Biological and Mechanistic Characterization of Novel Prodrugs of Green Tea Polyphenol Epigallocatechin Gallate Analogs in Human Leiomyoma Cell Lines

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ABSTRACT

Uterine fibroids (leiomyomas) are very common benign tumors grown on the smooth muscle layer of the uterus, present in up to 75% of reproductive-age women and causing significant morbidity in a subset of this population. Although the etiology and biology of uterine fibroids are unclear, strong evidence supports that cell proliferation, angiogenesis and fibrosis are involved in their formation and growth. Currently the only cure for uterine fibroids is hysterectomy; the available alternative therapies have limitations. Thus, there is an urgent need for developing a novel strategy for treating this condition. The green tea polyphenol epigallocatechin gallate (EGCG) inhibits the growth of uterine leiomyoma cells in vitro and in vivo, and the use of a green tea extract (containing 45% EGCG) has demonstrated clinical activity without side effects in women with symptomatic uterine fibroids. However, EGCG has a number of shortcomings, including low stability, poor bioavailability, and high metabolic transformations under physiological conditions, presenting challenges for its development as a therapeutic agent. We developed a prodrug of EGCG (Pro-EGCG or 1) which shows increased stability, bioavailability and biological activity in vivo compared to EGCG. We also synthesized prodrugs of EGCG analogs, compounds 2a and 4a, in order to potentially reduce their susceptibility to methylation/inhibition by catechol-O-methyltransferase. Here, we determined the effect of EGCG, Pro-EGCG, and 2a and 4a on cultured human uterine leiomyoma cells, and found that 2a and 4a have potent antiproliferative, antiangiogenic, and antifibrotic activities. J. Cell. Biochem. 9999: 1–13, 2016. © 2016 Wiley Periodicals, Inc.

KEY WORDS: UTERINE FIBROIDS; LEIOMYOMA; GREEN TEA; ECGG ANALOGS; PRODRUGS; THERAPY

Uterine fibroids, or uterine leiomyomas, are benign tumors grown on the smooth muscle layer (myometrium) of the uterus [Flake et al., 2003; Walker and Stewart, 2005]. Uterine fibroids are very common and present in up to 75% of reproductive-age women [Baird et al., 2003]. Uterine fibroids are comprised of smooth muscle cells and fibrous elements, although their etiology and...
biology are poorly understood. However, the literature strongly supports the role of hormonal factors (estrogens and progestogens) and accumulation of the extracellular matrix in favoring the growth of uterine fibroids. Furthermore, it is believed that uterine fibroid formation and growth involves dysregulated cellular proliferation, angiogenesis and fibrosis [Islam et al., 2014].

Currently the only cure for uterine fibroids is hysterectomy (removal of the uterus). About 200,000 myomectomy and hysterectomy procedures for uterine fibroids are performed each year in the United States, ranking them among the most common surgical procedures [Whitman et al., 2008; Cardozo et al., 2012]. There are two alternative procedures, approved by the Food and Drug Administration (FDA), uterine artery embolization and magnetic resonance-guided focused ultrasound surgery. However, these approaches have numerous limitations to wide spread use, and often new fibroids grow after such treatment [Taylor and Leppert, 2012; Segars et al., 2014]. Furthermore, the available drug therapies for uterine fibroids have limitations regarding efficacy or safety for long-term use [Taylor and Leppert, 2012; Segars et al., 2014]. Uterine fibroids, though not life-threatening, present a major public health challenge, with more than $4.1 billion in direct annual healthcare expenditures in the U.S. and annual societal costs greater than those for breast, colon or ovarian cancer [Cardozo et al., 2012]. Thus, there is an urgent need to develop novel strategies for treating this condition.

It has been reported that the green tea polyphenol epigallocatechin gallate (EGCG; Fig. 1A) inhibits the growth of uterine leiomyoma cells in vitro and in vivo [Ozercan et al., 2008; Zhang et al., 2010a,b], and the use of a green tea extract (containing 45% EGCG) suggests potential clinical activity as a safe therapeutic agent for symptomatic women with uterine fibroids [Roshdy et al., 2013]. However, EGCG has a number of shortcomings, including low stability, poor bioavailability, and high metabolic transformations under physiological conditions, presenting challenges for its development as a therapeutic agent [Johnson et al., 2010; Sang et al., 2011; Kanwar et al., 2012].

We have reported that EGCG is a proteasome inhibitor and tumor cell death inducer [Nam et al., 2001]. In an attempt to increase the stability of EGCG, we chemically converted its reactive hydroxyl groups into the corresponding acetates. We further demonstrated that this peracetate-protected EGCG (Pro-EGCG or 1, Fig. 1B) could be converted into EGCG under cell-free conditions and function as a prodrug of EGCG under cellular and in vivo conditions [Lam et al., 2004]. We also provided evidence that when breast cancer MDA-MB-231 cells were treated with Pro-EGCG, the EGCG metabolite was generated and accumulated intracellularly, accompanied by enhanced levels of proteasome inhibition, growth suppression, and apoptosis, as compared to cells treated with EGCG. To investigate the potential use of Pro-EGCG as a novel prodrug that converts to a proteasome inhibitor and antitumor agent in vivo, MDA-MB-231 tumors were induced in nude mice, followed by daily treatment with Pro-EGCG or EGCG for 31 days. The results of this in vivo study showed a significant inhibition of tumor growth by Pro-EGCG, as compared to EGCG, associated with increased proteasome inhibition and apoptosis induction in tumor tissues [Landis-Piwowar et al., 2007b]. The increased stability, bioavailability and biological activity of Pro-EGCG in vivo as compared to EGCG has been confirmed by independent research groups using various in vivo models [Lambert et al., 2006; Vyas et al., 2007; Lee et al., 2008; Meeran et al., 2011; Chiou et al., 2012, 2013; Wang et al., 2013].

It has been shown that EGCG is a substrate of the enzyme catechol-O-methyltransferase (COMT), which is involved in the metabolism of catecholamines and catechol-containing drugs [Miller et al., 2012]. The major site of EGCG methylation is the catechol moiety of the gallate ester ring affording the metabolite 4′′-O-methyl-EGCG (Fig. 1A) [Lu et al., 2003]. We further showed that the methylated metabolites of EGCG such as 4′′-O-methyl-EGCG have much lower proteasome inhibition activities than EGCG [Landis-Piwowar et al., 2007a]. Prodrugs of EGCG should be similarly affected by COMT under cellular conditions. We hypothesized that analogs of EGCG, such as compounds 2 or 4 (Fig. 1D), where one or two hydroxyl groups have been removed from the gallate ester moiety, may not be as susceptible to COMT-mediated methylation as EGCG. We thus designed and synthesized Pro-EGCG analogs 2a and 4a (Fig. 1C), prodrugs of the EGCG analogs 2 and 4. We previously showed that the racemic trans-substituted isomers of compounds 2a and 4a (Fig. 1C and D, lower panel) are more potent proteasome inhibitors and apoptosis inducers than Pro-EGCG and EGCG in cultured human T cell leukemia Jurkat cells [Kuhn et al., 2005]. In addition, these racemic trans-substituted isomers of compounds 2a and 4a were more potent than Pro-EGCG in inhibiting the proliferation of human breast cancer MCF-7 cells [Kuhn et al., 2005].

This study expands our understanding of the mechanism of action of Pro-EGCG and Pro-EGCG analogs and assesses whether Pro-EGCG and its analogs 2a and 4a display antiproliferative, antiangiogenic, and antifibrotic properties using human leiomyoma cell lines, as a prelude to advancing one or more compounds to human clinical trials.

MATERIALS AND METHODS

CHEMISTRY

Pro-EGCG or compound 1 (EGCG octaacetate) was synthesized according to literature procedure [Lam et al., 2004]. Compound 2a, the peracetate derivative of epigallocatechin (EGC) 3-hydroxybenzoate, had previously been synthesized using a semi-synthesis approach starting from EGC [Huo et al., 2008]. Because EGC is not readily available, we have developed an alternative semi-synthesis starting from the more readily available EGCG according to the scheme presented in the Supplemental Information. This approach has also been applied to the synthesis of compound 4a, the peracetate derivative of EGC 3,5-dihydroxybenzoate. The experimental details of the synthesis are provided in the Supplemental Information.

CELLS AND MATERIALS

Human uterine leiomyoma (UtLM) cells (GM10964) were purchased from Coriell Institute and transformed human uterine leiomyoma cells (UtLM-hI) transfected with human telomerase reverse transcriptase were provided as a kind gift from Dr. Darlene Dixon (NIEHS) [Carney et al., 2002]. Both cell lines were grown in complete MEM media supplemented with 10% fetal bovine serum (FBS), 0.1%
insulin, 0.2% hFGF-B, 0.1% GA-1000, and 0.1% hEGF (Lonza). Cell culture maintenance and experiments were performed at 37°C and 5% CO₂. (-)-Epigallocatechin-3-gallate (EGCG, 99% purity, Sigma–Aldrich), Pro-EGCG and analogs 2a and 4a were all dissolved in DMSO at 50 mM concentration as a stock solution, stored at −20°C, and diluted in cell culture medium when used. 3,5-Dinitrocatechol (DNC), a COMT inhibitor [Pérez et al., 1993], was purchased from Sigma–Aldrich, dissolved in DMSO at a 10 mM concentration as a stock solution, stored at −20°C, and diluted in cell culture medium as a 1 mM working solution. FBS was purchased from Life Technologies, dimethyl sulfoxide (DMSO) was from Fisher Scientific, and MEM media was from Gibco. Specific antibodies to PCNA, Caspase 3, Cyclin A, and β-actin were purchased from Santa Cruz Biotechnology, to poly ADP ribose polymerase (PARP), Akt and phospho-Akt from Cell Signaling, to VEGF-C and collagen type I (CoLI) from Abcam®, and to VEGF-R2 from Proteintech. The protein assay kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

**WHOLE CELL PROTEIN EXTRACT PREPARATION**

The treated cells were harvested by a scraper, centrifuged at 1,500 rpm and suspended in ice-cold cell lysis buffer (50 mM Tris-Cl, pH 7.4/150 mM NaCl/0.5% NP-40) for 30 min, followed by centrifugation at 12,000 rpm for 15 min. The clear supernatant was used as a whole cell protein extract.

**CELL VIABILITY ANALYSIS**

Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay, Sigma) as previously described [Nam et al., 2001]. Briefly, cells were plated at a density of 2 × 10³ cells/well in 96-well plates. After 24 h, the cells (in triplicate wells) were treated daily with EGCG, Pro-EGCG or Pro-EGCG analogs at various concentrations for 3 successive days. At the end of the experiment, the cells were incubated with 100 μl/well of 0.5% MTT solution for 4 h at 37°C. The MTT solution was removed and the dye was solubilized with 100 μl/well of DMSO for 10 min. The optical density (OD) of each well was measured.
with a spectrophotometer at 570 nm. Mean values of OD for each concentration were calculated.

**3-D SPHEROID FORMATION ASSAY**

Single cell suspensions of UtLM or UtLM-ht cells in serum-free medium containing supplements of B-27 (Gibco®) and EGF (Sigma) were plated in triplicate on low attachment six-well dishes (Corning) and treated daily with various concentrations of EGCG, Pro-EGCG, or Pro-EGCG analogs. After 6 days, primary spheres were photographed at 200× magnification and those with diameters greater than 125 microns were counted manually using an objective ruler, using spheroids in blank and DMSO-treated groups as controls.

**WESTERN BLOT ANALYSIS**

The cells were grown to ~80% confluency in 100 mm dishes, and treated with EGCG, Pro-EGCG, or Pro-EGCG analogs at various concentrations for 48 h, followed by harvesting and preparing whole cell extracts. Equivalent amounts of protein extracts (30–50 μg) were separated by NuPAGE Novex 10% Bis-Tris Gel (Invitrogen Life Technologies, Carlsbad, CA) under reducing conditions using 116 V for 120 min. The proteins were then electrophoretically transferred onto PVDF membranes (Millipore Corp., Billerica, MA) using the XCell II Blot Module (Invitrogen Life Technologies). After blocking nonspecific binding sites by incubation for 1 h with PBS-containing 5% fat-free milk and 0.1% Tween 20, the membranes were incubated with the primary antibodies overnight at 4°C. Immunologic detection was performed using the following primary antibodies: human proliferation cell nuclear antigen (PCNA) (1:500 dilution), Cyclin A (1:500), VEGF-C (1:300), VEGF-R2 (1:500), PARP (1:500), Caspase 3 (1:500), Akt (1:500), and pAkt (1:500). The membranes were then incubated for 1 h with horseradish peroxidase conjugated secondary antibodies, diluted at 1:5000 with blocking buffer. The antigen-antibody complexes were detected with the ECL chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ). The membranes were reprobed with a monoclonal antibody raised against β-actin (diluted 1:1000 to 5000, Sigma) as an internal control for protein loading and normalization between samples. Films exposed to blots were scanned and the optical densities of the positive signals were quantified.

**IMMUNOFLUORESCENCE ASSAY**

The UtLM-ht cells were seeded on cover slides using six-well plates and grown to 70–80% confluence, followed by treatment with EGCG, Pro-EGCG, Pro-EGCG analogs, or vehicle for 24 h. The dosage of each agent was selected based on a preliminary test for cell morphology and dose-range. The cells were then fixed in 4% formaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 15 min. The treated cells were stained for α-smooth muscle actin (αSMA) with overnight incubation in a refrigerator. The cells were washed with PBS and incubated for 1 h in a 1:200 dilution of the secondary antibody, a fluorescein-labeled anti-mouse antibody. After further washing, DAPI (1:200 dilution) was added in the dark. Following more washing, the slides were fixed in Eukitt® and placed in a dry chamber. The slides were dried and hardening mounting medium was applied. The slides were photographed at 200× magnification using a Leica microscope. The same field of view was photographed with and without primary antibodies to assess the staining signal.

**RNA ISOLATION AND ANALYSIS BY RT-PCR**

Total RNA was extracted from treated and control cells using the Qiagen RNeasy Mini Kit according to the manufacturer’s instructions (Qiagen, Courtaboeuf, France). One microgram of total RNA was used for reverse transcription using the RT first-strand kit. The PCR reactions were carried out in a final volume of 20 μL. The standard cycling condition was 50°C for 2 min and 95°C for 10 min, followed by 30 cycles of 95°C for 15 s and 60°C for 1 min. The products were visualized by agarose gel electrophoresis followed by ethidium bromide staining.

**WOUND HEALING ASSAY**

UtLM-ht cells at 90% confluency were scratched with a sterile 200 μL micropipette tip, and treated with EGCG, Pro-EGCG, Pro-EGCG analogs, or vehicle for 24 h. Cell morphologic evaluation was performed under a ZEISS AxionCam MRc phase-contrast microscope. The same fields of the wound margin were photographed at 100× magnification at 0 and 24 h. Wound areas were determined using ImageJ software and the results were calculated as a percentage of wound closure.

**IMAGE J ANALYSIS**

Image J analysis software (NIH) was used to perform data analysis for the wound healing assay.

**CELL-FREE PROTEASOME ACTIVITY ASSAY**

UtLM-ht cells were treated as indicated and then harvested, and used for whole-cell extract preparation. Whole-cell extracts (10 μg) were incubated with Suc-Leu-Leu-Val-Tyr-AMC (40 μmol/L) fluorogenic substrate at 37°C in 100 μL of assay buffer (50 mmol/L Tris-HCl, pH 8) for 2 h. After incubation, production of hydrolyzed 7-amino-4-methylcoumarin (AMC) was measured using a Victor3 Multilabel Counter with an excitation filter of 380 nm and an emission filter of 460 nm (Perkin-Elmer).

**RESULTS**

**PRO-EGCG ANALOGS POTENTLY INHIBIT PROLIFERATION OF HUMAN UTERINE LEIOMYOMA CELL LINES**

The formation and growth of uterine fibroids involves the cellular processes of proliferation, fibrosis, and angiogenesis [Islam et al., 2014], all of which have been shown to be inhibited by EGCG [Xu et al., 2009; Larsen and Dashwood, 2010; Matsuzaki and Darcha, 2014]. Since Pro-EGCG and related analogs have been demonstrated to be more potent than EGCG in various models [Lam et al., 2004; Kuhn et al., 2005; Lambet et al., 2006; Landis-Piwowar et al., 2007a, b; Vyas et al., 2007; Lee et al., 2008; Meieran et al., 2011; Chio et al., 2012, 2013; Wang et al., 2013], we initially compared the effect of Pro-EGCG and its analogs 2a and 4a, to EGCG on the proliferation of human uterine leiomyoma cell lines.

UtLM-ht (Fig. 2A) or UtLM (Fig. 2B) cells were treated with the solvent DMSO, EGCG, Pro-EGCG (1), analog 2a, or analog 4a, captured and merged using Nikon Advanced Research Imaging Software.
followed by the MTT assay. We found that all of the compounds inhibited the proliferation of both cell lines in a dose-dependent manner and the order of growth-inhibitory potency was the following: 2a, 4a > Pro-EGCG > EGCG. These results are generally consistent with those previously observed with human breast cancer MCF-7 cells involving the racemic trans-substituted isomers of 2a and 4a [Kuhn et al., 2005]. In our study, EGCG seems to have greater antiproliferative activity than a previous report [Zhang et al., 2010a], although our experimental protocol may differ.

Tumor stem cells have the characteristics of forming tumor-spheres [Chen et al., 2012]. We wondered whether cultured human uterine leiomyoma cells could form 3D spheres, and if so, whether

Fig. 2. Inhibition of human uterine leiomyoma cell growth and proteasome activity. UtLM-ht (A) or UtLM (B) cells, grown in a 96-well plate, were treated with either the control solvent DMSO, EGCG, Pro-EGCG, 2a, or 4a (at 5, 10, or 25 μM) daily for up to 3 days (with each drug repeatedly added every 24 h), followed by the MTT assay. UtLM-ht cells (C) were treated with either the control solvent DMSO, EGCG, Pro-EGCG, 2a, or 4a (at 25 μM) for 24 h, followed by preparation of whole cell extracts and performance of cell-free proteasomal chymotrypsin (CT)-like activity assay.
EGCG, Pro-EGCG and its analogs could have any inhibitory effects. A spheroid formation assay of UtLM-h (Fig. 3A and C) and UtLM cells (Fig. 3B and D) was performed and the effects of EGCG, Pro-EGCG, and Pro-EGCG analogs were determined. This assay has never been conducted previously with uterine leiomyoma cells. We report for the first time, that both uterine leiomyoma cell lines were able to form spheroid masses—large colonies of spheres formed from both cell lines under untreated or DMSO-treatment conditions by day 6 (Fig. 3A and B). In parallel, dishes treated daily with EGCG, Pro-EGCG, or analogs 2a or 4a for 6 days, decreased spheroids were observed in both numbers and size in both cell lines (Fig. 3A and B), with an order of inhibition of \(4a > 2a > \text{Pro-EGCG} > \text{EGCG}\) (Fig. 3C and D).

The above results suggest that EGCG, Pro-EGCG and Pro-EGCG analogs are able to inhibit proliferation of human uterine leiomyoma cell lines. To provide further evidence on a molecular level, we measured the effects of these agents on expression of cell cycle-specific proteins. PCNA and Cyclin A are two S-phase markers [Dou and Pardee, 1996]. UtLM-h (Fig. 4A) or UtLM (Fig. 4B) cells were treated with DMSO, EGCG, Pro-EGCG, and Pro-EGCG analogs 2a and 4a. We found that cell treatment resulted in a decrease of PCNA and Cyclin A protein levels as follows. Pro-EGCG analog 4a potently reduced the levels of both proteins in both cell lines. Pro-EGCG analog 2a preferentially reduced the levels of both proteins in the UtLM cell line, whereas Pro-EGCG preferentially reduced the levels of both proteins in the UtLM-h cell line. EGCG did not seem to affect PCNA and Cyclin A in the UtLM cell line and its effect in UtLM-h cells was less than the other studied compounds (Fig. 4). EGCG has previously been shown to reduce PCNA protein levels in cultured human...
leiomyoma cells, although the effect was only seen at concentrations of 50 μM and above [Zhang et al., 2010a].

**PRO-EGCG ANALOGS POTENTLY INDUCE APOPTOTIC DEATH OF HUMAN UTERINE LEIOMYOMA CELL LINES**

EGCG has been shown to induce apoptosis in various cancer cell lines [Chen et al., 2008]. We found that Pro-EGCG analogs 2a and 4a induced apoptosis-related morphological changes in both UtLM-ht and UtLM cell lines (data not shown). We next confirmed these observations by measuring the levels of PARP and pro-caspase-3 proteins [Yang and Dou, 2010]. We found that Pro-EGCG analogs 2a and 4a potently reduced pro-caspase 3 and PARP protein levels, indicating caspase activation, in both uterine leiomyoma cell lines and were more effective than EGCG and Pro-EGCG (Fig. 4). Interestingly, although this result is consistent with previous observations using the racemic trans-substituted isomers of 2a and 4a with human T cell leukemia Jurkat cells [Kuhn et al., 2005], a previous study with 2a in Jurkat cells indicated that it was less potent than Pro-EGCG in reducing PARP protein levels and inducing cell death, and equally potent in reducing caspase-3 protein levels [Huo et al., 2008]. However, a direct comparison of the biological activity of 2a and 4a with their trans-isomers has not yet been conducted. As the deacetylated metabolites of 2a and 4a are EGCG analogs, while the deacetylated metabolites of the trans-isomers are GCG analogs, one might expect the cis-isomers to have greater biological activity than the trans-isomers, based on previous comparisons between EGCG and GCG [Smith et al., 2002]. However, these observations may not apply across all assays comparing biological activity.

We found that expression of α-smooth muscle actin (αSMA) could also be used as a marker of apoptotic cell death (Fig. 5). UtLM-ht cells were treated with the control solvent DMSO or EGCG, Pro-EGCG, or Pro-EGCG analogs 2a or 4a, followed by

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**Fig. 4.** Reduction of protein biomarkers of cell proliferation, apoptosis and angiogenesis by Pro-EGCG analogs. UtLM-ht (A) or UtLM (B) cells were either untreated (NT) or treated with the control solvent DMSO, EGCG, Pro-EGCG, 2a, or 4a (at 15, 25, or 35 μM) for 48 h, followed by Western blotting using specific antibodies to PCNA (MW 37 kDa), Cyclin A (MW 54 kDa), Caspase-3 (MW 32 kDa), PARP (MW 116 kDa), VEGF-R2 (MW 152 kDa; the 200 kD-band might be a tetra-ubiquitinated form), VEGF-C (MW 46 kDa), pAkt and total Akt (MW 64 kDa) and Actin (MW 46 kDa). NT, no treatment; DM, DMSO. In (B), PARP, lane 1, the image was lost.

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immunostaining with a specific antibody to αSMA or DAPI. In control cells, αSMA is expressed mainly in the cytosol (red = αSMA; blue = nucleus) (Fig. 5A and D). EGCG treatment caused a decreased level of cytosolic αSMA expression and an increased level of nuclear αSMA expression (Fig. 5B). Treatment of Pro-EGCG induced a greater level of nuclear translocation of αSMA in almost all the cells, as compared to EGCG (Fig. 5E vs. B). Pro-EGCG analogs 2a and 4a induced apoptosis in all cells, as all of the apoptotic bodies expressed αSMA (Fig. 5C and F). The relative order of inducing αSMA nuclear expression and apoptosis was the following: 2a, 4a >> Pro-EGCG 1 > EGCG.

**PRO-EGCG ANALOGS INHIBIT EXPRESSION OF FIBROSIS BIOMARKERS IN HUMAN UTERINE LEIOMYOMA CELL LINES**

Fibrosis plays an important role in uterine leiomyoma formation and growth [Islam et al., 2014]. We determined the effect of EGCG, Pro-EGCG and Pro-EGCG analogs on the gene expression of two uterine fibrosis biomarkers—αSMA and collagen type I (Col-I). We found that Pro-EGCG analogs 2a and 4a potently reduced collagen type I and αSMA mRNA expression in the UtLM-ht cell line and were more effective than EGCG and Pro-EGCG (Fig. 6). EGCG has been shown to reduce these fibrosis markers in other models [Tipoe et al., 2010].

**PRO-EGCG ANALOGS INHIBIT EXPRESSION OF ANGIogenesis BIOMARKERS IN HUMAN UTERINE LEIOMYOMA CELL LINES**

Angiogenesis is necessary for the development of uterine fibroids [Islam et al., 2014]. We determined the effect of EGCG, Pro-EGCG and Pro-EGCG analogs on two angiogenesis biomarkers—vascular endothelial growth factor receptor 2 (VEGF-R2) and vascular endothelial growth factor C (VEGF-C) [Ebos and Kerbel, 2011]. The protein levels of these biomarkers have been previously shown to be reduced by EGCG in endometrial tissue [Xu et al., 2011].

UtLM-ht and UtLM cell lines were treated with DMSO, EGCG, Pro-EGCG, or Pro-EGCG analogs, followed by Western blot analysis using a specific antibody to VEGF-R2 or VEGF-C (Fig. 4). Our data shows that Pro-EGCG analogs 2a and 4a potently reduce protein levels of VEGF-R2/p152 in both cell lines (Fig. 4A and B). In addition, a ~200 kDa form of VEGF-R2 was detectable by this polyclonal antibody in the UtLM-ht cells after treatment with all of the compounds, with analog 2a being the most effective inducer (indicated by an arrowhead; Fig. 4A). VEGF-R2/p200 might be a tetra-ubiquitinated form of this protein prior to proteasomal degradation. Pro-EGCG analog 4a potently reduced VEGF-C levels in both cell lines. Pro-EGCG analog 2a preferentially reduced VEGF-C levels in the UtLM cell line, while Pro-EGCG preferentially reduced this level in the UtLM-ht cell line. EGCG did not seem to markedly affect VEGF-C levels in either cell line. This profile is similar to that discussed previously with regard to PCNA and Cyclin A and suggests that the mechanism of action of each compound varies depending on the cellular environment.

**PRO-EGCG ANALOGS INHIBIT MIGRATION OF HUMAN UTERINE LEIOMYOMA CELL LINES**

To study whether EGCG, Pro-EGCG, and Pro-EGCG analogs can affect migration of uterine leiomyoma cells, we performed a scratch-wound-healing assay with UtLM-ht (Fig. 7) and UtLM cells (data not shown). At time zero, a scratch was produced in all the dishes, which were then either not treated or treated with DMSO or each agent at 25 μM, followed by a 24-h recovery period. The untreated UtLM-ht cells were able to migrate into the “gap” produced by the scratch and in the presence of DMSO, the migration was almost complete (90% recovery) (Fig. 7). We found that all of the tested compounds were able to inhibit the migration of UtLM-ht cells and the Pro-EGCG analogs 2a and 4a were the most effective (Fig. 7). A similar result was observed when UtLM cells were used (data not shown).

**PRO-EGCG ANALOGS INHIBIT ENZYMATIC ACTIVITIES LINKED TO DIRECT MOLECULAR TARGETS OF EGCG**

EGCG potently inhibits proteasomal chymotrypsin-like activity [Nam et al., 2001; Smith et al., 2002]. We have previously shown that the racemic trans-substituted isomers of compounds 2a and 4a are more potent proteasome inhibitors than Pro-EGCG and EGCG in cultured human T cell leukemia Jurkat cells [Kuhn et al., 2005]. We assessed the levels of proteasome inhibition in UtLM-ht cells and determined that the relative levels of potency in this cell line were: 4a > 2a, Pro-EGCG > EGCG (Fig. 2C). Previous studies in Jurkat T cells showed that 2a was less potent than Pro-EGCG in proteasome inhibition [Huo et al., 2008], possibly due to cellular environment and COMT activity levels. We must also consider that proteasome inhibition and other biological activity comparisons between EGCG and GCG [Smith et al., 2002] may not apply widely to all analogs of EGCG and GCG. Assessing COMT activity levels directly in the cell lines and comparing the proteasome-inhibitory activity of 2a and 4a with their trans-isomers directly would help to further support these observations.

![Fig. 5. Analysis of αSMA biomarker in human uterine leiomyoma UtLM-ht cell line by immunohistochemistry. Cells were treated with the control solvent DMSO (A and D) or 25 μM of EGCG (B), Pro-EGCG (E), 2a (C), or 4a (F) for 24 h, followed by immunostaining with a specific antibody to αSMA (red) or DAPI (4',6-diamidino-2-phenylindole) for nucleus (blue), and visualized with a Ti-E fluorescence microscope.](image-url)
Unlike other proteasome inhibitors, EGCG has been found to inhibit Akt kinase activity leading to reduced tumor cell growth [Hao et al., 2007]. EGCG has been demonstrated to be an ATP-competitive inhibitor of PI3K (as well as mTOR) within physiologically relevant concentrations, directly binding to the kinase domain active site [Van Aller et al., 2011].

We assessed whether Pro-EGCG and its analogs could inhibit Akt activation in human uterine leiomyoma cell lines. UtLM-ht (Fig. 4A) or UtLM (Fig. 4B) cells were treated with either control DMSO, EGCG, Pro-EGCG, or Pro-EGCG analogs 2a or 4a, followed by Western blotting with specific antibodies to pAkt and total Akt protein. We found that the order of the inhibition of pAkt but not total Akt protein expression by these compounds is: 4a, 2a > Pro-EGCG > EGCG (Fig. 4). Therefore, Pro-EGCG and its analogs are able to inhibit the Akt pathway in these human uterine leiomyoma cell lines.

COMT INHIBITION ENHANCES THE ANTIPROLIFERATIVE ACTIVITY OF PRO-EGCG IN HUMAN UTERINE LEIOMYOMA CELLS

Our data suggest that Pro-EGCG analogs 2a and 4a have more potent antiproliferative, antifibrotic, and antiangiogenic activity than Pro-EGCG and EGCG in human uterine fibroid cell lines. We then investigated the underlying molecular mechanisms.

One plausible mechanism for this increased activity might be related to a reduced susceptibility to methylation in the presence of...
COMT, due to the absence of the catechol structure in the ester D ring (Fig. 1). To test this hypothesis, we used a known inhibitor of COMT, DNC [Pérez et al., 1993]. Human UtLM-ht cells were pre-treated with 10 μM DNC or control solvent DMSO for 2 h, followed by co-treatment with either DMSO, EGCG, Pro-EGCG, or Pro-EGCG analogs, and the MTT assay. We observed that DNC treatment significantly increased the antiproliferative potency of EGCG and Pro-EGCG, but had a lesser effect on the antiproliferative activity of the Pro-EGCG analogs (Fig. 8). These data support our hypothesis, and suggest that UtLM-ht cells express active COMT, and that COMT is involved in regulating the sensitivity of cells to EGCG-based therapy. Zhang et al. [2014] also demonstrated the presence of active COMT in a human uterine leiomyoma cell line; however, their conclusions are obfuscated by their use of non-physiological concentrations of EGCG (100 μM). Consistent with the study reported here, we have previously shown that DNC treatment increases the biological activity of EGCG and Pro-EGCG in human breast cancer MDA-MB-231 cells—a cell line with a high level of COMT activity [Huo et al., 2010; Landis-Piwowar et al., 2010]. Other COMT inhibitors have also been reported to synergistically enhance EGCG’s growth inhibition of cancer cells [Forester and Lambert, 2014].

**DISCUSSION**

In the current study we tested whether Pro-EGCG and its analogs can be more potent antiproliferative, antifibrotic, and antiangiogenic agents than EGCG in cultured human leiomyoma cell lines. We also investigated the involved mechanisms of action, all toward the goal of moving these agents to clinical trials for treating symptomatic women with uterine fibroids.

Overall, our data is consistent with the hypothesis that the metabolites of Pro-EGCG analogs 2a and 4a have reduced susceptibility to COMT-mediated methylation as compared to EGCG (Figs. 1 and 8), whereby such methylation could be responsible for decreased biological activity. This could account for an increased activity of compounds 2a and 4a over Pro-EGCG and EGCG in all of the assays studied in this report. A previous case-control study of breast cancer in Asian-American women [Wu et al., 2003] revealed a reduced risk of breast cancer in women who consumed green tea and who also carried the low activity COMT polymorphism, while breast cancer risk did not differ between tea drinkers and non-tea drinkers among those who were homozygous for the high activity COMT allele. A plausible explanation for this difference is that in women with the high activity COMT allele, EGCG may have been converted by COMT into methylated EGCG metabolites. Previous studies have shown that methylated EGCGs have reduced biological activity [Landis-Piwowar et al., 2007a].

In the current study, cell proliferation was measured by growth of human uterine leiomyoma UtLM and UtLM-ht cell lines in both 2D and 3D culture models. The Pro-EGCG analogs 2a and 4a demonstrated greater antiproliferative activity than Pro-EGCG and EGCG (Figs. 2–4). The Pro-EGCG analogs 2a and 4a were also shown to be more potent apoptosis inducers in these leiomyoma cell lines when compared to Pro-EGCG and EGCG, as evidenced by increased levels of apoptotic bodies (Fig. 5), morphological changes (not shown), and decreased levels of caspase-3 and PARP protein expression (Fig. 4). We also found that both Pro-EGCG analogs are potent inhibitors of fibrosis biomarkers when compared to Pro-EGCG and EGCG (Fig. 6). Furthermore, Pro-EGCG analog 4a may have potent antiangiogenic activity, based on its ability to reduce protein levels of VEGF-R2 and VEGF-C in both UtLM and UtLM-ht cell lines (Fig. 4). While Pro-EGCG analog 2a demonstrated reduced protein levels of VEGF-R2 and VEGF-C in UtLM cells and VEGF-R2 in UtLM-ht cells, it had little effect on VEGF-C expression in UtLM-ht cells for unknown reasons (Fig. 4). Finally, both Pro-EGCG analogs potently inhibited migration of the uterine leiomyoma cells (Fig. 7).
Additional angiogenesis and fibrosis assays are needed in order to confirm the activity of compounds 2a and 4a as angiogenesis and fibrosis inhibitors.

Akt (protein kinase B) is a serine-threonine kinase that is activated by phosphoinositide-3-kinase (PI3K). Phosphorylated Akt (pAkt) triggers a number of downstream signals, regulating multiple cellular processes including proliferation, apoptosis, angiogenesis and fibrosis [Naumann, 2011; Makker et al., 2012]. The proapoptotic, antiproliferative, antiangiogenic, and antifibrotic properties of EGCG have all been shown to be associated (at least in part) with its inhibition of Akt signaling [Tang et al., 2003; Zhang et al., 2006; Qin et al., 2007]. Pro-EGCG has previously been shown to inhibit Akt signaling in leukemia cell lines and in vivo models [Lam et al., 2004; Chiu et al., 2012; Chiu et al., 2013]. In this report, we describe that Pro-EGCG analogs 2a and 4a are more potent inhibitors of Akt phosphorylation than Pro-EGCG and EGCG in human uterine leiomyoma cell lines (Fig. 4). As described above, this mechanism can (at least partially) account for the activity of these Pro-EGCG analogs in assays and affecting biomarkers associated with cell proliferation, apoptosis, angiogenesis and fibrosis. For example, it has been shown that transcription of CoLi and oSMA is regulated by the Akt signaling pathway in leiomyoma cells [Salama et al., 2012]. It has been shown that Akt signaling is required for proliferation of human uterine leiomyoma cells [Yin et al., 2007]. Also Akt signaling has been shown to play a role in regulating VEGF expression [Jiang and Liu, 2008].

The antiproliferative and proapoptotic effects of Akt pathway inhibition may be (at least partially) effected by downstream cascades involving elevation of p27 and IκBα levels [Lee et al., 2011; Hussain et al., 2012]. Both p27 and IκBα protein levels are also enhanced by proteasome inhibition [Landis-Piwowar et al., 2006]. Therefore, inhibition of chymotrypsin-like proteasome activity and Akt signaling are likely to result in synergistic proapoptotic and antiproliferative effects [Mimura et al., 2014]. Pro-EGCG, and the racemic trans-substituted isomers of 2a and 4a have all been shown to elevate p27 and IκBα protein levels [Kuhn et al., 2005]. Consistently, Pro-EGCG, 2a and 4a inhibited proteasome activity in human uterine leiomyoma cells (Fig. 2C).

We should also acknowledge that an alternate hypothesis may involve a potent inhibition of COMT by 2a and 4a (and synergizing less with DNC) in mediating some or all of the reported biological effects. At present, we do not favor this hypothesis because COMT activity has not yet been associated with all of the observed biological effects of 2a and 4a. In addition, the ability of EGCG to inhibit COMT activity has not yet been observed in vivo (see Lorenz et al., 2014). Nevertheless, further investigation of the effect of compounds 2a and 4a on signaling pathways associated with inhibition of Akt, proteasome and COMT are warranted in the future.

In summary, we found that the Pro-EGCG analogs 2a and 4a may have reduced susceptibility to COMT-mediated methylation and are proteasome and Akt pathway inhibitors. These mechanisms can create synergies and result in enhanced antiproliferative, antiangiogenic, and antifibrotic properties as compared to Pro-EGCG in human uterine leiomyoma cell lines. We hope to advance this research with more mechanistic and in vivo investigations.

The successful completion of this study has several potential advantages. First, these prodrugs should retain the established safety profile of EGCG. Second, these prodrugs should be more effective than EGCG or green tea extracts. Third, the Pro-EGCG analogs 2a and 4a may have efficacy in a broader patient population than EGCG (or green tea extracts) including both high-activity and low-activity COMT phenotypes. And finally, the inclusion of these prodrugs in the management of symptomatic women with uterine fibroids may have the potential for long-term use avoiding hysterectomy and preserving women’s reproductive functions in the absence of irreversible negative consequences.

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REFERENCES


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