Bayraktar, S.; Pezzuto, J. M.; Shin, D. M.; Khuri, F. R.; Aggarwal, B. B.; Surh, Y.-J.; Kucuk, O.; Imsumran, A.; Makonkawkeyoon, L.; Pompimon, W.; Lee, T. R. Ginger extract inhibits human telomerase reverse transcriptase and c-Myc expression in A549 lung cancer cells. J. Med. Food 2010, 13, 1347–1354.

(14) Hemann, M. T.; Strong, M. A.; Hao, L.-Y.; Greider, C. W. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell 2001, 107, 67–77.

(15) Zhao, Y.; Li, W.; Liang, W.; Van Bremeren, R. B. Identification and Quantification of Gingerols and Related Compounds in Ginger. Diet and Nutrition Forum. 2010, 3, 18572–18580.

(16) Alibakhshi, A.; Ranjbari, J.; Pilehvar-Soltanahmadi, Y.; Nasiri, M.; Mollazade, M.; Zarghami, N. An update on phytochemicals in molecular target therapy of cancer: Potential inhibitory effect on telomerase activity. Curr. Med. Chem. 2016, 23, 2380–2393.

(17) Nasani, L.; Seimiya, H.; Tsuurno, T. Telomerase inhibition, telomere shortening, and senescence of cancer cells by tea catechins. Biochem. Biophys. Res. Commun. 1998, 249, 391–396.

(18) Khaw, A. K.; Hande, M. P.; Kalthur, G.; Hande, M. P. Curcumin inhibits telomerase and induces telomere shortening and apoptosis in brain tumour cells. J. Cell. Biochem. 2013, 114, 1257–1270.

(19) Gurung, R. L.; Lim, S. N.; Khaw, A. K.; Soon, J. F. F.; Shenoy, K.; Mohamed Ali, S.; Jayapal, M.; Sethu, S.; Baskar, R.; Hande, M. P. Thymoquinone induces telomere shortening, DNA damage and apoptosis in human glioblastoma cells. PLoS One 2010, 5, e12214.

(20) Meena, J.; Rudolph, K. L.; Günes, C. Telomere dysfunction, chromosomal instability and cancer. Recent Results Cancer Res. 2015, 200, 61–79.
(38) Baena Ruiz, R.; Salinas Hernández, P. Cancer chemoprevention by dietary phytochemicals: Epidemiological evidence. *Maturitas* 2016, 94, 13–19.
(39) Nazim, U.; Jeong, J.-K.; Seol, J.-W.; Hur, J.; Eo, S.-K.; Lee, J.-H.; Park, S.-Y. Inhibition of the autophagy flux by gingerol enhances TRAIL-induced tumor cell death. *Oncol. Rep.* 2015, 33, 2231–2236.
(40) Hung, J.-Y.; Hsu, Y.-L.; Li, C.-T.; Ko, Y.-C.; Ni, W.-C.; Huang, M.-S.; Kuo, P.-L. 6-Shogaol, an active constituent of dietary ginger, induces autophagy by inhibiting the AKT/mTOR pathway in human non-small cell lung cancer A549 cells. *J. Agric. Food Chem.* 2009, 57, 9809–9816.
(41) Eren, D.; Betul, Y. M. Revealing the effect of 6-gingerol, 6-shogaol and curcumin on mPGES-1, GSK-3β and β-catenin pathway in A549 cell line. *Chem. Biol. Interact.* 2016, 258, 257–265.
(42) Warin, R. F.; Chen, H.; Soroka, D. N.; Zhu, Y.; Sang, S. Induction of lung cancer cell apoptosis through a p53 pathway by [6]-shogaol and its cysteine-conjugated metabolite M2. *J. Agric. Food Chem.* 2014, 62, 1352–1362.
(43) Kang, C. G.; Lee, H.-J.; Kim, S.-H.; Lee, E.-O. Zerumbone suppresses osteopontin-induced cell invasion through inhibiting the FAK/AKT/ROCK pathway in human non-small cell lung cancer A549 cells. *J. Nat. Prod.* 2015, 79, 156–160.
(44) Vichai, V.; Kirtikara, K. Sulfon rhodamine B colorimetric assay for cytotoxicity screening. *Nat. Protoc.* 2006, 1, 1112–1116.
(45) Szatmari, I.; Aradi, J. Telomeric repeat amplification, without shortening or lengthening of the telomerase products: a method to analyze the processivity of telomerase enzyme. *Nucleic Acids Res.* 2001, 29, No. E3.
(46) Taka, T.; Huang, L.; Wongnopavich, A.; Tam-Chang, S.-W.; Lee, T. R.; Tuntiwechapikul, W. Telomere shortening and cell senescence induced by perylene derivatives in A549 human lung cancer cells. *Bioorg. Med. Chem.* 2013, 21, 883–890.
(47) OECD Guidelines for the Testing of Chemicals. No. 425: Acute Oral Toxicity-Fixed Dose Procedure, 2008.
(48) Punvittayagul, C.; Sankam, P.; Taya, S.; Wonggroomchai, R. Anticlastogenicity and anticarcinogenicity of purple rice extract in rats. *Nutr. Cancer* 2016, 68, 646–653.