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# Epigallocatechin gallate (EGCG), a major component of green tea, is a dual phosphoinositide-3-kinase/mTOR inhibitor

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ABSTRACT

The PI3K signaling pathway is activated in a broad spectrum of human cancers, either directly by genetic mutation or indirectly *via* activation of receptor tyrosine kinases or inactivation of the PTEN tumor suppressor. The key nodes of this pathway have emerged as important therapeutic targets for the treatment of cancer. In this study, we show that (−)-epigallocatechin-3-gallate (EGCG), a major component of green tea, is an ATP-competitive inhibitor of both phosphoinositide-3-kinase (PI3K) and mammalian target of rapamycin (mTOR) with $K_i$ values of 380 and 320 nM respectively. The potency of EGCG against PI3K and mTOR is within physiologically relevant concentrations. In addition, EGCG inhibits cell proliferation and AKT phosphorylation at Ser473 in MDA-MB-231 and A549 cells. Molecular docking studies show that EGCG binds well to the PI3K kinase domain active site, agreeing with the finding that EGCG competes for ATP binding. Our results suggest another important molecular mechanism for the anticancer activities of EGCG.



## 1. Introduction

Natural product EGCG is the major catechin of green tea, one of the most common beverages consumed in the world. Numerous studies have shown that EGCG has notable anticancer and cancer preventive effects against a variety of cancer types [1–3]. In particular, recent reports from ongoing clinical studies showed that EGCG and other green tea extracts possess remarkable chemopreventive activities [4,5]. Many mechanisms have been proposed for the cancer preventive activities of EGCG. However, it is still unclear which of these mechanisms play an important role in inhibiting carcinogenesis or cancer cell growth. In this study, we show that EGCG is a potent inhibitor of phosphoinositide-3-kinase (PI3K) and mammalian target of rapamycin (mTOR), two important components of the PI3K/Akt/mTOR signaling pathway.

The PI3K/AKT/mTOR signaling pathway is one of the most commonly activated signaling pathways in human cancer [6]. The reported somatic alterations that result in pathway activation include loss of tumor suppressor PTEN function, amplification or mutation of PI3K and AKT, and activation of growth factor receptors [7]. The important nodes of this pathway therefore have emerged as key therapeutic targets for the treatment of cancer. Numerous inhibitors of PI3K isoforms, AKT, and mTOR are currently in clinical development for the treatment of multiple cancer types [8]. Among the compounds currently being tested that target this pathway, probably the most interesting are dual PI3K-mTOR inhibitors. PI3Ks and mTOR belong to the PI3K superfamily, where the members possess structurally similar kinase domains. Several reported PI3K inhibitors also inhibit the catalytic activity of mTOR complexes. In this study, we show that natural product EGCG is a genuine inhibitor of PI3K and mTOR with $K_i$ values in the range of 300 nM and targets the PI3K/AKT/mTOR pathway in cancer cells. These findings shed new light on the molecular mechanism for anticancer and cancer preventive effects of EGCG.

## 2. Materials and methods

### 2.1. Reagents

EGCG was obtained from Sigma Chemical Co. (St. Louis, MO, USA) (purity > 92% by LC-MSMS). Primary antibodies to Akt and phosphorylated Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, MA), and secondary antibodies were purchased from GE Healthcare UK limited (United Kingdom). The bicinchoninic acid protein assay kit was obtained from Pierce

*Abbreviations:* EGCG, (−)-epigallocatechin-3-gallate; PI3K, phosphoinositide-3-kinase; mTOR, mammalian target of rapamycin; PI(3,4,5)$P_3$, phosphatidylinositol-3,4,5-triphosphate; ATP, adenosine 5′-triphosphate; mTORC2, mammalian target of rapamycin complex 2; FRAP1, FK506 binding protein 12-rapamycin associated protein; PDK1, phosphoinositide-dependent kinase 1..

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Biotechnology (Rockford, IL). Human breast tumor cell line BT-474 was purchased from American Type Culture Collection (Manassas, VA). Rabbit anti-Rictor (A300–458) and rabbit anti-Raptor (A300–553A) were purchased from Bethyl Laboratories. Protein G Sepharose (10-1242) from came from Zymed, and AlphaScreen SureFire p-Akt (S473) Assay Kit (TGRAS500) was purchased from PerkinElmer. PI3K enzymes p110α, p110β and p110δ, co-expressed with His-tagged p85α in baculovirus were cloned and purified as previously described [9]. Akt1, Akt2, Akt3, and mTOR kinase activity kit were purchased from Invitrogen. Rabbit anti-mTOR (04-385) and anti-phospho-serine antibody were purchased from Millipore, the Lance Eu-W1024-labeled Anti-mouse IgG from PerkinElmer, and the Streptavidin-XL665 from Cisbio.

### 2.2. PI3K assays

PI3K enzyme activities were measured in a scintillation proximity assay as previously described [10]. For $IC_{50}$ determinations, reactions were performed in 96 well plates in a final volume of 25 μl. Reactions were carried out at 25 °C in 10 mM MOPS, pH 6.5, 40 μM $PIP_2$, 25 mM $MgCl_2$, 30 μM ATP and 0.25 μCi of [$\gamma^{33}$P]-ATP final concentrations. Final, nominal enzyme concentrations were 4, 6, 7, and 12 nM for PI3Kα, β, δ, and γ respectively. Reactions were started by the addition of ATP and were quenched at 30 min with 25 μl of 50 mM EDTA. Yttrium silicate wheatgerm agglutinin SPA beads were suspended in 250 mM potassium phosphate buffer, pH 7.6, and added to stopped reactions to reach a final concentration of 2 mg/ml. Inhibitors were diluted serially in DMSO and added to wells prior to all other reaction components.

### 2.3. mTOR assays

mTOR kinase activity was determined in a Lanthascreen format using GFP- 4EBP1 as substrate and FRAP1 kinase domain (invitrogen). Product detection was quantified by time-resolved fluorescence using a terbium conjugated phospho-specific antibody. Final reactions include 20 mM HEPES (pH 7.5), 2 mM DTT, 1% DMSO, 0.1 mM CHAPS, 2 mM $MnCl_2$, 550nM GFP-4EBP1, and 500 pM mTOR in 10 μl final volume. For $IC_{50}$ determinations, ATP was fixed at the apparent $K_M$ concentration and catechins were tested at 11 concentrations using 3-fold dilutions. Reactions were started by addition of ATP and quenched with 5 μl of 40 mM EDTA.

mTORC1 and mTORC2 immunoprecipitates were prepared from HEK293 MSRII cells in lysis buffer containing 40 mM HEPES 7.5, 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 50 mM NaF, 1 EDTA-free protease inhibitor tablet, 1 mM sodium orthovanadate and 1 mM DTT. Thawed HEK293 MSRII cells were spun at 1000 RPM, 6 min., 4 °C and the pellet was washed with ice-cold DPBS twice. The washed pellet was resuspended in ice-cold lysis buffer and allowed to rest on ice for 15 min. The sample was then centrifuged at 12,000 RPM for 20 min at 4 °C, and then the clarified lysate was transferred to a new tube. A BCA assay was then used to adjust total protein concentration of the lysate to 3 mg/mL in lysis buffer. The lysate was then "pre-cleared" by rotating at 4 °C for 30 min with 2.17 μL of protein G Sepharose suspension (washed w/lysis buffer) per mg of raw lysate protein. The lysate was then centrifuged at 3700 RPM for 5 min at 4 °C, and the pre-cleared lysate was transferred to a clean tube. Then, 1.5 μg of Bethyl rabbit anti-Raptor or 0.4 μg of rabbit anti-Rictor IgG were added per mg lysate protein, and the samples were rotated for 90 min at 4 °C. Next, 26.7 μL of protein G Sepharose suspension per mg lysate protein were washed three times in lysis buffer and added to the mixture of lysate and antibody. Following the final wash the bead suspension was brought to a final volume of 100 μL per 0.46 mg (100 μL of IP enzyme bead suspension considered 1× enzyme from previous experiments) of original raw lysate protein in this buffer and split into aliquots then frozen in liquid $N_2$ and stored at −80 °C. mTORC1 activity was assayed using a LanthaScreen protocol (invitrogen). mTORC2 activity was assayed using an AlphaScreen format (Perkin–Elmer).

### 2.4. Akt kinase assay

Akt kinase activity was determined in a TR-FRET assay format using biotinylated peptide biotin-Ahx-KKLNRTLSFAEPG as substrate. Product detection was quantified by measuring the resonance energy transfer from the phospho-serine specific antibody bound IgG-Eu-Chelate to the streptavidine-XL665. Final reactions include 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 10 μM ATP, 1 μM biotin-Ahx-KKLNRTLSFAEPG, and Akt enzymes.

### 2.5. Data analysis

$IC_{50}$ values were converted to apparent $K_i$ values by means of the Cheng–Prusoff equation (Eq. (1)). $K_i$ values reported are the mean ± standard deviation of two independent measurements

$$IC_{50} = \left(1 + \frac{S}{K_m}\right) K_i \tag{1}$$

For determining the mode of inhibition, ATP was tested at eight concentrations using 1.6-fold dilutions. EGCG was tested at four concentrations using 3-fold dilutions. Initial velocity data were fit to Eq. (3) which describes competitive inhibition.

$$v = \frac{V_{\max} \cdot [S]}{K_m \cdot \left(1 + \frac{[I]}{K_i}\right) + [S]} \tag{2}$$

### 2.6. Cell proliferation assay

MDA-MB-231 and A549 cells were purchased from American Type Culture Collection (ATCC) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin. Cells were plated in duplicate in 96 well plates. After 24 h, DMSO or EGCG was added and cells were grown for 72 h followed by the addition of 50% by volume of CellTiterGlo (Promega) reagent.

### 2.7. AKT phosphorylation assay

MDA-MB-231 and A549 cells were maintained in 5% $CO_2$ humidified atmosphere at 37 °C in DMEM medium. EGCG was dissolved in PBS (pH 7.4) and used for the treatment of cells. To study the effect of EGCG on IGF-1-induced Akt protein expression and phosphorylation, exponentially growing MDA-MB-231 and A549 cells were serum starved for 24 h followed by pretreatment with EGCG for 1 h in serum-free medium. Afterward, pretreated cells were cultured in serum-free medium in the presence of IGF-1(0.1 μg/mL) and EGCG for indicated concentration. Akt protein levels were determined by Western blot analysis. The cells were lysed in lysis buffer [20 mM Tris(pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, and 1:100 phosphatase inhibitor (Pierce)] and scraped from the culture dishes. The cell lysate was centrifuged at 13,000 rpm for 10 min. The supernatant was collected, and the protein concentration was determined by bicinchoninic acid reagent kit (Pierce). The protein samples were resolved by electrophoresis in 10% SDS–PAGE gels and transferred to PVDF membrane (Bio-Rad). Membranes were blocked and then incubated overnight with primary antibodies against Akt and phospho-Akt at Ser473. Mem-