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14. ABSTRACT The size and morphology of chitosan-based nanoparticles encapsulating EGCG (CHI-EGCG-NPs) were examined by transmission electron microscopy (TEM). This technique allowed us to directly visualize the CHI-EGCG-NPs. It was found that the nanoparticles had a size of around 200 nm, which is further supported by what we observed earlier with our dynamic light scattering (DLS) data. Additionally, from the TEM picture, it is clear that the nanoparticles are spherical in shape. We also studied the release kinetics of EGCG from CHI-EGCG-NPs in simulated gastric juice and simulated intestinal fluid. It became clear from the data that the release of EGCG from these nanoparticles is very slow in acidic medium, even at 24 hrs only ~10% of EGCG was released. On the other hand, release of intestinal fluid is much faster, and around 50% of EGCG was released in 24 hrs. We have earlier shown that treatment with CHI-EGCG-NPs (3 and 6 mg/kg body wt.) resulted in significant inhibition of tumor growth in athymic nude mice implanted with 22Rv1 cells. Further extending this work, we report here that in tumor tissues of mice treated with both doses of CHI-EGCG-NPs as compared to group treated with EGCG and controls, there was significant (i) reduction in Ki-67 and proliferating cell nuclear antigen (PCNA) (ii) induction of poly (ADP-ribose) polymerases (PARP) cleavage, (iii) activation of caspases and, (iv) increase in the protein expression of Bax and decrease in Bcl2. Through this study, we propose a novel preventive and therapeutic modality using EGCG that addresses issues related to bioavailability, that is a major reason for its limited success in humans.

15. SUBJECT TERMS
Nanochemoprevention; EGCG; CHI-EGCG-NPs; athymic nude mice; prostate cancer; transmission electron microscopy

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INTRODUCTION:

Prevention of cancer by natural agents is often restricted by the lack of delivery of desired levels in target tissue thus limiting their bioavailability and clinical outcome. Hence, newer approaches are required to improve bioavailability and to decrease toxicity of natural agents to accomplish maximum response. Among all natural agents, green tea has shown promise in preclinical, epidemiological and initial clinical studies, especially for prostate cancer (PCa). Much of the effects of green tea are considered to be exerted by its major polyphenol (-) epigallocatechin-3-gallate (EGCG). We reported significant dose-advantage of polylactic acid-polyethylene glycol (PLA-PEG) encapsulated EGCG (nanoEGCG) over non-encapsulated EGCG (1), for PCa in cell-culture and xenograft model. However, PLA-PEG is unstable in acidic environment and is therefore not recommended for oral consumption, a desired route for chemopreventive agents. We developed a formulation of chitosan-based nanoparticles encapsulating EGCG (CHI-EGCG-NPs). Despite extensive efforts that have been put to prevent cancer through the use of bioactive food components and other agents, lack of delivery of desired levels thus limiting their bioavailability, remains a challenging issue for effective clinical outcome (2-4). There is a strong unfulfilled need in the biomedical community to find novel tools for making prevention and treatment of cancer a success. Therefore, in order to achieve maximum response of a natural agent, novel strategies are urgently needed to enhance the bioavailability, effectiveness and to reduce perceived toxicity. It is expected that nanotechnology will continue to have a profound and positive impact on human health. Many nanotechnology-based diagnostic and treatment modalities already are in use, with many others at various stages of pre-clinical and clinical testing. Several nanotechnology platforms hold great promise for diagnosis and treatment of cancer. We propose that nanotechnology will serve as a cornerstone in cancer chemoprevention. The concept of nano-chemoprevention (i.e. encapsulation of chemopreventive agents in nanoparticles) possesses strong merit and rationale for conducting additional detailed *in vivo* studies in appropriate animal models with relevance to human disease. As with many other nanoparticles, the advantage of using PLA-PEG nanoparticles lies in their high surface area to volume ratio, which presumably allows them to upload more EGCG while maintaining the small size. This is particularly important because the enhanced conjugation of nanoparticles with agents often results in the generation of nanoparticles that are sparingly taken up by the diseased cells, owing to their bigger size and, thereby, undermining their medical usefulness. Also, the therapeutic/clinical importance of PLA-PEG nanoparticles relies on the fact that, being biodegradable, they rarely exhibit any toxicity.

BODY:

Size measurement by Transmission Electron Microscopy (TEM):

The size and morphology of CHI-EGCG-NPs were further examined by transmission electron microscopy (TEM) using a JEOL JEM-100CX transmission electron microscope (JEOL, USA, Inc., Peabody, MA, USA). One drop of the CHI-EGCG-NPs solution was mounted on a thin film of amorphous carbon deposited on a copper grid (300 mesh). The solution was air dried and the sample was examined directly.

As reported last year, CHI-EGCG-NPs were further characterized by TEM. This technique allowed us to directly visualize the CHI-EGCG-NPs. As shown in Figure 1, the nanoparticles have a size of around 200 nm. Additionally, from the TEM picture it is clear that the nanoparticles are spherical in shape.

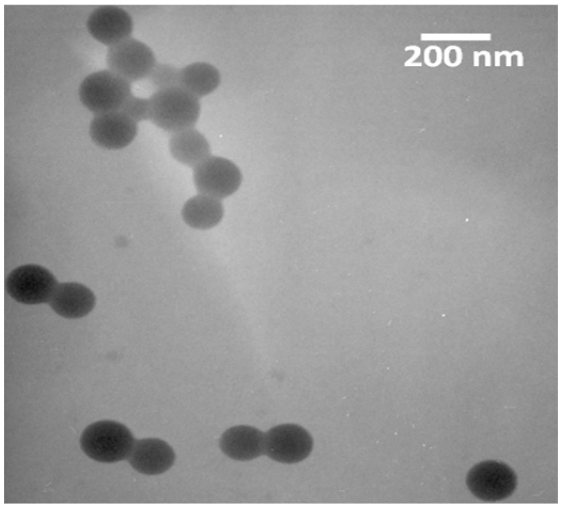


Figure 1: Transmission Electron Microscopy Images showing the spherical size of CHI-EGCG-NPs.

Release kinetics studies:

We have studied the release kinetics of EGCG from CHI-EGCG-NPs in simulated gastric juice and simulated intestinal fluid. For this cumulative release kinetic study, a known amount of nanoparticles encapsulating EGCG was suspended in 15 ml of simulated gastric juice/simulated intestinal juice. The solution was kept in an incubator at room temperature (25°C). At predetermined intervals of time, 500 µl of the solution was filtered through Millipore centricon tubes containing a 100 KD membrane to separate the released EGCG from the nanoparticles. The amount of free EGCG present in the filtrate was determined by LC/MS. A graphical representation comparing the release profiles in simulated gastric juice and simulated intestinal fluid is shown in Figure 2.

In the case of oral delivery of an active biomaterial/natural product the degradation starts in the stomach, due to the acidic pH. One of the most important factors to be kept in mind for carrier-mediated drug delivery through an oral route is that the carrier system must not degrade/release at acidic pH, releasing the entire drug. At the same time, it has to retain its capability to release the drug at neutral pH. To test the stability and release kinetics of CHI-EGCG-NPs we studied the release kinetics in simulated gastric juice and simulated intestinal fluid. It is clear from the Figure 2 that the release of EGCG from these nanoparticles is very slow in acidic medium; even at 24 hrs only ~10% of EGCG was released. On the other hand, release of intestinal fluid is much faster, and around 50% of EGCG was released in 24 hrs. Then there was a steady release of EGCG. This is strong evidence that these nanoparticles are capable of avoiding degradation in the stomach, they do not release EGCG and they are capable of releasing EGCG in a neutral medium.

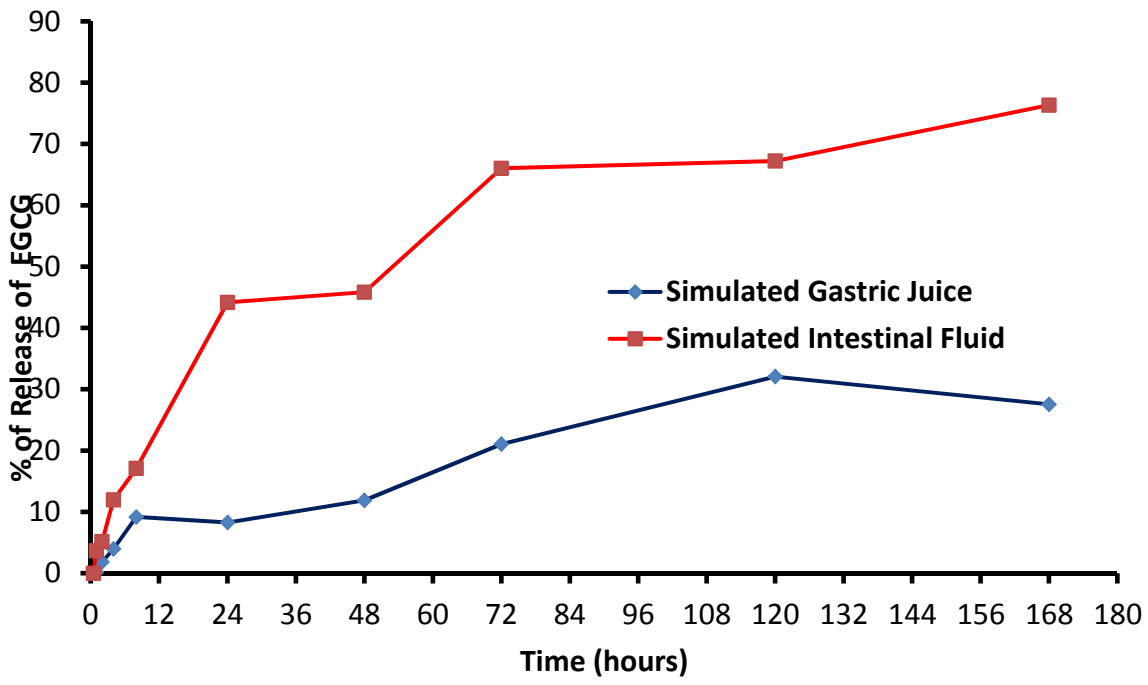


Figure 2: Release kinetics study of EGCG from CHI-EGCG-NPs in simulated gastric juice and simulated intestinal fluid.

Analysis of EGCG by LC/MS

Stock solutions of EGCG were obtained by dissolving EGCG in 50% methanol. The calibration curve for EGCG was obtained by spiking stock EGCG solutions of different concentrations in bovine plasma. At least two calibration curves were acquired to achieve a more reliable technique (Figure 3). With the help of this standard curve, we can measure the amount of EGCG in the range of 0.8-31.28 ng/ml. In brief, the calibration curve was obtained by diluting bovine plasma (1:3 w/NS) and spiked with EGCG solution ranging from 0.8 to 31.25 ng/ml. For sample analysis, 10 µl of an internal standard (ethyl gallate) was added to each sample before extraction. A total amount of 100 µl of spiked serum sample was extracted from each sample with ethyl acetate and acetonitrile. The supernatant was dried and the residual was resuspended with 40% acetonitrile, and 25 µl was injected for the LC/MS/MS assay. In all cases, an internal standard was used to normalize the amount of EGCG recovery from the plasma.

Figure 3 shows the calibration curves obtained from EGCG standard solution at different concentrations (0.8-31.25 ng/ml). These standard curves were used to quantify the amount of EGCG in the mice serum at various time points (of the pharmacokinetics studies). The experiments for the standard curves were in duplicate to test the accuracy of our method. From Figure 3, it is observed that the two curves are nearly identical to each other, indicating our method is accurate.

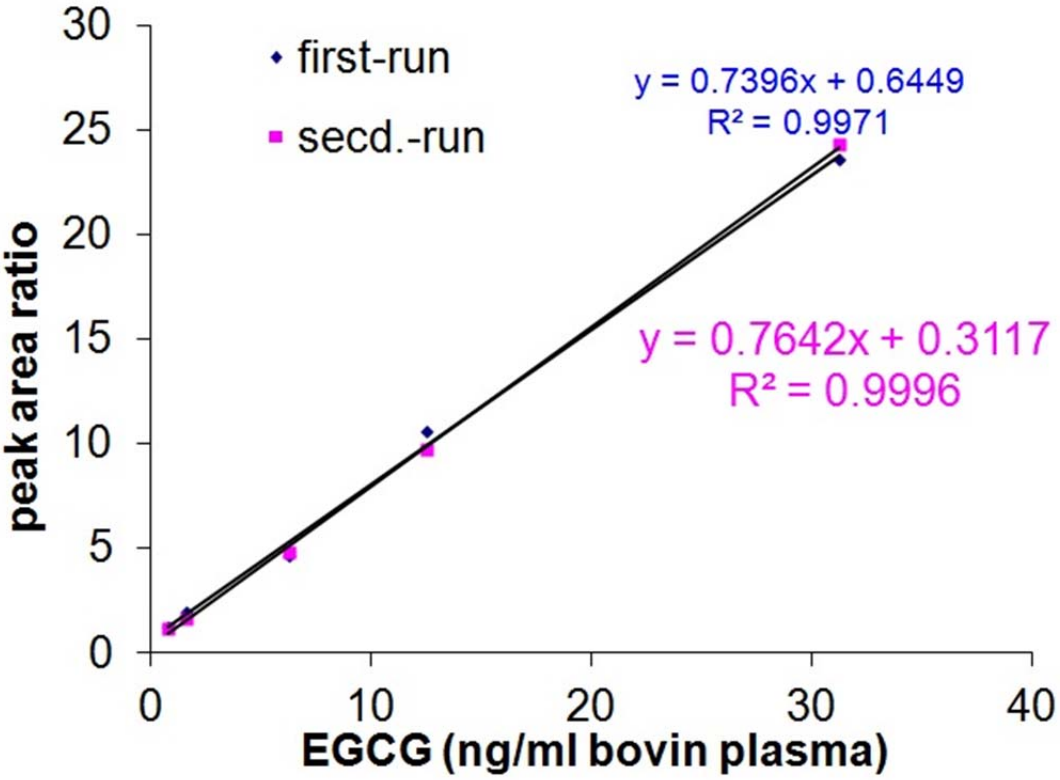
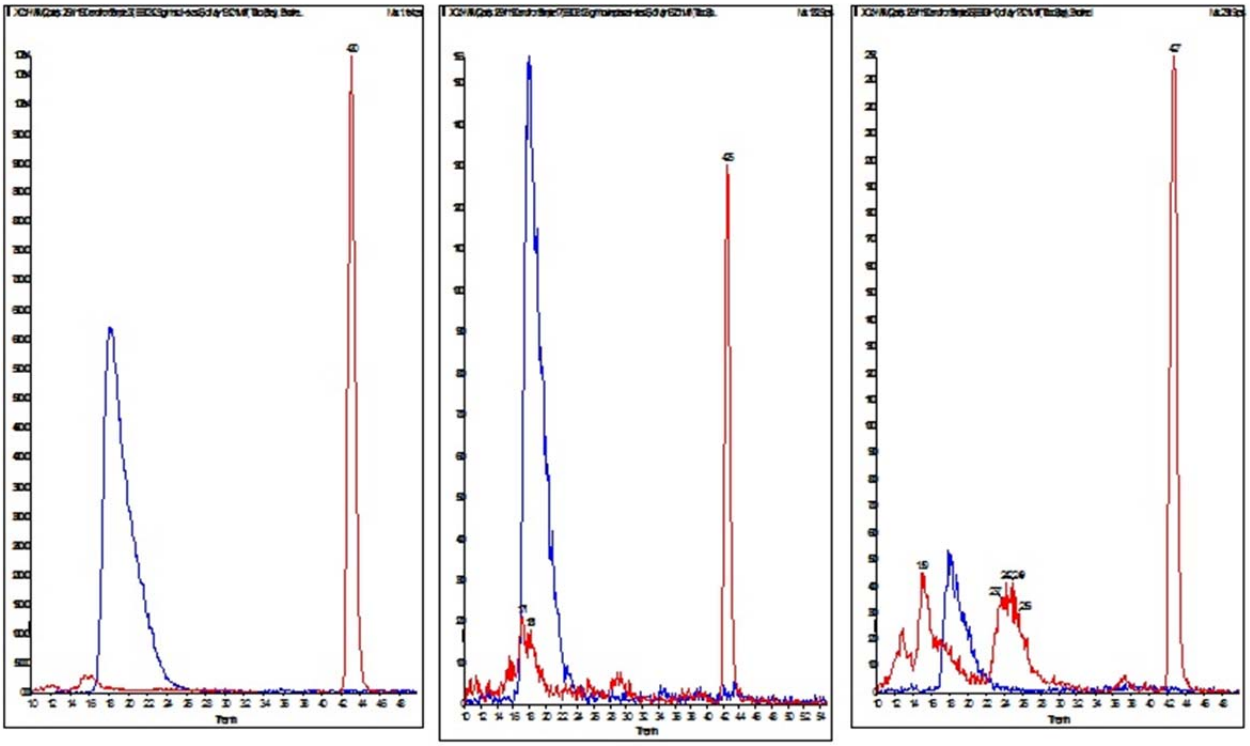


Figure 3: Calibration curves generated with bovine plasma. EGCG ranges from 0.8-31.25 ng/ml. The calibration samples were measured twice.

The HPLC was a Shimadzu Prominence LC system containing a CBM-20A system controller, two LC-20AD pumps, a SIL-20AC autosampler, and a CTO-20AC oven. For the analysis of EGCG, a Waters SunfireTM C18, 3.5 µm particle size, 3.0 × 150 mm column at and an isocratic elution method with mobile phases of 40% acetonitrile and 0.1% formic acid was used The flow rate was set at 0.35 ml/min and the eluent was directed to the mass spectrometer ion source without splitting. An API 4000 triple-quadruple mass spectrometer (Applied Biosystems, MDS SCIEX Ontario, Canada) equipped with a Turbo V Source and Turbo Ion Spray was coupled to the HPLC. The mass spectrometer was operated in negative mode and selective ion monitoring was used for qualitative analyses of EGCG (EGCG, m/z 457), and multiple reaction monitoring was used for quantitative analyses of EGCG with mass transitions 457→168.9m/z (Figure 4).

Figure 4 shows a typical chromatogram (LC/MS) of EGCG (blue line) in various physiological conditions. Figure 4 (i) shows the chromatogram of standard EGCG solution (12.5 ng/ml); Figure 4 (ii) shows the chromatogram of EGCG in bovine plasma (6.25 ng/ml), and Figure 4 (iii) shows the chromatogram of EGCG in mouse serum (1.17 ng/ml). In all cases, an internal standard of ethyl gallate (red line in the chromatogram) was used for normalization of the data.

All these chromatograms demonstrate our ability to detect EGCG in different physiological conditions at varying concentrations.



i) EGCG 12.5 ng/ml in standard solution	ii) EGCG 6.25 ng/ml in bovine plasma	iii) EGCG 1.17 ng/ml in mouse serum

Figure 4: Typical chromatograms of EGCG in various matrixes detected by LC/MS

Athymic nude mice study:

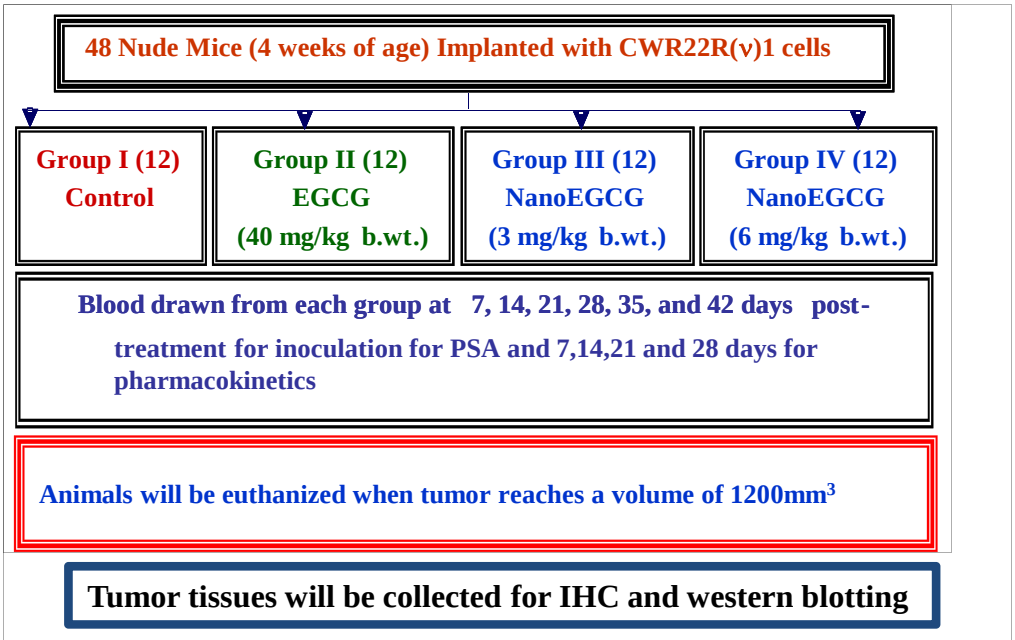


Fig. 5. Schematic representation of tumor studies in nude mice

Design: Forty eight male athymic nude mice (4 weeks of age) were housed four/cage and fed *ad libitum* with autoclaved semi-purified, AIN-76 B-40 diet. A total of 1 million 22Rv1 cells (in 50 µl DMEM + 50 µl Matrigel) were implanted by a sub-cutaneous injection on left and right sides, below the shoulders (2 tumors per mouse). **Treatment of nude mice**: Mice implanted with cells were randomly distributed into four groups of twelve each (Figure 5). Group I received void nano particles and served as the control. Group II received EGCG (40 mg/kg b.wt). Group III received nanoEGCG (3 mg/kg b. wt.) and Group IV received nanoEGCG (6 mg/kg b. wt.). Treatments were by oral intubation and started one day post cell inoculation, 5 times a week (Monday-Friday) until tumors reach a targeted volume of 1200 mm^3. The animals were also evaluated for body weight, consumption of food and apparent signs of toxicity. At weekly intervals phlebotomy was performed to obtain sera for PSA estimation by ELISA and for pharmacokinetics study. Tumor tissues were collected at the end of the protocol for immunohistochemical and western blotting for evaluation of cell proliferation markers (Ki-67 and PCNA) and apoptotic markers (PARP, Bax, Bcl2 and caspases). **Analysis of serum EGCG concentrations**: Serum samples from EGCG and nanoEGCG treated mice were subjected to HPLC and GC mass spectrometry for analysis of pharmacokinetic distribution of EGCG and bioavailability.

Though we have developed a sensitive method to measure the amount of EGCG levels in blood plasma, there was little detectable EGCG found in the plasma after 7 days (Figure 6). The probable reason may be that the serum was collected too late after the oral administration of EGCG/CHI-EGCG-NPs, and that most of the EGCG was secreted out of the body or was converted to other metabolites.

	dosage (mg/Kg)	code of mouse	EGCG con. (ng/ml)
Day-7	control	mouse-1	BLOQ
		mouse-2	0.14
		mouse-3	0.03
	3	mouse-4	BLOQ
		mouse-5	BLOQ
		mouse-6	BLOQ
	6	mouse-7	BLOQ
		mouse-8	BLOQ
		mouse-9	BLOQ
	40	mouse-10	BLOQ
		mouse-11	BLOQ
		mouse-12	BLOQ
Day-14	control	EGCG-II-1	BLOQ
		EGCG-II-2	BLOQ
		EGCG-II-3	BLOQ
	3	EGCG-II-4	BLOQ
		EGCG-II-5	0.91
		EGCG-II-6	0.55
	6	EGCG-II-7	BLOQ
		EGCG-II-8	0.12
		EGCG-II-9	BLOQ
	40	EGCG-II-10	1.17
		EGCG-II-11	0.16
		EGCG-II-12	0.14

	dosage (mg/Kg)	code of mouse	EGCG con. (ng/ml)
Day-21	control	EGCG-III-1	BLOQ
		EGCG-III-2	BLOQ
		EGCG-III-3	BLOQ
	3	EGCG-III-4	BLOQ
		EGCG-III-5	60.18
		EGCG-III-6	BLOQ
	6	EGCG-III-7	BLOQ
		EGCG-III-8	BLOQ
		EGCG-III-9	BLOQ
	40	EGCG-III-10	BLOQ
		EGCG-III-11	BLOQ
		EGCG-III-12	BLOQ
Day-28	control	EGCG-IV-1	BLOQ
		EGCG-IV-2	BLOQ
		EGCG-IV-3	BLOQ
	3	EGCG-IV-4	BLOQ
		EGCG-IV-5	BLOQ
		EGCG-IV-6	BLOQ
	6	EGCG-IV-7	BLOQ
		EGCG-IV-8	BLOQ
		EGCG-IV-9	BLOQ
	40	EGCG-IV-10	BLOQ
		EGCG-IV-11	BLOQ
		EGCG-IV-12	BLOQ

Figure 6: EGCG concentration in blood plasma after oral administration of EGCG and EGCG encapsulated nanoparticles (CHI-EGCG-NPs). (BLOQ: below limit of quantification)

Effect of CHI-EGCG-NPs on apoptosis in tumor tissues of athymic nude mice

Protein extraction and western blotting

For western blotting, 30-50 µg protein was resolved over 8-12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% nonfat dry milk/1% Tween 20; in 20 mM TBS, pH 7.6) for 1 hr at room temperature, incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for one and half hr to overnight at 4°C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate obtained from Amersham Life Science Inc. (Arlington Height, IL, USA) and detected by chemiluminescence and autoradiography using Bio-Rad Gel-Doc (Bio-Rad Laboratories Inc., Hercules, CA).

As shown by western blots in Figure 7, PARP cleavage analysis showed that the full size PARP (116 KD) protein was cleaved to yield an 85 KD fragment on treatment with CHI-EGCG-NPs. There was also a significant increase in the protein expression of Bax whereas; the protein expression of Bcl-2 was significantly decreased by CHI-EGCG-NPs in a dose-dependent fashion. We also found the activation of casapse-3, -8 and -9 in tumor tissues of mice treated with both doses of CHI-EGCG-NPs as compared to group treated with EGCG and control (Figure 7).

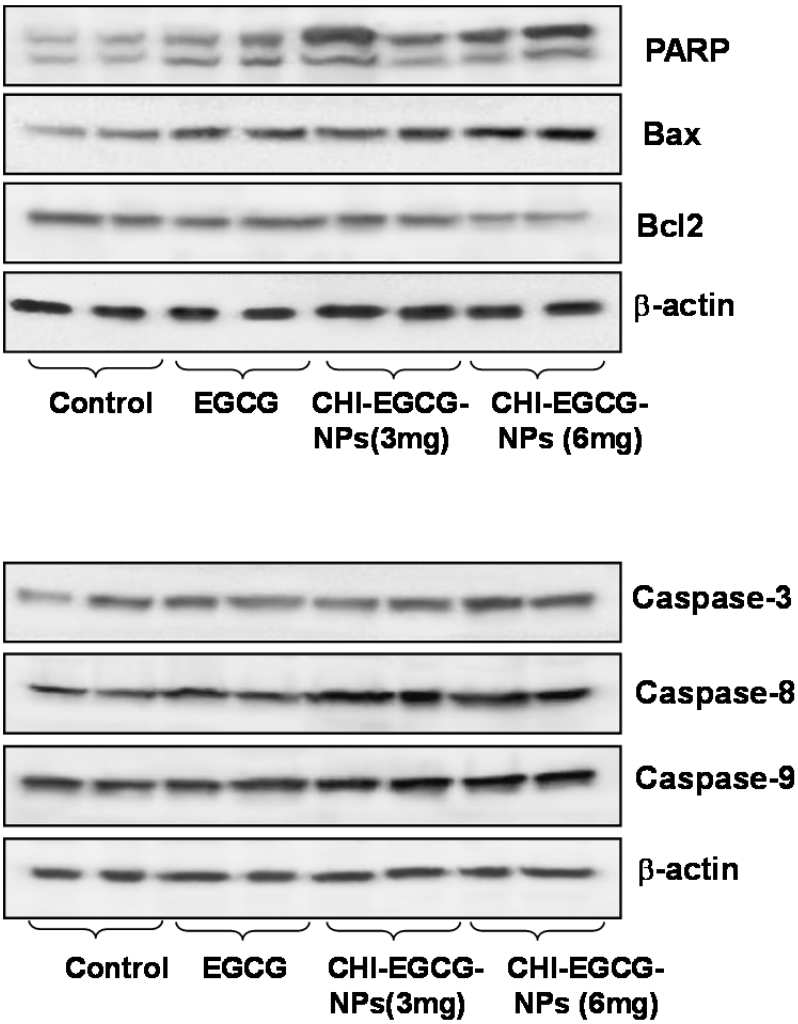


Figure 7. Effect of CHI-EGCG-NPs on the protein expression of PARP, Bax, Bcl2 and caspases-3, -8 and -9 in tumor tissues of athymic nude mice. As detailed in Figure 5, mice were treated with EGCG (40 mg/kg b.wt.), CHI-EGCG-NPs (3 mg/kg b.wt.) and CHI-EGCG-NPs (6 mg/kg b. wt.). Tissue lysate was prepared and 40 μg protein was subjected to SDS-PAGE followed by western blot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the western blot and reprobing it for β-actin.

Effect of CHI-EGCG-NPs on cell-proliferation markers in tumor tissues of athymic nude mice

Immunohistochemical analysis: Sections (5 mm 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.). Images were captured with an attached camera linked to a computer.

Ki-67 and PCNA are well known markers of cellular proliferation. We observed that there was markedly less expression of Ki-67 and PCNA in tissues of mice treated with CHI-EGCG-NPs than group treated with EGCG and control (Figure 8).

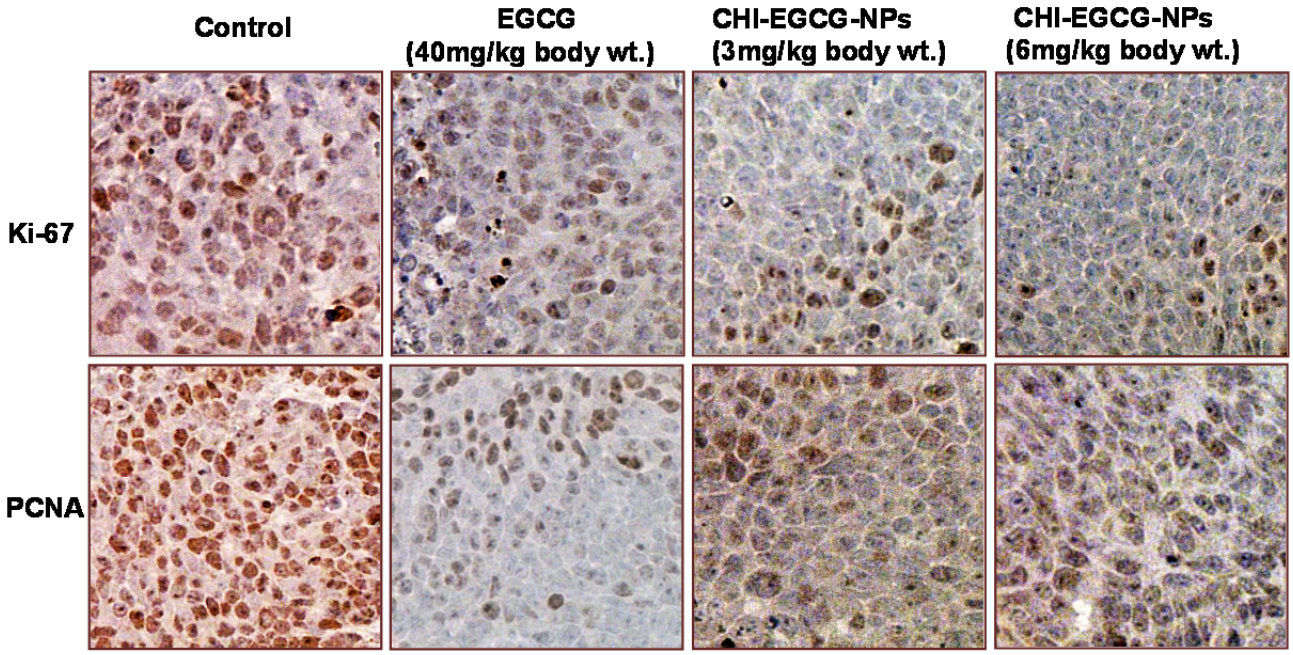


Figure 8. Effect of CHI-EGCG-NPs on cell-proliferation markers in tumor tissues of athymic nude mice. As detailed in Figure 5, mice were treated with EGCG (40 mg/kg b.wt.), CHI-EGCG-NPs (3 mg/kg b.wt.) and CHI-EGCG-NPs (6 mg/kg b. wt.). Tumor tissues were stained using Ki-67 and PCNA antibodies and counterstaining was performed with hematoxylin.

Effect of CHI-EGCG-NPs on angiogenesis markers in tumor tissues of athymic nude mice

Angiogenesis is crucial for the growth of tumors and metastasis and is strictly controlled by a highly coordinated process that is regulated by many molecules. Among them, platelet-derived endothelial cell adhesion molecule (CD31) and vascular endothelial growth factor (VEGF) are most common markers of tumor-associated angiogenesis.

By immunohistochemical analysis of the tumor tissue samples from athymic nude mice, we found more profound expression of CD31 and VEGF positive cells in control and EGCG treated groups than in CHI-EGCG-NPs treated groups (Figure 9).

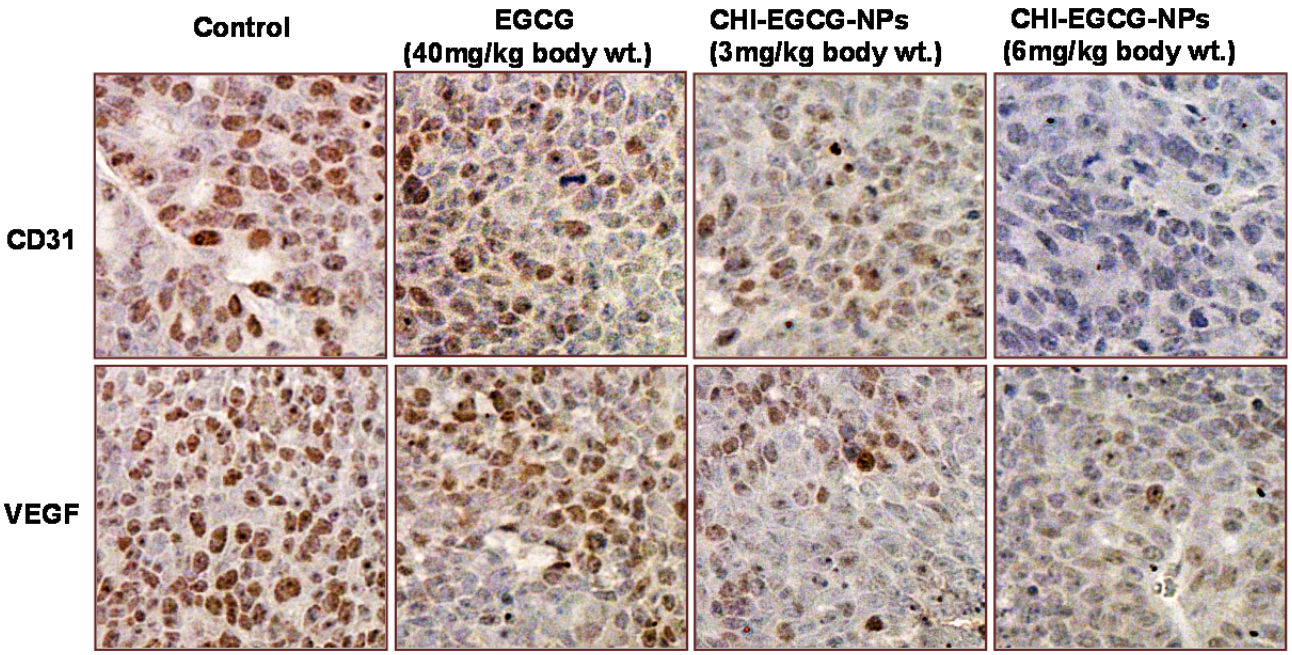


Figure 9. Effect of CHI-EGCG-NPs on angiogenesis markers in tumor tissues of athymic nude mice. As detailed in Figure 5, mice were treated with EGCG (40 mg/kg b.wt.), CHI-EGCG-NPs (3 mg/kg b.wt.) and CHI-EGCG-NPs (6 mg/kg b. wt.). Tumor tissues were stained using CD31 and VEGF antibodies and counterstaining was performed with hematoxylin.

KEY RESEARCH ACCOMPLISHMENTS:

- Prevention of cancer by natural agents is often restricted by the lack of delivery of desired levels in target tissue thus limiting their bioavailability and clinical outcome. Hence, newer approaches are required to improve bioavailability and to decrease toxicity of natural agents to accomplish maximum response. Among all natural agents, green tea has shown promise in preclinical, epidemiological and initial clinical studies, especially for PCa.

- As reported last year, CHI-EGCG-NPs were further characterized by TEM. This technique allowed us to directly visualize the CHI-EGCG-NPs. As shown in Figure 2, the nanoparticles have a size of around 200 nm; this is further supported by our earlier DS data. Additionally, from the TEM picture it is clear that the nanoparticles are spherical in shape.

- To test the stability and release kinetics of CHI-EGCG-NPs we studied the release kinetics in simulated gastric juice and simulated intestinal fluid. It is clear from the Figure 3 that the

release of EGCG from these nanoparticles is very slow in acidic medium; even at 24 hrs only ~10% of EGCG was released. On the other hand, release of intestinal fluid is much faster, and around 50% of EGCG was released in 24 hrs. Then there was a steady release of EGCG. This is strong evidence that these nanoparticles are capable of avoiding degradation in the stomach and that they do not release EGCG. And, they are capable of releasing EGCG in a neutral medium.

- Through the studies in athymic nude mice, we established the anti-tumor efficacy of oral CHI-EGCG-NPs vs. EGCG alone. Oral administration of CHI-EGCG-NPs to athymic nude mice implanted with human PCa cells resulted in:

- Induction of apoptosis as determined by cleavage of PARP, up regulation of Bax and down regulation of Bcl2 proteins (Figure 7)

- Induction of active caspases-3,-8 and -9 (Figure 7)

- Inhibition of cell-proliferation markers (Figure 8)

- Inhibition of the markers of angiogenesis (Figure 9)

ONGOING ANIMAL STUDIES:

We are currently investigating if supplementation of oral nanoEGCG will inhibit the development of PIN lesions in the Nkx3.1/Paten mutant mice via modulations in i) pro-inflammatory milieu, and ii) oxidative stress in the prostate. Further, we will determine the preventive and therapeutic efficacy of oral nanoEGCG and to identify the stage of prostate cancer development that is most vulnerable to the anti-cancer effects of oral nanoEGCG in the transgenic TRAMP mice.

REPORTABLE OUTCOMES:

Khan N, Adhami VIM, Siddiqui IA, Bareli DJ, Mouse SA and Hasan Mukhtar. Oral administration of naturally occurring chitosan based nanoformulated green tea polyphenol EGCG effectively inhibits prostate cancer cell growth in a xenograft model. The 103rd Annual Meeting of the American Association for Cancer Research, Chicago, IL, USA, Proc Amer Assoc Cancer Res, 53: 5438, 2012.

CONCLUSION:

Based on our promising data, we suggest that the concept of nano-chemoprevention (i.e. encapsulation of chemopreventive agents in nanoparticles) possesses strong merit and rationale for conducting additional detailed *in vivo* studies in appropriate animal models with relevance to human disease. As with many other nanoparticles, the advantage of using PLA-PEG nanoparticles lies in their high surface area to volume ratio, which presumably allows them to upload more EGCG while maintaining the small size. This is particularly important because the enhanced conjugation of nanoparticles with agents often results in the generation

of nanoparticles that are sparingly taken up by the diseased cells, owing to their bigger size and, thereby, undermining their medical usefulness. Also, the therapeutic/clinical importance of PLA-PEG nanoparticles relies on the fact that, being biodegradable, they rarely pose any toxicity. Through this study, we propose a novel preventive and therapeutic modality using EGCG that addresses issues related to bioavailability, that is a major reason for its limited success in humans.

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APPENDICES:

None.