Potent antitumour activity of (–)epigallocatechin gallate: indications from in vitro, in vivo and in silico studies

Samarendra Narayanan1,*, Balaji Ramchandran2, Satheesh Rajendiran2, Sarbani Chandra2, Atul Tiwari2, Ravisankar Rajarethinam3 and Ramesh Kureeckal Vasudev1

1Department of Biotechnology, Center for Post-graduate Studies, Jain University, Bengaluru 560 011, India
2Department of Biology, Syngene International Limited, Jigani Link Road, Bengaluru 560 011, India
3Institute of Molecular and Cell Biology, Biopolis, Singapore

Antitumour efficacy of (–)-epigallocatechin-3-gallate (EGCG) was evaluated in vitro against the cancer cell lines BxPC-3 (pancreatic cancer), A549 (lung cancer), SH-SY5Y (neuroblastoma), MDA-MB-231 and MCF-7 (breast cancer); in vivo in nude mice by tumour growth inhibition of pancreatic cancers (BxPC-3, MIAPaCa-2), breast cancer (MDA-MB-231) and in silico by docking studies. EGCG significantly inhibited these cancer cell lines in vitro and showed significant tumour reduction in vivo. EGCG docked on to the Her-2 receptor (1N8Y) and the tubulin dimer receptor at a site other than the existing docetaxel ligand. Overall our results suggest that EGCG has potent antineoplastic activity.

Keywords: Antitumour efficacy, breast cancer, docking, epigallocatechin gallate, lung cancer.

Cancer is the deadliest of all diseases known to mankind. Despite significant advances in cancer therapy in recent years, the mean survival time, even after aggressive therapy, remains low. According to the Global Cancer Report (GLOBOCAN), in 2012 there were 14.1 million new cases worldwide1. Presently various anticancer drugs are administered in combination with radiation therapy. Not only is the treatment for cancer expensive, but it also has severe side effects.

Pancreatic cancers, a group of extremely aggressive human cancers, have been reported to cause 330,391 deaths worldwide annually1, while the overall 5-year survival rate remains less than 5% (ref. 2). Conventional treatments have little impact on the progression of pancreatic cancers1. Breast cancer is the leading type of cancer in women, accounting for 25% of all cases, and causing 522,000 deaths worldwide annually1. Treatment is often accompanied by severe side effects. Prostate cancer, one of the most aggressive cancers in men with a 5-year survival rate of less than 5%, is resistant to conventional chemotherapeutic agents. It has high morbidity and an estimated annual mortality of more than 307,481 deaths1. Small-cell lung cancer is a highly malignant cancer that most commonly arises within the lung and occasionally in the cervix, prostrate and gastrointestinal tract. Relapse is common with a median survival time of only 12–18 months1.

Epigallocatechin gallate (EGCG), a green tea polyphenol, is known to inhibit cell proliferation and induce apoptosis in a variety of human neoplasms4–6. Recently, some compounds from natural products targeting tubulin have been discovered such as epothilone, paclitaxel, colchicines and vindesine7–9. Previous studies on EGCG have attempted to examine the antitumour activity employing in vitro or in vivo methods6,10.

We report here, the anticancer activity of EGCG in addition to the in vitro, in vivo and immunohistochemical (IHC) studies conducted, and the in silico aspects of inhibition of HER 2 tyrosin kinase receptor and tubulin dimer receptor 1 TUB (already containing docetaxel ligand) with the help of docking studies. Besides studying the docking of EGCG with Her2 receptor (1N8Y), we also examine the in silico aspect of microtubule polymerization for the inhibition of tumour cell growth. In the mitotic phase of the cell cycle, microtubules maintain dynamic equilibrium with tubulin dimers by assembling the tubulin into microtubules or, conversely, disassembling microtubules to tubulin7. Disruption of the dynamic equilibrium can induce cell-cycle arrest and ultimately lead to apoptosis.

Materials and methods

EGCG (Carbosynth, Berkshire, UK) was dissolved in sterile 1 mM phosphate buffered saline (PBS) to obtain a clear solution.

*For correspondence. (e-mail: sam19narayan@yahoo.co.in)
**Cell culture**

Cancer cell lines procured from the American Type Culture Collection (ATCC) (Manassas, VA, USA) included BxPC-3, MIAPaCa-2 (human pancreatic adenocarcinoma); A549 (human lung carcinoma); Caco-2 (human colorectal adenocarcinoma); MDA-MB-231, MCF-7 (human breast adenocarcinoma) and SH-SY5Y (human neuroblastoma). These cell lines were cultured in DMEM (GIBCO, Life Technologies, NY, USA), supplemented with 10% foetal bovine serum (FBS) and penicillin–streptomycin. The cells were subjected to media change every 2–3 days, sub-cultured after 80% confluence was attained and then dislodged using 0.25% trypsin–EDTA solution.

**Cell proliferation/cytotoxicity assay**

Here, 100 μl of cells was seeded at a density of 5000 cells/well in a black 96-well clear bottom plate (Sigma Aldrich, MO, USA) and allowed to adhere in a humidified incubator at 37°C and 5% CO₂ for 2–3 h before addition of the EGCG. The initial concentration of IC₅₀ was finalized based on results from a three concentration screening test carried out earlier. EGCG was diluted as required and 100 μl of the solution was added to the cells. Respective controls for the vehicle and 100% cytotoxicity were also added. The plates were incubated in a humidified incubator at 37°C and 5% CO₂ for 48 h. Post-incubation, the medium was discarded from the plates by gentle flicking, and replaced with 100 μl of 1× Cell Titer-Blue® (Promega, WI, USA) solution. The plates were further incubated at 37°C for 1 h and subsequently read on a molecular reader (Molecular Devices Flex Station III, Molecular Devices, CA, USA) using excitation and emission wavelengths of 560 and 590 nm respectively. Viability of the cells (%) was determined by dividing the blank-corrected average relative fluorescence units (RFUs) for each concentration over the blank-corrected average RFU of the vehicle control. IC₅₀ was determined using GraphPad Prism Software (GraphPad SoftwareInc, CA, USA). Three replicates were performed for each dilution and the average was taken for comparison.

**Human tumour xenografts**

Athymic female nude mice (Hsd: Athymic Nude-Foxn1tm1) were used as controls. In the second xenograft experiment, mice (n = 5) bearing the MIAPaCa-2 tumours were administered EGCG at a dose of 80 mg/kg, i.p. on days 0, 3, 6 and 9 (Q3D× four doses). In the third xenograft experiment with mice bearing MDA-MB-231 tumours, EGCG was administered at a dose of 90 mg/kg, i.p. on day 0, 3, 6 and 9 (Q3D× four doses). Animals were maintained in a controlled environment in individually ventilated cages.

All procedures were performed in a bio-safe cabinet following sterile techniques. BxPC-3, MIAPaCa-2 and MDMAB-231 cell lines with 70–80% confluent and >90% viability were chosen for the study. Then 5 × 10⁶ cells were suspended in 200 μl of cold PBS or serum-free media containing 50% matrigel kept in an ice-bath. The above-mentioned cell lines were propagated in the animals by injecting them subcutaneously in the flanks or at the back of the animals. The implanted area was monitored for growth of tumour. Once the tumour attained palpable and required volume, the animals were randomized based on tumour volume and dosing was initiated. The mice were weighed and tumour volume was determined by measuring the dimensions of the tumour in two mutually perpendicular directions with vernier calipers on the day of randomization (day 0) and once every three days thereafter. Cage-side observations were recorded and change in body weight (%) of individual mice was calculated. Animals were observed individually for visible clinical signs of morbidity and mortality. The animals were euthanized after final observation and the tissues were collected for histopathology and immunohistochemical (IHC) staining.

**Antitumour activity**

Mean volume of the tumour on EGCG-treated mice (T) on any observation day was compared to mean tumour volume of control mice (C). Antitumour activity on a particular day was taken as a ratio of T to C expressed as percentage, i.e. (T/C) × 100.

**Tumour growth inhibition**

On each observation day, tumour growth inhibition (TGI) was calculated as (1 – T/C) × 100.

**Immunohistochemistry**

BxPC-3 tumour tissue from euthanized control as well as EGCG-treated animals was subjected to IHC study using.
antibody generated against Ki-67, a mitotic (proliferative) marker, following a slightly modified protocol based on Lee et al.\textsuperscript{10}. Tumour tissue was fixed in 10% buffered neutral formalin for one day, post-fixed with 70% alcohol, then processed in an automatic tissue processor (Leica Biosystem), progressively dehydrated using ascending grades of isopropyl alcohol and finally cleared with xylene. Dehydrated tissue was embedded in paraffin block, sectioned at 3–5 μm size, taken onto a poly-L-lysine slide and IHC staining was performed. After deparaffinization, heat-induced epitope antigen retrieval was carried out by treating the slides with 10 mM citrate buffer, pH 6.0, for 10 min at 121°C in a decloaking chamber (DC2002 INTL, Biocare Medicals, USA). Endogenous peroxidase was quenched using 3% H2O2 and blocked at room temperature for 15 min, followed by protein block (background punisher) carried out for 15 min at room temperature. The tissue sections were incubated with primary antibody, anti-human Ki-67 rabbit monoclonal antibody (CRM 325, ABC Biocare Medicals, USA) for 1 h in a humidified chamber at room temperature. Negative controls were run in parallel and treated with PBS instead of a specific antibody solution under identical conditions. MACH 4 detection kit (M4U534G, H, L MM) was used for the detection of Ki-positive reaction in the cells. MACH 4 probe was added and incubated for 10 min. Following incubation, MACH 4 HRP polymer was added and the colour of the sections was visualized by treatment with diaminobenzidine (DAB). The sections were counterstained with Mayer’s hematoxylin followed by dehydration, clearing and mounting. The evaluation of tumour cells expressing Ki-67 was performed at 40× magnification by an independent observer blinded to the protocol. For each slide, 15 representative photomicrographs at high magnification were taken, and positive-stained cancer cells and total number of cells were counted using Leica Analysis Suite (LAS) image processing program (Leica Microsystems, Germany). Ki-67 proliferation index was calculated as the percentage of positively stained cells to total cells. The tumour tissue was also analysed for the presence of necrosis with Leica analysis software.

In silico studies

Two-dimensional structure of EGCG was drawn using GAUSSVIEW package (Gaussian Inc, CT, USA) followed by optimization of the geometry using GAUSSIAN package as detailed elsewhere\textsuperscript{12}. Hatree–Fock theory with ‘6-31++g (d, p)’ as the basis set was used for optimizing the structures. Standard orientation of EGCG after convergence to its global energy minima was visualized using ARGUS package (Argus Lab, WA, USA) before the docking exercise\textsuperscript{13}, and was saved in PDB format. Geometrically optimized EGCG was later docked using Hex ver. 6.0 software\textsuperscript{14} onto the crystal structure of epidermal growth factor receptor from Rattus norvegicus (PDB ID: lN8Y)\textsuperscript{15}. The optimized EGCG structure was also docked onto the crystal structure of αβ tubulin heterodimer, a structural subunit of microtubules from Sus scrofa complexed with docetaxel (PDB ID: 1TUB)\textsuperscript{16}. Docking energy in each case was calculated based on the shape and electrostatics using default grid spacing of 6.0 Å.

Statistical analysis

All comparisons of mean antitumour activity and mean TGI were done using Student’s t-test and GraphPad Prism v5. P value less than 0.05 indicates statistically significant differences between groups (95% CI).

Results

EGCG inhibits growth of cancer cells

Figure 1 depicts the growth inhibition curves for various tumour cell lines by EGCG. Table 1 shows the 50% inhibition concentration (IC$_{50}$) values for each of these cell lines. The dose–response curves indicate that EGCG is cytotoxic to all the cancer cell lines examined and inhibits the growth of these cells in a dose-dependent manner.

EGCG inhibits tumour growth in mouse models

Based on the observed inhibition of the growth of these cancer cell lines in vitro, anticancer activity of EGCG was evaluated in xenograft-bearing nude mice models induced by subcutaneous injection of the pancreatic cancer cell lines, BxPC-3 and MIAPaCa-2, and breast cancer cell line MDA-MB-231.

BxPC-3: Figure 2a shows the mean body weight of control and EGCG-treated mice harbouring BxPC-3 tumour, while Figure 2b depicts tumour volume determined on various observation days. There was no loss of body weight in vehicle control and EGCG-treated mice during the experimental period. All animals were active and healthy. EGCG therapy was relatively well tolerated at the tested dose level with no mortality. Moreover, there were no visible signs of abnormal behaviour or any adverse clinical symptoms during treatment. EGCG showed a strong antitumour activity in vivo when injected in nude mice at a dose of 80 mg/kg. Treatment with EGCG resulted in an optimal ratio (T/C) of mean tumour volume of treated mice (T) to that of control mice (C), of 19% on day 15. TGI for the EGCG group at the tested dose level was found to be 80.9% (day 15, $P < 0.001$).

MIAPaCa-2: As shown in Figure 3a, there was no significant loss of body weight in vehicle control and
EGCG-treated group during the experimental period. EGCG therapy was relatively well tolerated at the tested dose level with no mortality. Moreover, there were no visible signs of abnormal behaviour or any adverse clinical symptoms during treatment. Figure 3b shows the tumour growth curve for control as well as EGCG-treated group. EGCG demonstrated significant ($P < 0.001$) antitumour activity against MIAPaCa-2 xenograft tumour model. Treatment with EGCG was continued only up to day 9, but post-treatment observation was continued up to day 24. The results show an optimal $T/C$ of 44.5% on day 24. TGI for the EGCG group at the tested dose level was found to be 54.59% (day 45, $P < 0.001$).

**Histopathology and immunohistochemistry**

Histopathological examination of the tumour tissue (BxPC-3) obtained from all the animals showed encapsulation under the subcutaneous tissue (Figure 5a and b). Pleomorphic malignant epithelial cells were arranged in cord or acinar pattern. These tumour cells were surrounded by abundant fibrous stroma. Individual tumour cells contained vacuolation in the cytoplasm. The tumour

<table>
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<th>Cell line</th>
<th>IC50 ($\mu$M)</th>
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<tr>
<td>BxPC-3</td>
<td>245.9</td>
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<tr>
<td>A549</td>
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<tr>
<td>Caco-2</td>
<td>228.5</td>
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<tr>
<td>SH-SY5Y</td>
<td>75.1</td>
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<tr>
<td>MDA-MB-231</td>
<td>206.8</td>
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<td>MCF7</td>
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**MDA-MB-231:** EGCG was relatively well tolerated at the tested dose level (90 mg/kg) with no mortality. There was no significant loss in body weight in vehicle control as well as EGCG-treated group during the experimental period (Figure 4a). Based on cage side observations, there were no visible signs of abnormal behaviour or clinical symptoms in the EGCG-treated group. In the dosage regime studied, EGCG treatment demonstrated significant ($P < 0.001$) antitumour activity (Figure 4b) against MDA-MB-231 xenograft tumour model, with an optimal $T/C$ of 45.4% on day 45. TGI for the EGCG group at the tested dose level was found to be 54.59% (day 45, $P < 0.001$).
also showed the presence of keratinized epithelial cells forming the pearls. We observed that the area of necrosis was more predominant in EGCG-treated animals than the control group. The average area of necrosis was 2.86 ± 0.94 mm² in the control animals, while it increased to 6.64 ± 2.14 mm² in EGCG-treated animals. Figure 5c shows the mean area of necrosis as measured by image analysis.

Figure 6a and b shows the IHC staining of BxPC-3 tumour tissues from control as well as EGCG-treated animals. The expression of Ki-67 positive cells was significantly lower (P < 0.05) in EGCG-treated animals (777.97 ± 84.90/mm²) than control animals (1388.32 ± 117.04/mm²; Figure 6c). EGCG significantly inhibited tumour cell growth in the xenograft animal model (56%). This effect was further demonstrated by the finding that the proliferation index significantly reduced (P < 0.05) to 22% after EGCG treatment (Figure 6d) in comparison to saline control (46%).

In silico docking studies

Figure 7 shows the energy-minimized structure of EGCG. Table 2 lists the results of minimization using Gaussian package. Convergence was achieved for all the parameters after 11 cycles.

The geometrically optimized ligand EGCG was docked successfully onto the tyrosine receptor kinase (Herceptin, PDBID: 1N8Y) using the HEX docking program (Figure 8a). The docking energy was −302.09 kcal mol⁻¹. The neighbouring residues for EGCG, as depicted in Figure 8b, are:

\[ \text{asn}^{298}, \text{gln}^{299}, \text{glu}^{300,383,384}, \text{val}^{301}, \text{thr}^{302}, \text{ala}^{318}, \text{arg}^{319,408,411}, \text{val}^{320}, \text{cys}^{321,346}, \text{lys}^{347,348}, \text{ile}^{349,385,409}, \text{phe}^{350} \text{and leu}^{382} \]

EGCG was also docked onto the experimentally determined structure of tubulin αβ dimer complexed with TXL from S. scrofa (PDB ID: 1TUB) (Figure 9a). Geometrically optimized EGCG was able to dock at a site different from that of docetaxel. The docking energy was −302.6 kcal mol⁻¹. EGCG interacts with the neighbouring residues in both A and B chains (Figure 9b), while docetaxel interacts with residues from the B chain only (Figure 9c). The neighbouring residues observed in the
Figure 4. Effect of EGCG on MDA-MB-231 tumour induced in nude mice at the tested dose of 90 mg/kg (day 45, $P < 0.001$): (a) mean body weight and (b) tumour volume of EGCG-treated and control animals.

Figure 5. Effect of EGCG on the growth of BxPC-3-induced pancreatic xenograft in nude mice. Tumour cells showing area of necrosis (N) in tumour cells in (a) saline control and (b) EGCG-treated animals (H&E stain, x4). (c) Area of necrosis (mean ± SE) in saline control and EGCG-treated mice as measured by image analysis.

Table 2. Energy minimization parameters of EGCG

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<th>Parameter</th>
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<tr>
<td>Predicted change in energy</td>
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</table>

Optimization completed
Stationary point found

The neighbouring residues in the vicinity (within 6 Å) of EGCG (in the docked structure) are as follows:

A chain: gly$^{95,106,410}$, lys$^{96}$, glu$^{97,113,411}$, ala$^{99}$, arg$^{105}$, tyr$^{108}$, thr$^{109}$, ile$^{110}$.

B chain: met$^{1}$, arg$^{2}$, glu$^{3}$, asp$^{130,163}$, lys$^{131}$, leu$^{132}$, glu$^{133}$, gly$^{134}$, glu$^{160}$, tyr$^{161}$, pro$^{162}$, arg$^{164,253}$, ile$^{165}$.

The neighbouring residues in the vicinity of TXL (Figure 9 c) belong to the B chain only and are

B chain: lys$^{19,372}$, glu$^{22}$, val$^{23}$, asp$^{26,226}$, phe$^{53,272}$, leu$^{217,219,227,230,275,371}$, thr$^{213,276}$, gly$^{225,273,370}$, his$^{229}$, ser$^{232,236,277}$, ala$^{233}$, pro$^{274,359,360}$, arg$^{278,320,364}$, gln$^{281}$, asn$^{282}$.

In silico studies confirm the docking of EGCG with 1N8Y as well as 1TUB receptors to a similar extent, as indicated by the calculated docking energies.

Discussion

In this study EGCG shows significant antitumour activity as seen from the results of in vitro, in vivo, IHC and in silico docking studies. The observation of significant TGI, 80.9% against BxPC-3, 55.5% against MIAPaCa-2 and 55.6% against MDA-MB-231 tumours strongly indicates that EGCG could be considered as a valuable adjunct to the battery of frontline anticancer compounds. The histological observations of significant destruction of BxPC-3 tumour cells, are evidenced by increased necrosis in EGCG-treated animals compared to untreated.
Figure 6. Representative photomicrographs of tumour cells showing the effect of EGCG on the proliferation of Ki-67 marker, in BxPC-3 induced mouse xenograft. (a) Vehicle control and (b) EGCG-treated mice (immunoperoxidase stain, x40). c. Expression of Ki-67 is significantly reduced (56%) in EGCG-treated mice ($P < 0.05$).

Figure 7. Geometrically optimized EGCG, $[E(RHF) = -1667.05 \text{ U after 11 cycles}]$.

control. The results of immunohistochemistry show significantly reduced expression of the proliferation marker Ki-67 in tumours of nude mice treated with EGCG, compared to untreated controls. Further, results of the docking studies corroborate the in vitro, in vivo and IHC findings as demonstrated by docking of EGCG to the Her2 receptor (1N8Y) with a good docking energy and in the case of the microtubule receptor, 1TUB, at a site away from that of docetaxel. Further, EGCG is also observed to interact with neighbouring residues in both A
and B chains while docetaxel is seen interacting only with residues from the B chain, which may indicate a potentially complementary action in binding to the microtubule network.

EGCG, the major component of green tea, is known to be capable of inhibiting the growth of a variety of human cancer cells via the induction of apoptosis. The finding of marked cell destruction is well supported by the observation of reduced number of viable cells on treatment with epigallocatechin, in human lung cancer H1299 cells in culture and in xenograft tumour reported by Li et al. In the present study, morphometric evaluation of cell death clearly indicates the effect of EGCG on viable cells. Studies have also shown that polyphenols from green tea possess antitumour and anti-metastatic properties in animal xenograft and allograft models, suggesting a possible therapeutic potential. Vu et al. have also supported possible use of EGCG in reducing metastasis of pancreatic cancer, although EGCG alone has limited effect on cell growth inhibition.
Treatment for neuroblastoma is usually through multi-therapy with radiation, surgery and chemotherapy. Canete et al. reported poor response to treatment and the urgent need for new approaches to therapy for this type of cancer. In the present study, EGCG has shown strong inhibition of the neuroblastoma cell line, SHY5Y, in the *in vitro* model. It has also shown a dose-dependent inhibition of A549, a highly malignant, small-cell lung cancer cell line, and deserves further evaluation in combination therapy.

**Summary**

EGCG has been evaluated earlier by *in vitro* and *in vivo* studies. Here, in addition to the above, we also conducted IHC and docking studies. Taken together, the results indicate that EGCG demonstrates significant antitumour activity, which may open new vistas in combination anti-cancer therapy.

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