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## Novel D-ring Analog of Epigallocatechin-3-gallate Inhibits Tumor Growth and VEGF Expression in Breast Carcinoma Cells

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**Abstract.** *The cancer chemopreventive activity of green tea and its major polyphenolic constituent, epigallocatechin-3-gallate (EGCG) have been attributed to its antioxidant, antiproliferative and antiangiogenic effects. Several new molecular targets for EGCG's anticarcinogenic activity have been proposed in the recent literature. However, the understanding of the molecular mechanisms of EGCG's activity in vivo have been confounded by its low oral bioavailability and low plasma levels. Studies of EGCG would be greatly aided by the availability of synthetic analogs of EGCG designed to understand the contributions of the A, B, and D-rings and the phenolic hydroxyl groups of EGCG to its molecular mechanisms of action. We recently reported the de novo synthesis of a D-ring analog of EGCG, with the objective of using such analogs to understand the molecular mechanisms of EGCG action. We report here the first studies with a synthetic D-ring analog of EGCG. We examined the ability of the synthetic D-ring analog to inhibit tumor cell proliferation in breast carcinoma cells. We also investigated the effect of the analog on stress-induced VEGF production in breast carcinoma cells using Northern analysis and quantitative RT-PCR. We report here that the synthetic D-ring analog inhibits breast cancer cell growth in vitro with potencies equivalent to those of EGCG. Our results also show that, like EGCG, the synthetic analog inhibits hypoxia- and serum starvation-induced production of VEGF mRNA in breast cancer cells. Such synthetic analogs are valuable for understanding the structure-function relationship of EGCG and identifying relevant mechanisms of the chemopreventive action of EGCG.*

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Green tea has been shown to have cancer preventive activity at various organ sites in several rodent models (1). The cancer preventive properties of green tea are mainly associated with its polyphenolic components, the catechins, of which the four major ones are epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epicatechin (EC). EGCG is the most abundant and the most chemopreventive component of green tea (Figure 1). EGCG itself has shown cancer preventive activity in several animal models of breast, prostate, skin and lung cancers (2,3).

Extensive investigations into the chemopreventive mechanisms of EGCG show that it inhibits carcinogenesis at all stages *viz.* initiation, promotion and progression (4). EGCG has been shown to inhibit carcinogen activation (5), inhibit cell proliferation (6), induce apoptosis (7) as well as potentially inhibit angiogenesis and invasion (8,9). Green tea polyphenols and EGCG in particular, are strong antioxidants (10). The chemopreventive activity of EGCG is postulated to stem from its potent antioxidative activity (11), that affords protection against cellular damage and signaling pathways that lead to malignant transformation (12). On the other hand, several studies have implied that changes in signaling and gene expression by EGCG are associated with the generation of H<sub>2</sub>O<sub>2</sub> by EGCG under *in vitro* conditions (13,14).

The understanding of the relevant molecular mechanisms of the chemopreventive action of EGCG is further confounded by recent reports of putative specific molecular targets for EGCG. Nam *et al.* (15) reported that EGCG inhibits the chymotrypsin-like and caspase-like activity of the proteasome. EGCG was also shown to inhibit the anti-apoptotic Bcl-2 family of proteins (16). Fang *et al.* (17) reported that EGCG inhibits the DNA-methylating enzyme DNMT, whereas Tachibana *et al.* (18) have proposed the ECM-associated protein laminin as a receptor for EGCG. All these studies have corroborated their hypothesis by extensive molecular modeling studies and have proposed detailed molecular interactions of EGCG with the

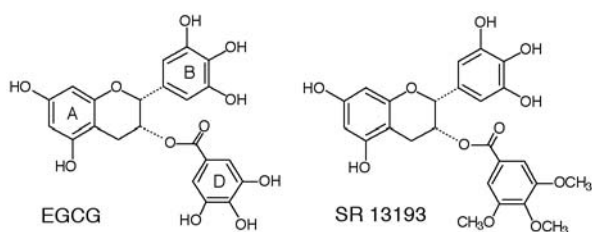


Figure 1. Structure of epigallocatechin-3-gallate and synthetic D-ring analog, SR 13193.

respective targets. However, without structure-activity studies using synthetic analogs of EGCG, it is difficult to conclusively validate the nature of the interactions between EGCG and the putative targets at the molecular level.

Thus far, it has been difficult to understand whether the broad anticarcinogenic activity of EGCG is due to its antioxidant activity or due to interactions with specific molecular targets. This understanding would be greatly facilitated by using structural analogs of EGCG that are designed to answer such questions. We recently reported a versatile chemical synthesis of EGCG using a *de novo* approach that allows us to vary the A, B and D-rings of EGCG independently of each other (19). We also reported the synthesis of a D-ring analog of EGCG, SR 13193 (Figure 1). We report here the first studies with this synthetic analog of EGCG. We studied the effect of the D-ring analog, SR 13193, on *in vitro* cell growth of breast cancer cells. We further studied the effect of the analog on a key molecular event that has been implicated in the antiangiogenic activity of EGCG, the induction of the potent angiogenic factor VEGF in tumor cells. EGCG has been shown to inhibit VEGF-induced angiogenesis in the chick chorioallantoic membrane (CAM) assay (8). Sartippour *et al.* (20) have shown that green tea extract and EGCG decrease VEGF mRNA levels in MDA-MB-231 breast cancer cells. Masuda *et al.* (21) have also shown that EGCG decreases VEGF promoter activity and protein levels in MDA-MB-231 cells *via* the inhibition of EGFR-related signaling pathways. Jung *et al.* (22) have further shown that EGCG inhibits VEGF mRNA levels and VEGF promoter activity induced by serum deprivation in human colon cancer cells. We, therefore, examined our synthetic D-ring EGCG analog, SR 13193, for its effect on hypoxia- and serum starvation-induced VEGF expression in breast cancer cells.

We report here that the D-ring methylated analog SR 13193 is as potent as EGCG itself in inhibiting the proliferation of breast cancer cells *in vitro*. Furthermore, it is equally effective against hormone-responsive MCF-7 cells and hormone-insensitive MDA-MB-231 cells. We

also found that the D-ring analog inhibits VEGF expression in breast cancer cells to the same extent as that observed with EGCG.

## Materials and Methods

**Materials.** MCF-7 and MDA-MB-231 human breast cancer cells were obtained from ATCC. EGCG was purchased from Sigma (St. Louis, MO, USA). The D-ring analog SR 13193 was synthesized in our laboratory by our published procedure (19).

**Cell culture and cell treatment.** MCF-7 and MDA-MB-231 cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine serum. Cells were maintained in a 5% CO<sub>2</sub> incubator at 37°C. Drug treatments were carried out when cells were 70-80% confluent.

**Inhibition of cell proliferation.** MCF-7 or MDA-MB-231 cells at 80% confluence were harvested *via* trypsinization and re-seeded in 96-well plate at 2,000 cells/well in 200  $\mu$ L of DMEM supplemented with 10% fetal bovine serum. Cells were allowed to attach for 24 h prior to the initiation of drug treatment. SR 13193 and EGCG were dissolved in DMSO and diluted with growth medium to reach the appropriate concentrations for treating the cells. The final DMSO concentration in each well did not exceed 0.25%. SR 13193 or EGCG were added to each well in 10  $\mu$ L aliquots. Control wells received 10  $\mu$ L of vehicle only. The medium in each well was changed on the third day and fresh test solutions added. On Day 6, the medium was replaced with fresh medium without test compound, followed by 15  $\mu$ L of Tetrazolium Blue reagent (Promega, Madison, WI, USA). The cells were cultured for an additional 4 h and 100  $\mu$ L of Solubilization/Stop solution (provided by Promega) was added to each well. The plate was then kept at room temperature overnight in a plastic bag containing paper towels soaked in water. The formazan product formed was read on an ELISA plate reader at 575 nm. The optical density obtained for treated samples relative to that of the vehicle control was used to determine the percent inhibition. The growth inhibition IC<sub>50</sub> was calculated from the percent inhibition at five concentrations. The IC<sub>50</sub> values represent the mean of three independent experiments run in triplicate.

**Hypoxia exposure.** Hypoxia experiments were performed as described previously (23). Briefly, cells were plated in 60-mm plates and placed inside specially designed aluminum chambers attached to a 5% CO<sub>2</sub>/N<sub>2</sub> manifold on a vacuum line. The chambers were kept in a circulating water bath at 37°C. Atmospheric oxygen was extracted by 5 cycles of pumping to a fixed pressure followed by filling with 5% CO<sub>2</sub>/N<sub>2</sub> ( $pO_2 \leq 0.01\%$  relative to air at 21%). Oxygen levels within this apparatus were calibrated by using a polarographic oxygen electrode (Controls Katharobic, Gulph Mills, PA, USA) in an attached test chamber. The chambers were sealed by closing the valves to the manifold and incubated at 37°C for 16 h.

**Northern blot analysis.** Total RNA was isolated using QIAGEN RNeasy mini kit (QIAGEN Inc, Valencia, CA, USA) following the manufacturer's recommended procedure and quantified spectrophotometrically. Five  $\mu$ g of total RNA was electrophoresed in 1% agarose gels containing 6% formaldehyde. Gels were stained with ethidium bromide to visualize the positions of 28S and 18S

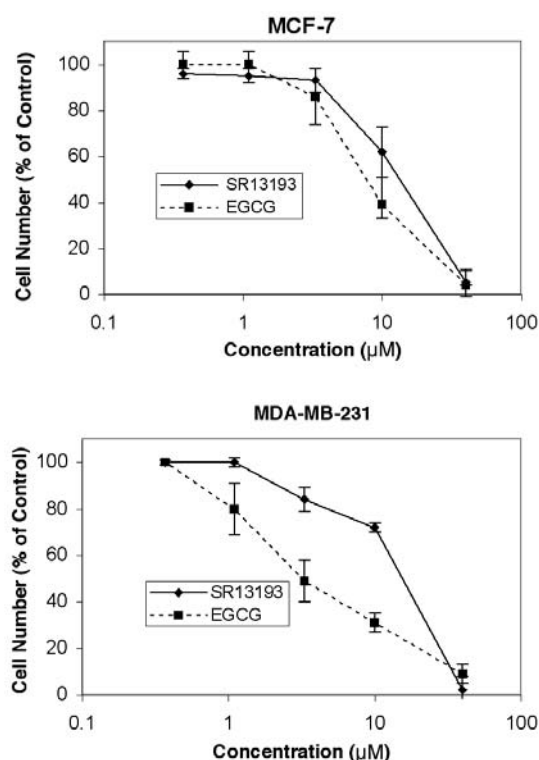


Figure 2. Inhibition of breast cancer cell growth *in vitro* by EGCG and synthetic analog SR 13193. MCF-7 and MDA-MB-231 cells were incubated with various concentrations of test compounds as described in Materials and Methods. The values represent the mean  $\pm$  SD of three independent experiments.

RNA. The RNAs were transferred to nylon membranes (Amersham Hybond N) by capillary blotting and fixed to the filter by exposure to UV light. The blots were probed with a  $^{32}$ P-labeled 660 bp human cDNA sequence corresponding to the coding region of VEGF<sub>165</sub> gene. Hybridizations were carried out at 60°C in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 0.1% SDS and 0.3 mg/ml salmon sperm DNA. Filters were washed by 1 x SSC, 0.1% SDS, twice at room temperature for 15 min and once at 65°C in 0.1 x SSC, 0.1% SDS for 1 h. Filters were exposed to X-ray film at -70°C (Coronex Hi-Plus). Hybridized bands were quantified by analyzing the images using an AlphaImager™ S-3400 (Alpha Innotech Corp., San Leandro, CA, USA).

**Quantitative polymerase chain reaction (PCR).** One µg of total RNA was reverse transcribed using random primers (Life Technologies, Inc.) and Moloney murine leukemia virus RNase H- reverse transcriptase (Life Technologies, Inc.), 1 mM dNTPs, 10 mM dithiothreitol and 20 units of RNasin (Promega Corp, Madison, WI, USA) in a 100 µl reaction. The reaction was incubated at 25°C for 10 min, 48°C for 40 min and 4°C for 5 min. We used the TaqMan Universal PCR master mix of PE Applied Biosystems (Foster City, CA, USA) following their recommended procedures.

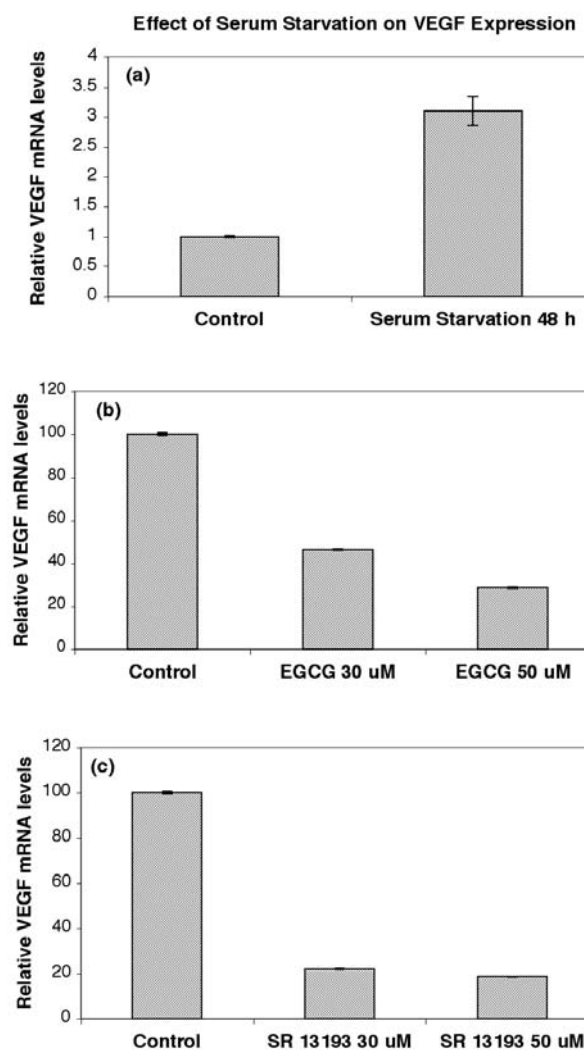


Figure 3. Induction effect of serum starvation on VEGF mRNA (a) and inhibition of VEGF induction by EGCG (b) and analog SR 13193 (c) using quantitative RT-PCR. MCF-7 cells were treated with appropriate concentrations of the compounds for 48 h in serum-free medium. Cells were lysed and total RNA was isolated as described in Materials and Methods. Human malate dehydrogenase (MDH) gene was used as internal control to normalize the test results.

TaqMan probes were designed based on the supplier's specifications using the Primer Express program and the chromophores FAM (6-carboxy-fluorescein) and TAMRA (6-carboxy-tetramethyl-rhodamine) as reporter and quencher dyes, respectively. An ABI PRISM 7700 Sequence detection system was used for thermal cycling. The optimum probe and primer concentrations were obtained by following thermal cycling steps: hold 2 min at 50°C, hold 10 min at 95°C, and each of 50 cycles of melting 15 sec at 95°C and annealing and extension 1 min at 60°C. The reactions were carried out in 96-well plates in triplicates. Data

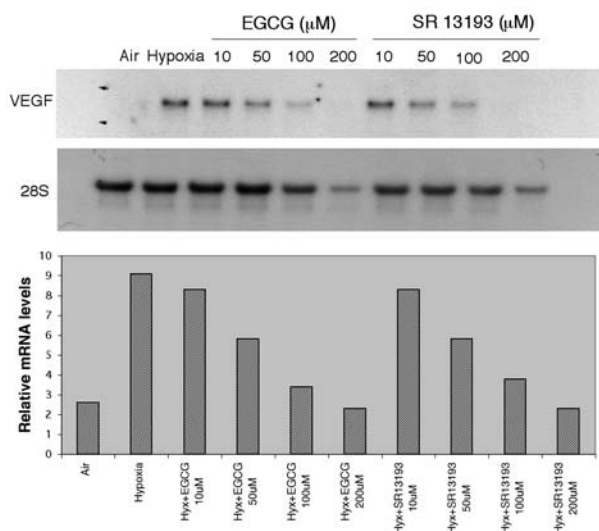


Figure 4. Effect of EGCG, SR 13196 and SR 13913 on the hypoxia-induced expression of VEGF. MCF-7 cells were treated with EGCG and SR 13193 at appropriate concentrations. Cells were subjected to hypoxia for 16 h. Total RNA was extracted and subjected to Northern blotting analysis. A 660 bp cDNA corresponding to the coding region of human VEGF<sub>165</sub> was used as probe. 28S RNA was used to normalize the data.

were analyzed using Sequence Detector version 1.6.3 program. The human malate dehydrogenase (MDH) gene was used as internal control to normalize the test results.

## Results

*D-ring EGCG analog SR 13193 inhibits proliferation of human breast cancer cells in vitro.* SR 13193 is a synthetic analog of EGCG, in which all three phenolic hydroxy groups of the D-ring galloyl group are methylated (Figure 1). We investigated the effect of this D-ring modification on the antiproliferative activity of SR 13193, compared to EGCG, in human breast cancer cells. Both hormone-responsive MCF-7 as well as hormone-insensitive MDA-MB-231 cells were used for this study. As shown in Figure 2, SR 13193 inhibits cell proliferation of breast cancer cells with potencies similar to EGCG. The IC<sub>50</sub> for MCF-7 cell growth inhibition by SR 13193 was  $11.4 \pm 1.1 \mu\text{M}$ , whereas the IC<sub>50</sub> for EGCG was  $12.5 \pm 0.79 \mu\text{M}$ . The IC<sub>50</sub> values for SR 13193 and EGCG were  $8.8 \pm 0.56 \text{ mM}$  and  $7.8 \pm 1.6 \text{ mM}$  in MDA-MB-231 cells, respectively. These results indicate that this modification of the D-ring does not affect the antiproliferative activity of EGCG. These results also show that the activity of EGCG and the D-ring analog are independent of the hormonal status of the breast cancer cells. We next investigated the effect of SR 13193 on molecular events affected by EGCG.

SR 13193 and EGCG inhibit the cellular stress-induced induction of VEGF, a potent angiogenic factor. VEGF is one of the most potent angiogenic factors implicated in cancer progression and angiogenesis. VEGF secreted by tumor cells stimulates angiogenesis and promotes tumor growth. VEGF expression is increased in tumor cells exposed to stress such as serum starvation (24) and hypoxia (25). EGCG was shown to decrease the expression of VEGF mRNA in human colon carcinoma cells exposed to serum starvation (22). We investigated the effect of SR 13193 and EGCG on the induction of VEGF mRNA by serum deprivation in MCF-7 breast cancer cells using quantitative RT-PCR. Serum starvation for 48 h causes a 3-fold induction of VEGF mRNA (Figure 3a). SR 13193 suppresses the induction of VEGF mRNA to control levels in MCF-7 breast cancer cells at concentrations of 30  $\mu\text{M}$  and 50  $\mu\text{M}$  (Figure 3c), more effectively than EGCG at the same concentrations (Figure 3b).

SR 13193 and EGCG inhibit hypoxia-induced production of VEGF mRNA in breast cancer cells. Since hypoxia is also known to stimulate the expression of VEGF in tumor cells (25), we investigated the effect of SR 13193 and EGCG on the hypoxia-mediated induction of VEGF in MCF-7 cells. Hypoxia exposure resulted in a 7-fold induction of VEGF mRNA in MCF-7 cells (Figure 4). Both EGCG and the analog SR 13193 inhibited the induction of VEGF mRNA by hypoxia at 50  $\mu\text{M}$  and 100  $\mu\text{M}$ .

Our results indicate that this modification of the D-ring of EGCG does not adversely affect the inhibitory potential of EGCG against *in vitro* tumor cell proliferation or its ability to inhibit the induction of the key angiogenic factor, VEGF.

## Discussion

The molecular mechanisms for the chemopreventive activity of EGCG appear to be multifaceted (4). On one hand, the anticarcinogenic activity of EGCG has been attributed to the antioxidative potential of its phenolic hydroxyl groups, whereas on the other hand, several recent reports have postulated discrete molecular targets for EGCG itself. The question of whether it is the antioxidant activity of EGCG or its interaction with specific targets that is responsible for its anticarcinogenic activity can be resolved by evaluating carefully designed synthetic analogs of EGCG that are specifically modified to answer these questions. We have addressed this issue by devising a chemical synthesis of racemic EGCG that allows us to vary the different rings of EGCG using a *de novo* approach (19). Using our approach, we have synthesized several synthetic EGCG analogs modified in the A, B and D-rings of EGCG. Here we report our studies with the D-ring analog, SR 13193. Our results show that SR 13193 is as potent as EGCG in inhibiting the proliferation of human breast cancer cells *in*



*vitro*. Our study also demonstrates that SR 13193 is as effective as EGCG in inhibiting the induction of VEGF mRNA produced by hypoxia and serum starvation.

There are very few structure-function studies of EGCG with respect to its molecular mechanism of action and these are limited to the available naturally occurring epimeric catechin analogs of EGCG or analogs obtained by minor modifications of EGCG itself (15-17). There have been some studies on the effect of the structure of the various green tea catechins on their antioxidant potential (10). Guo *et al.* (26) have shown that the free radical scavenging activity of 3-galloylated catechins such as EGCG were stronger than their non-galloylated counterparts (that do not possess a galloyl D-ring, Figure 1). However, Valcic *et al.* (27) studied the detailed mechanism of the antioxidant activity of EGCG and have provided convincing evidence that it is the gallate B-ring of EGCG, and not the galloyl D-ring, that is the primary site for the antioxidant reactions of EGCG. The D-ring may take part in the antioxidant reactions at higher concentrations of reactive oxygen species, but it is the B-ring that primarily takes part in the scavenging reactions of reactive oxygen species (27).

Our results with the synthetic D-ring modified analog, SR 13193, appear to be consistent with the observations of Valcic *et al.* (27), that it is the B-ring phenolic hydroxyls of EGCG and not the D-ring hydroxyls that take part in antioxidant reactions of EGCG. SR 13193 is modified in the D-ring but still possesses the B-ring phenolic hydroxyls (Figure 1). The D-ring modification in SR 13193, therefore, does not appear to affect the growth inhibitory activity of EGCG. Further studies on the comparison of the antioxidant potential of SR 13193 with EGCG should give valuable insights into the contribution of the antioxidant activity to the anticarcinogenic potential of EGCG. Studies with analogs such as SR 13193 on other molecular targets of EGCG will also clearly give insights into the structure-function relationships of the various phenolic hydroxy groups in the A, B and D-rings of EGCG and the chemopreventive mechanism of EGCG.

Inhibition of angiogenesis is of great interest as a mechanism of chemoprevention (28). Therefore, therapies that modulate the expression of angiogenic factors such as VEGF are of great interest as potential antiangiogenic chemopreventive agents. Analogs like SR 13193, that act by inhibiting VEGF induction, further offer promise for chemopreventive development, not only because of their molecular mechanisms of action, but perhaps their structural differences compared to EGCG may overcome the pharmacokinetic and bioavailability issues that have plagued the use of EGCG as an effective chemopreventive agent.

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