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Excellent anti-proliferative and pro-apoptotic effects of (−)-epigallocatechin-3-gallate encapsulated in chitosan nanoparticles on human melanoma cell growth both in vitro and in vivo

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Abstract

Earlier we demonstrated the anti-proliferative and pro-apoptotic effects of green tea polyphenol epigallocatechin-3-gallate (EGCG) on human melanoma cells (*Int J Cancer*. 2005; 114(4): 513-21). The doses used in this study were not physiologically attainable and for chemoprevention the preferred route of administration is oral consumption. To overcome these shortcomings, and taking advantage of our novel concept of nanochemoprevention (*Cancer Res*. 2009;69(5):1712-6), we developed a nanotechnology based oral delivery system to encapsulate EGCG. Here, using human melanoma Mel 928 cells we demonstrate 8-fold dose advantage of this nanoformulation over native EGCG. Further, nano-EGCG treated cells showed marked induction of apoptosis and cell cycle inhibition along with the growth of Mel 928 tumor xenograft. Nano-EGCG also inhibited proliferation (Ki-67 and PCNA) and induced apoptosis (Bax, PARP) in tumors harvested from the treated mice. These observations warrant further *in vivo* efficacy studies of nano-EGCG in robust animal models of human melanoma.

From the Clinical Editor: This team of investigators developed a nanotechnology based oral delivery system to encapsulate EGCG, a green tea-derived polyphenol in chitosan nanoparticles. Using human melanoma cells, an eight-fold dose advantage was demonstrated over native EGCG, leading to measurable apoptosis induction and proliferation inhibition, warranting further in vivo investigations.

Key words: Melanoma; Nanotechnology; EGCG; Chitosan

Melanoma is the most deadly form of skin cancer, accounting for greater than 76% of skin cancer deaths despite the fact that it only accounts for less than 5% incidence rate.[1] Unlike other common types of cancers, the incidence of melanoma continues to rise and the American Cancer Society estimates that ~76,690 new melanomas will be diagnosed in 2013 and about 9480 people are expected to die of melanoma[1] (http://www.skincancer.org/Skin-Cancer-Facts/#melanoma). Melanomas are also well known for their capability to resist apoptosis induction[2-4] which however has been observed to circumvent by the use of some dietary food components.[5-10] One such agent which is highly explored and tested in a variety of cancer models is green tea polyphenol -(−)epigallocatechin-3-gallate (EGCG) (Figure 1 inset), which has been shown to impart anti-proliferative and chemopreventive effects against several cancers including skin cancer.[11-14]

We have earlier demonstrated the anti-proliferative and pro-apoptotic effects of EGCG on human melanoma cells.[2,5] These studies were accomplished with doses that are not achievable at physiologically attainable levels of EGCG and thus we intensified our efforts and introduced a novel concept of '*nanochemoprevention*'[15] and then prepared a nanoformulation of EGCG suitable for oral consumption[16] by encapsulating natural

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biopolymer chitosan nanoparticles which were stable in the acidic environment of the stomach and resulted in steady and sustained release of EGCG. Chitosan nanoparticles have their characteristic mucoadhesive properties and they also adhere to the gastrointestinal tract for a longer time. Thus, chitosan nanoparticles' ability to increase retention time, ultrafine size, and ability to release the drug for a longer time make them ideal oral delivery vehicle.

Under this study, we report that the EGCG released from this nanosystem is as potent as its native counterpart although at a much lower concentration in Mel 928 human melanoma cells. Also, the antitumor efficacy of this uniquely formulated nano-EGCG was assessed in subcutaneously implanted tumor xenograft in athymic nude mice and the data suggested a significant improvement of therapeutic benefit against melanoma tumors compared to the native agent.

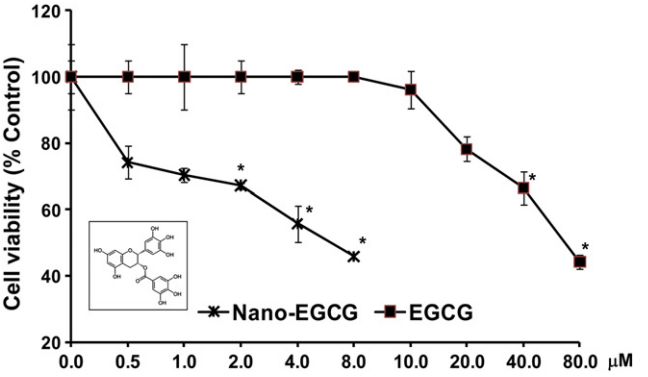

Figure 1. Comparative effects of nonencapsulated EGCG and nano-EGCG treatment on cell viability. Mel 928 cells were treated with EGCG and nano-EGCG for 24 h, and cell growth was determined by MTT assay. Points, mean of three separate experiments wherein each treatment was repeated in 10 wells; vertical bars, SE. *, $P < 0.001$ compared with the vehicle-treated controls. The experiment was repeated four times with comparable results. Inset: Structure of EGCG.

Methods

Materials

Anti- Bax, Bcl2, active caspases-3 and 9 and PARP antibodies were obtained from Cell Signaling Technology (Beverly, MA). Cyclin D1 and D3 antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Ki-67 and PCNA antibodies were purchased from Abcam (Cambridge, MA). Anti-mouse and anti-rabbit secondary antibody horse radish peroxidase conjugates were obtained from Cell Signaling Technology (Beverly, MA). BCA Protein assay kit was obtained from Pierce (Rockford, IL). Novex precast Tris–glycine gels were from BioRad (Hercules, CA). EGCG, Pentasodium tripolyphosphate hexahydrate (TPP), heptafluorobutyric acid (HFBA), formaldehyde, acetonitrile, and cellulose dialysis tubing were purchased from Sigma Aldrich Co (St. Louis, MO). Water soluble chitosan was obtained from Polysciences Inc. (Warrington, PA). The human melanoma Mel 928 cells were a kind gift from Prof. Paul Robbins (NCI, Bethesda).

Cell viability

Cell viability was determined after 48 h by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.[17] Briefly, cells were plated at 5×10^3 per well in 1 ml complete culture medium in 24 well plate. Cells (60% confluent) were treated with native EGCG (in PBS) and nano-EGCG with the indicated doses. 48 h post-treatment the medium was removed and cells were washed with PBS and incubated for 90 min with MTT reagent (300 μL, 0.5 mg/ml final concentration in medium). The MTT solution was removed and the formazan crystals were dissolved in DMSO and absorbance was recorded at 570 nm on a microplate reader. Experiment was repeated three times with similar results. The effect of each treatment was assessed as percentage of cell viability in which not untreated control was taken as 100% viable.

Cell cycle analysis

Mel 928 cells were treated with EGCG (in PBS) or nano-EGCG for 24 h and processed for labeling with fluorescein-tagged dUTP nucleotide and propidium iodide using the APO-DIRECT kit (Phoenix Flow Systems, CA) and analyzed using Modfit software. Briefly, the treated cells were fixed in 1% paraformaldehyde (methanol free) in PBS for 60 min on ice, centrifuged (5 min; 300×g), washed with PBS and pelleted again. Cells were resuspended in 70% ice cold ethanol and kept on ice for 30 min. The cells were pelleted (5 min; 300×g) and washed twice in the wash buffer provided in the kit. DNA labeling solution containing TdT enzyme and FITC dUTP was used to label the cells for 60 min at 37 °C. Cells were again washed twice by centrifuging in wash buffer and finally the pellet was resuspended in PI/RNase staining buffer provided in the kit and analyzed using a FACSCalibur.

Protein extraction and immunoblotting

The treated cells or tumor tissue samples were homogenized on ice by sonication in ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, pH 7.4) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA). The homogenate was then centrifuged at 14,000 g for 25 min at 4 °C, and the supernatant was collected, aliquoted and stored at −80 °C. For immunoblotting, 30-50 μg protein was resolved over 8-12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (7% non-fat dry milk/1% Tween 20; in 20 mM TBS, pH 7.6) for 1 h at room temperature, incubated with appropriate primary antibody in blocking buffer overnight at 4 °C, followed by incubation with horseradish peroxidase conjugate secondary antibody and detected by chemiluminescence and auto radiography using Bio-Rad Gel-Doc System (Bio-Rad Laboratories Inc., Hercules, CA).

In vivo tumor xenograft study

Athymic (nu/nu) male nude mice (Harlan Laboratories, Madison, WI) were housed under pathogen-free conditions with a 12-h light/dark cycle. Thirty two male athymic nude mice (3-5 weeks of age) were housed four/cage and fed ad libitum with autoclaved diet and

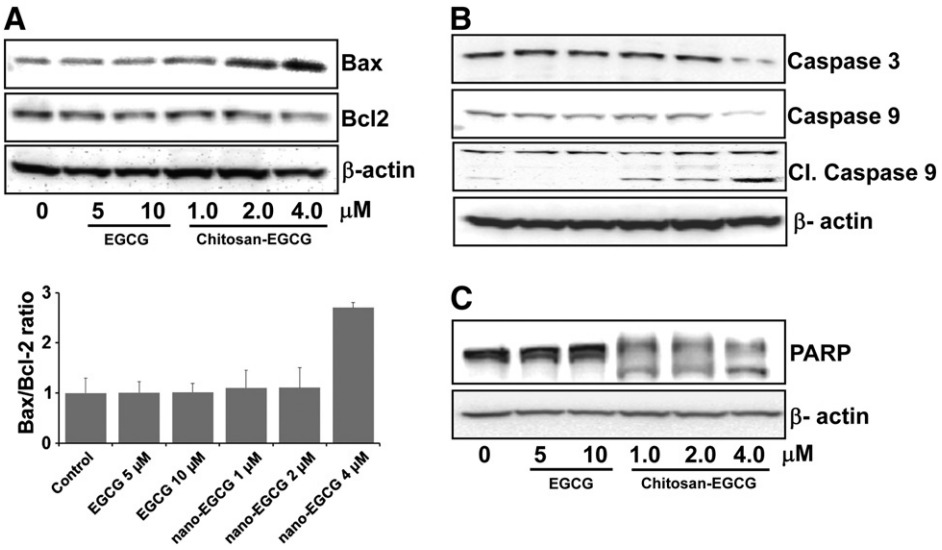

Figure 2. Comparative effects of nonencapsulated EGCG and nano-EGCG on apoptotic biomarkers. **(A)** Protein expression of Bax, Bcl2, and the Bax/Bcl2 ratio. **(B)** Protein expression of caspases 3, 9 and cleaved caspase 9. **(C)** Protein expression of cleaved PARP. The cells were treated with each agent and harvested 24 h after treatments. Details of the experiments are described in Materials and methods. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Each experiment was repeated thrice with similar results.

water. A total of 1×10^6 Mel 928 cells (in 50 μl DMEM + 50 μl Matrigel) were implanted sub-cutaneously on left and right flanks. Mice implanted with the cells were randomly divided into four groups of eight each. One day post inoculation, animals of group I received void chitosan nano particles or PBS (4 mice each; 100 μl treatment volume) and served as control, group II received EGCG (1 mg/mice in 100 μl PBS), group III received EGCG (100 μg/mice in 100 μl PBS) and group IV received nano-EGCG (100 μg/mice; 120 μl treatment volume). All treatments were provided by oral intubation and five times a week. Treatments were continued until tumors in the individual groups reached a targeted volume of around 1300 mm^3. The tumor size was measured by calipers, and the volume was calculated using the formula $V = 0.5328 \times L \times B \times H$ (mm^3).[18] At the end of experiment, the animals were sacrificed and tumors were harvested and either snap-frozen or processed directly for further analyses. The animals were also evaluated for body weight, consumption of food to access apparent signs of toxicity.

Ethics statement

The studies were conducted according to the Institutional guidelines for the care and use of animals and were approved by Animal Care and Use Committee, School of Medicine and Public Health, University of Wisconsin-Madison.

Immunohistochemical analysis

Sections (5 μm thick) were cut from paraffin embedded tumor tissues. Immunostaining was performed using specific antibodies with appropriate dilutions and was replaced with either normal host serum or block for negative controls, followed by staining with appropriate HRP-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counter stained with a weak solution of hematoxylin stain. The stained slides were dehydrated and mounted in permount and visualized on Nikon Eclipse Ti system (Nikon Instruments, Inc., Melville, NY). Images were captured with an attached camera linked to a computer.

Statistical analysis

In all statistical analysis, the significance was set at a probability of $P < 0.05$. All results were reported as the means ± standard error (SEM). Statistical analysis was performed by Student's *t* test for two groups, and one-way ANOVA for multiple groups, followed by Newman–Keuls test if $P < 0.05$.

Results

Nano-EGCG decrease Mel 928 cell viability

To test the biological efficacy of our nanoconjugate system, we first compared the effectiveness of nano-EGCG versus nonencapsulated EGCG on proliferative ability in human melanoma Mel 928 cells. The cells were treated for 48 h with nano-EGCG, and/or native EGCG and evaluated for cell viability using the MTT assay. Treatment of cells with nanoparticles alone had negligible effect, thereby confirming the lack of any toxicity of nanoparticles (data not shown). Nano-EGCG, however, in comparison to native EGCG, produced remarkable cell growth inhibitory effects with about 8-fold better efficacy. As shown in Figure 1, the IC_{50} of nano-EGCG was approximately 7 μM while that of native EGCG was around 53 μM. Similar effects were seen at 72 h post-treatment suggesting a sustained release of EGCG from the nanoparticles (data not shown).

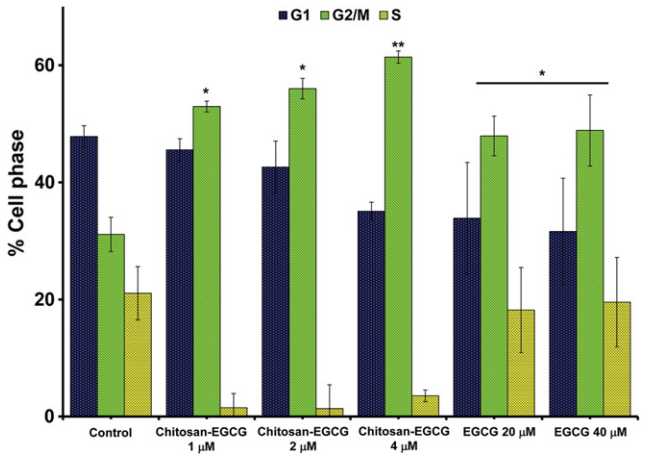

Figure 3. Comparative effects of nonencapsulated EGCG and nano-EGCG on cell cycle. Cells treated with EGCG or nano-EGCG were collected and stained with PI using the Apo-Direct Kit. Following FACS analysis, cellular DNA histograms were analyzed by ModFitLT V3.0. The data are representative of triplicate experiments. Bars, mean of one experiment wherein each treatment was repeated in quadruplicate; vertical bars, SE. *, $P < 0.05$; **, $P < 0.001$ compared with the cells in the same phase of cell cycle in vehicle-treated control group. The experiment was repeated four times with comparable results.

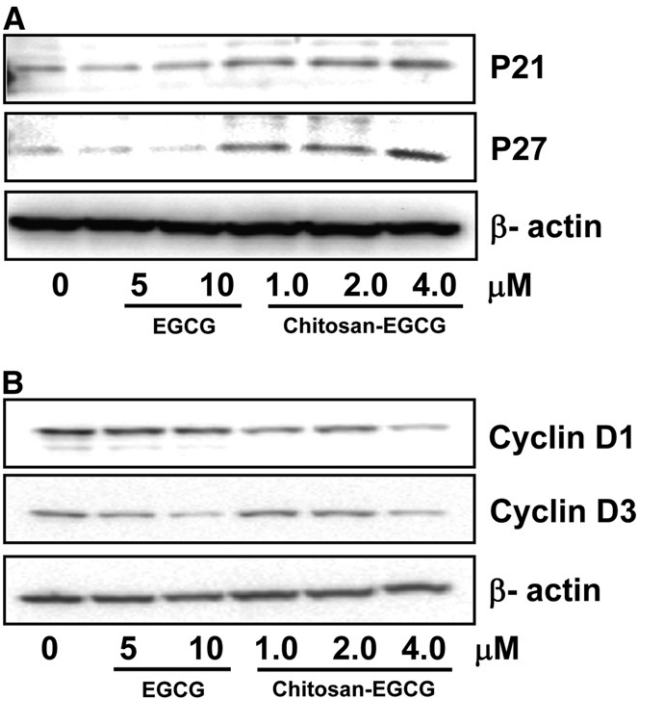

Figure 4. Comparative effects of nonencapsulated EGCG and nano-EGCG on cell cycle biomarkers. **(A)** Protein expression of P21 and P27. **(B)** Protein expression of Cyclin D1 and D3. The cells were treated with each agent and harvested 24 h after treatments. Details of the experiments are described in Materials and methods. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Each experiment was repeated thrice with similar results.

Nano-EGCG modulates intrinsic apoptotic pathway in Mel 928 cells

To determine if EGCG retains its mechanistic identity when encapsulated in chitosan nanoparticles, we studied several molecules that have been demonstrated to be modulated by EGCG in variety of cancer models. First we determined the effects of nano-EGCG on molecules known to be modulated by native EGCG and demonstrated to be involved in the initiation and execution of apoptosis. Using western blot analysis, we determined the effect on two key proteins Bax and Bcl-2 from the Bcl-2 family. The cells maintain a fine balance between these two proteins and any imbalance can be critical for the cells to undergo apoptosis.[19,20] Nano-EGCG treatment to Mel 928 cells resulted in a significant increase in pro-apoptotic Bax with an associated decrease in anti-apoptotic Bcl-2, thereby shifting the Bax/Bcl-2 ratio in favor of apoptosis (Figure 2, *A*). We observed a ratio of over 2.5 at 4 μM dose of nano-EGCG as compared to 10 μM of the native agent. We used a non-effective dose of native EGCG to draw a parallel with the doses of our nanoformulation. Since caspases play an important role in apoptosis, we further determined the protein expression of caspase 3 and 9 and observed a significant inhibition of both these proteins at the highest dose of nano-EGCG. We also observed a cleavage of caspase 9 which was dose dependent in the nano-EGCG treated cells (Figure 2, *B*). Next to further verify the apoptosis induction by nano-EGCG we looked at poly(ADP-ribose) polymerase (PARP) cleavage which is a marker for apoptosis. PARP has the ability to induce programmed cell death, via the production of PAR, which stimulates mitochondria to release apoptosis-inducing factor.[21] We observed a significant increase in the cleavage of the protein in cells treated with different concentrations of nano-EGCG. We found that full size PARP protein (116 KD) was cleaved to yield 85 KD fragment following nano-EGCG treatment (Figure 2, *C*). Importantly, these responses were observed at a concentration of nano-EGCG which otherwise does not produce any effects when delivered in the native form. All these data further support a remarkable dose advantage when EGCG was delivered in chitosan nanoparticles.

Nano-EGCG causes cell cycle arrest in Mel 928 cells

In the next set of experiments to investigate if the induction of apoptosis is mediated through cell cycle arrest, we performed DNA cell cycle analysis to determine the effect of nano-EGCG treatment on distribution of cells in various phases of cell cycle. As shown in Figure 3, nano-EGCG treatment to the Mel 928 cells resulted in dose dependent accumulation of cells in G2/M phase of the cycle. We observed 54, 58, and 65% cells in G2/M phase at 1, 2, and 4 μg/ml dose as compared to 45 and 47% with 20 and 40 μg/ml dose of native EGCG which was significant ($P < 0.05$ as compared to control group at 1 and 2 μM and $P < 0.001$ as compared to control at 4 μM dose). This is an important observation considering the fact that inhibition of cell cycle is largely appreciated as a target for management of cancers.

Nano-EGCG induced cell cycle arrest is mediated via induction of p21 and p27 and concomitant inhibition of Cyclin D1 and D3

In the next set of experiments we examined the effects of nano-EGCG to Mel 928 on key cell cycle regulatory molecules operative in G1 phase of the cell cycle. Studies have shown that p21/CIP1

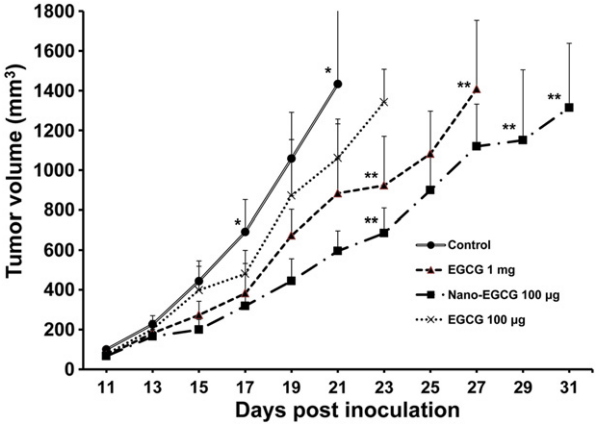

Figure 5. Comparative effects of nonencapsulated EGCG and nano-EGCG on tumor growth in a xenograft model. Details of the experiments are given in Materials and methods. Points, tumor volume (mm^3) of eight mice; vertical bars, SE. *, $P < 0.05$ compared with the data from control group at previous time point. **, $P < 0.01$ compared with the control group at the respective time point.

and p27/KIP1 are the master regulators of the cell cycle and apoptosis, thus we examined the protein expression of these molecules and observed a significant and dose dependent increase in both proteins with nano-EGCG while no change was seen with native EGCG at the tested concentrations (Figure 4, *A*). We further determined the protein expression of cyclins D1 and D3 which are thought to be regulated by p27. We observed a dose dependent inhibition of these proteins with native EGCG and nano-EGCG. The effects observed with nano-EGCG were more extensive and were observed at a much lower dose (Figure 4, *B*).

Nano-EGCG inhibits the growth of Mel 928 tumors in athymic nude mice

To establish the in vivo relevance of our in vitro data we implanted Mel 928 tumor xenografts on athymic$^{nu/nu}$ mice. The effect of oral administration of native EGCG (1 mg/mice in 100 µl PBS) and nano-EGCG (100 µg/mice) was tested on the growth of tumors in this mouse model. As shown in Figure 5, the targeted tumor volume of 1400 mm^3 was reached on day 32 post implantation in nano-EGCG treated group as compared to day 27 in EGCG treated group despite the fact that 10 fold higher dose of the native compound was used. In the group where 100 µg dose of native EGCG (equal to the nano-EGCG dose given in 100 µl PBS) was used the targeted volume was reached in only 22 days. In untreated control group of mice, however, the targeted tumor volume was reached within 20 days (Figure 5). At this point the calculated volume in the EGCG treated group was 820 mm^3 while in the nano-EGCG treated group was around 650 mm^3. These data clearly indicated that nano-EGCG imparts a more efficient tumor inhibition with a distinct dose advantage over its native counterpart. Throughout the experiment we did not see any noticeable difference in body weight gain and food and water consumption between control animals and treated groups suggesting that our nanoformulation or native EGCG does not cause any apparent toxicity to the animals (data not shown).

Induction of apoptosis in tumor tissues isolated from nano-EGCG treated mice

To establish the mechanism behind the observed inhibition of tumor xenograft growth we investigated the tumor lysates for key proteins affected during apoptosis. As shown in Figure 6, *A*, nano-EGCG treatment to the animals resulted in a significant increase in pro-apoptotic Bax with a concomitant decrease in anti-apoptotic Bcl-2, thereby shifting the Bax/Bcl-2 ratio in favor of apoptosis which was over 8 fold as compared to the control while the ratio was around 5 in the native EGCG treated group. Further, we also observed an increase in the cleavage of PARP protein in the tumors isolated from EGCG and nano-EGCG treated mice.

Induction of cell cycle inhibition in tumor tissues isolated from nano-EGCG treated mice

To further elucidate the mechanism behind the inhibition of tumor growth, the tumor lysates were tested for key proteins that regulate the cell cycle mechanism. Cyclin-dependent kinases (CDKs) are a family of protein kinases which play an important role in regulating the cell cycle and also involve in regulating transcription, mRNA processing, and the differentiation.[22,23] As shown in Figure 6, *B*, we observed a significant inhibition in the protein expression of CDK 4 and 6, two proteins which play important role in G1 phase of cell cycle.

Inhibition of proliferation in tumor tissues isolated from nano-EGCG treated mice

In our last set of experiments we looked at the inhibition of cell proliferation markers since these could have direct relevance to inhibition of tumor growth. We determined the protein expression of Ki-67 and PCNA which are considered as the most promising markers of cell proliferation. These are also considered to be involved in regulation of cell cycle and Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). During interphase, Ki-67 can be exclusively within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. As shown in Figure 6, *C*, we observed an expected significant presence of these proteins in the nuclei of control group while treatment with both native EGCG and nano-EGCG imparted a significant inhibition in the nuclear expression of these proteins.

Discussion

Despite significant efficacy in preclinical settings, green tea has demonstrated only limited efficacy in clinical settings mainly due to limited bioavailability and perceived toxicity.[15,16,24-26] To circumvent the issue we suggested the use of nanotechnology for cancer chemoprevention and proposed the concept of 'nanochemoprevention'.[15] After our initial proof-of-principle study, this concept was utilized by laboratories worldwide and at present many bioactive agents have shown promising efficacy for chemoprevention in nanotechnology settings.[15,24,27-30] One disadvantage of using the nanoformulation based on PLGA-PVA, used in our initial study, was its unstable nature in acidic

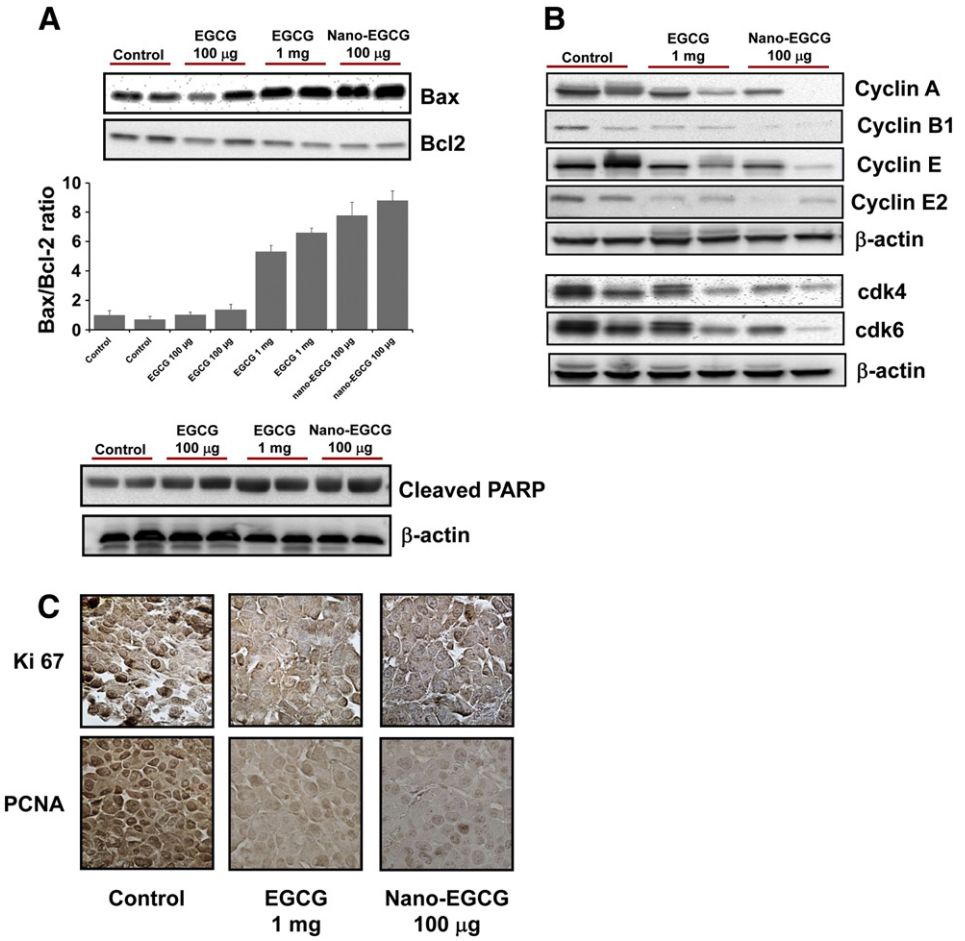

Figure 6. Comparative effects of nonencapsulated EGCG and nano-EGCG on markers of apoptosis, cell cycle and proliferation markers in tumors isolated from nude mice. **(A)** Protein expression of Bax, Bcl2, and the Bax/Bcl2 ratio and PARP. **(B)** Protein expression of Cyclins A, B1, E, E2, CDK 4 and 6. The cells were treated with each agent and harvested 24 h after treatments. Details of the experiments are described in Materials and methods. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Each experiment was repeated twice with similar results. **(C)** Effect of the treatments on expression of Ki-67 and PCNA in tumor tissues isolated from athymic nude mice. Tumor sections were stained using specific antibodies as detailed in Materials and methods. Counterstaining was performed with hematoxylin. Scale bar, 50 μm. Photomicrographs (magnification, ×20) show representative pictures from two independent samples.

environment and therefore it could not be utilized for oral consumption. To overcome this obstacle, we recently developed an oral formulation of EGCG employing a naturally occurring polymer chitosan, which we observed to result in a steady and sustained release of EGCG in the plasma of mice.[16] In this study we present convincing data suggesting the efficacy of chitosan nanoparticles encapsulating EGCG in melanoma cancer model and demonstrate that our novel nanoformulation is not only diverse in its specificity but could in fact be opening novel avenues in the field of nanochemoprevention.

The biological efficacy shown by green tea or its individual polyphenol EGCG in variety of cancer models, both in vitro[31-34] and animal models[35-37] is well known. One can hardly argue the true potential of EGCG in cancer prevention and/or treatment but in clinical settings only limited studies[16,38-41] were able to show that green tea has potential. Limited bioavailability of its active agent(s) could be the major drawback associated with lack of remarkable effects of green tea in clinical settings. In addition, limited absorption of bioactive components in the gastrointestinal tract could be an additional reason behind these limited effects. We, in a recent study have clearly demonstrated that nano-EGCG is released slowly in simulated acidic pH of the gastric juice while in simulated intestinal fluid the release was faster.[16] This is an important observation since oral delivery is the preferred route for chemopreventive protocols and also for anticancer drug in humans.

In this study we demonstrate that our unique oral delivery ready nanoformulation of EGCG demonstrates substantial efficacy in melanoma cancer model and has great potential for further advancement for use in clinical settings. First we demonstrated the cell growth inhibitory potential of our chitosan based nanoformulation containing EGCG and further proved its several fold dose advantage over native EGCG. Next to test if EGCG encapsulated in the nanoformulated concoction retains its mechanistic identity, we tested several molecules established to be modulated by EGCG in cancer models. The cell growth inhibitory effects were observed to be linked to induction of apoptosis as we demonstrate that the key regulators of apoptosis were modulated by the treatment. We

observed a significant change in the expression of Bax and Bcl-2 proteins, both of which are considered the driving force of apoptosis.[37] Change in the ratio of Bax to Bcl-2 is known to lead to destabilization of mitochondria which leads to release of apoptosis inducing factors. These factors induce the intrinsic apoptotic pathway and result in initiation of caspase proteolytic cascade and cleavage of PARP protein into its fragments.[37] Our observations suggest significant cleavage in whole PARP into its 85 KD fragment along with the activation of the caspase cascade.

It is well established that uncontrolled cell growth as a result of defects in cell cycle and/or apoptotic machinery is in part responsible for development of variety of cancers. Thus any agent that can arrest cell in the cell cycle is considered very effective. Inhibition of cell growth and initiation of apoptosis are highly correlated with arrest of cells in G1, S or G2/M phase.[42,43] G2/M transition provides an important checkpoint in cell cycle. This checkpoint ensures that cells don't initiate mitosis before they have a chance to repair the damaged DNA after replication.[44] Our treatment resulted in majority of the cells arrest in G2/M phase. Further, we examined the key proteins modulated in the cell cycle arrest and observed that cyclins and p21 and p27, the master regulators of cell cycle were efficiently modulated by nano-EGCG. The apoptotic induction and cell cycle regulation effects of our nanoformulation suggested that the encapsulated EGCG retains its mechanistic identity in demonstrating the anticancer efficacy.

The final goal of our study was to check the in vivo efficacy of our nanoformulation in human melanoma cell xenograft model system. Our data clearly suggested that nano-EGCG inhibits the growth and progression of tumor xenografts implanted on nude mice even at 10-fold lower dose than the native agent. To draw a parallel we used an equivalent dose (100 μg/mice) of the native agent and observed that this group of animals did have some inhibition of tumor growth however the change from the control group was not statistically significant. The time to reach 1000 mm^3 in control group was recorded to be ~19 days. The most effective tumor growth inhibitory response was seen in nano-EGCG group where this volume was reached in ~27 days while somewhat similar response (~25 days) was seen in native EGCG group but at 10 fold higher dose. We further looked at induction of apoptosis and inhibition of cell cycle in tumors isolated from the animals to define the mechanism behind tumor growth inhibition. We observed that key proteins related to both induction of apoptosis (Bax, Bcl-2, and PARP) and inhibition of cell cycle (cyclins and CDKs) were efficiently modulated in the tumors. Lastly we looked at the protein expression of PCNA and Ki-67, key cell proliferation markers in the tumors isolated from the animals. As tumor cell proliferation is a central feature of melanoma progression, these molecules are very important in their roles in melanomas. PCNA is typically overexpressed in cells in early G1 and S phase of the cell cycle while Ki-67 marks the cells in all the phases of cell cycle apart from G0 phase.[45,46] As expected we observed that both these proteins are abundant in the nuclei of untreated control while treatment with both native EGCG and nano-EGCG significantly inhibits the level of expressions of these proteins suggesting a marked inhibition of cell growth in the tumors treated with our agents.

Taken together, our study suggested that this oral ready nanoformulation of EGCG was effective in both in vitro and in vivo animal model systems of malignant melanoma. This clearly suggests that using this system green tea polyphenols could be provided at physiologically achievable concentrations. The outcome from this study could thus have a direct practical implication and translational relevance to human melanoma patients.

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