Growth inhibition and cell cycle arrest effects of epigallocatechin gallate in the NBT-II bladder tumour cell line

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OBJECTIVES

To examine the growth inhibition and cell cycle arrest effects of epigallocatechin gallate (EGCG), a major constituent of green tea polyphenols, on the NBT-II bladder tumour cell line.

MATERIALS AND METHODS

Growth inhibition and cell cycle arrest effects of EGCG were evaluated by the tetrazolium assay, flow cytometry and apoptotic DNA ladder tests. The cell cycle-related oncogene and protein expressions in NBT-II bladder tumour cells, when incubated with EGCG, were detected with reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis.

RESULTS

EGCG inhibited growth of the NBT-II bladder tumour cells in a dose- and time-dependent manner. Flow cytometry showed a G0/G1 arrest in cells when cultured with EGCG at doses of 10, 20 or 40 μmol/L for 48 or 72 h. The apoptotic DNA ladder test showed that EGCG at 10 μmol/L induced early apoptosis after 48 h of incubation. A down-regulation of cyclin D1 was detected by RT-PCR when the cells were incubated with EGCG (20 μmol/L for 48 h. EGCG also down-regulated protein expression of cyclin D1, cyclin-dependent kinase 4/6 and phosphorylated retinoblastoma protein, in both a time- and dose-dependent manner, when detected by Western blot.

CONCLUSION

EGCG had growth inhibition and cell-cycle arrest effects in NBT-II bladder tumour cells by down-regulating the cyclin D1, cyclin-dependent kinase 4/6 and retinoblastoma protein machinery for regulating cell-cycle progression.

KEYWORDS

epigallocatechin gallate, cyclin D1, cell cycle, green tea, bladder cancer

INTRODUCTION

Green tea is a popular beverage in Asia; epidemiological studies suggest that drinking green tea reduces the risk of cancer developing in the prostate, bladder, stomach, oesophagus and lung [1–5]. Based on many *in vivo* and *in vitro* studies [6–10], it is suggested that the biological activity of green tea is mediated by its major polyphenolic constituent, epigallocatechin gallate (EGCG), which is a potent antioxidant. More recently, EGCG was reported to cause cell-cycle arrest in various mouse, rat and human cell lines. This effect of EGCG was proposed to be a result of its regulation of cell-cycle related oncogenes and proteins, particular cyclin D1 and its cyclin-dependent kinases (cdk4 and cdk6) [11–13]. In this study, the growth inhibition and effects on cell-cycle arrest of EGCG in NBT-II bladder tumour cells were investigated.

MATERIALS AND METHODS

EGCG (>98% purity) was purchased from Sigma Co. Ltd, USA. The primary antibodies (cyclin D1, cdk4, and cdk6) were obtained from Santa Cruz Biotechnology Co. Ltd, USA, while the primary antibodies for phosphorylated retinoblastoma protein (ppRb) and β-actin were purchased from StressGen Biotechnologies Corp., Canada, and Sigma, respectively. All other chemicals used were of the highest purity commercially available.

The NBT-II cell line was derived from a bladder tumour induced with N-butyl-N-(4-hydroxybutyl) nitrosamine in Wistar rats, and purchased from the American Type Culture Collection (Rockville, USA). Cells were propagated into minimum essential medium (containing 2 mmol/L L-glutamine and Eagle's balanced saline adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, and 1.0 mmol/L sodium pyruvate) with 10% fetal bovine serum, 100 U/mL penicillin G and 100 μg/mL streptomycin in an incubator of 37 °C, 65% humidity and 5% carbon dioxide.

EGCG dissolved in PBS (50 mmol/L, pH 7.4) was incubated with cells at the desired doses for 24, 48 and 72 h, whereas cells treated only with PBS served as the control. Growth inhibition was assayed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) reduction. NBT-II cells were plated onto 96-well plates at a density of 5000 cells/well and treated with EGCG (10, 20, 40 μmol/L) 24 h later. At the end of the treatment period (24, 48 or 72 h), MTT was added to each well at a final concentration of 0.5 mg/mL. After 3 h of incubation at 37 °C, isopropanol was added, and the absorbance of each well at 570 nm

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Gene	Sequence	Size, bp
CCND1	5′-GAGACCATCCCCCTGACGGC-3′	
	5′-TCTTCCTCCTCCTCGGCGGC-3′	484
CDK4	5′-ACGGGTGTAAGTGCCATCTG-3′	
	5′-TGGTGTCGGTGCCTATGGGA-3′	464
CDK6	5′-CGAATGCGTGGCGGAGATC-3′	
	5′-CCACTGAGGTTAGAGCCATC-3′	499
GAPDH	5′-TCCCATCACCATCTTCCA-3′	
	5′-ACTCACGCCACAGTTTCC-3′	379

TABLE 1
The sense and antisense primers used for RT-PCR

measured in a microplate reader (MRX, Dynex Technologies, USA). The mean absorbance of six individual wells was used to calculate the relative growth.

For flow cytometry, NBT-II cells were synchronized in G1 by serum starvation for 36 h before EGCG (10, 20, 40 μmol/L) treatments for 24, 48 or 72 h. Cells treated with or with no EGCG were detached with trypsin and washed with PBS. A DNAcon3 kit (Dako, Italy) was used for DNA staining. Briefly, 1 mL propidium iodide was added into each DNAcon3 test-tube containing dehydrated buffer mixture. After 5 min, 2×10^6 cells were added to each tube and incubated at 4 °C for 1 h until analysis. Flow cytometry was performed with a Beckman-Coulter FACScan, and the cell-cycle distribution analysed using ModFit LT software (Beckman).

For the apoptotic DNA ladder test, NBT-II cells (2×10^6) were incubated with EGCG (5, 10, 20, 40 μmol/L) or vehicle for 24 or 48 h. The cells were collected and resuspended in 200 μL PBS. An apoptotic DNA Ladder Kit (Roche Molecular Diagnostics, Germany) was used to purify nucleic acids from different samples, in accordance with the manufacturer's instructions. Purified DNA (3 μg) was loaded onto a 1% agarose-DNA gel containing 0.3 μg/mL ethidium bromide in Tris-boric acid-EDTA buffer (pH 8.3, containing 89 mmol/L Tris, 89 mmol/L boric acid and 2 mmol/L EDTA). The bands were visualized after 1.5 h with an ultraviolet transilluminator (Geldoc 1000, Biorad Laboratory, USA).

For RT-PCR of cell cycle-related oncogenes, total RNA was isolated from cells incubated with EGCG (20 μmol/L) or vehicle using TRIzol reagent (Gibco Laboratory, USA) according to the manufacturer's instructions. Single-strand cDNA was synthesized from 5 μg of RNA using 0.5 nmol random primers, 15 U ThermoScrip RT in 4 μL 5 × cDNA synthesis buffer, 40 U Rnaseout, and 10 nmol dNTP Mix (all from Gibco) in a total volume of 10 μL. RT was conducted for 10 min at 25 °C and 1 h at 50 °C and the samples subsequently heated for 5 min to terminate the RT reaction. With the cDNA obtained a PCR reaction was conducted using 4 μL of the RT reaction mix, to which 2.5 U Tag DNA polymerase, 50 pmol sense and 50 pmol antisense primers (all from Invitrogen, USA) were added. The final reaction volume was 50 μL. The tubes were then incubated in a Gene AMP PCR System 9700 (Perkin-Elmer, Norwalk, USA) at 95 °C for 5 min to denature the primers and cDNA. The cycling programme comprised 20–30 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The sequences of primers and resulting PCR products [14] are shown in Table 1. Finally, 10 μL of PCR product was loaded onto a 1.5% agarose gel and the bands visualized using an ultraviolet transilluminator (Geldoc 1000).

Based on the results obtained in cell growth and cell-cycle analysis, NBT-II cells were incubated with EGCG at 20 or 40 μmol/L for 48 h, while the incubation with 20 μmol/L EGCG was further extended to 72 h. Cells were lysed with lysis buffer (0.1 mol/L Tris-HCl, pH 7.5 containing leupeptin 1 μg/μL, pepstain A 1 μg/μL and phenylmethylsulphonyl fluoride 50 μg/mL (Sigma) over ice for 30 min, and then centrifuged at 14 000 ***g*** for 15 min at 4 °C. The supernatant was collected for protein determination. Protein samples (50 μg) were separated on SDS-PAGE electrophoresis gels and transferred to nitrocellulose membranes. Membranes were blocked with Tris buffered saline containing 10% nonfat milk for 2 h at room temperature and incubated with appropriate primary monoclonal or polyclonal antibodies (β-actin, cyclin D1, ppRb, cdk4 and cdk6) overnight. The membranes were then incubated for 2 h with respective secondary antibody-horseradish peroxidase conjugates (Biorad) and washed. The bound antibody was visualized with the ECL-Western Blotting Detection Kit (Amersham, UK) and autoradiographed, then quantified by densitometric analysis using the Multi-Analyst Imaging System (Biorad).

STATISTICS

The two-tailed unpaired Student's *t*-test was used to compare the mean absorbance of cells in six individual wells which were treated with different concentrations of EGCG (10, 20, 40 μmol/L) or vehicle in the MTT assay, with $P < 0.05$ considered to indicate statistical significance.

RESULTS

The effect of EGCG on the growth of NBT-II bladder tumour cells was evaluated at different dosages and incubation times; 10, 20 or 40 μmol/L for 24, 48 or 72 h inhibited cell growth in a dose- and time-dependent manner (Fig. 1). Incubation with EGCG at doses of 10, 20 or 40 μmol/L for 48 or 72 h, but not 24 h, caused significantly lower cell viability than in the control ($P < 0.01$).

Flow cytometry showed a G0/G1 arrest in cells cultured with 10, 20 or 40 μmol/L EGCG for 48 or 72 h (Fig. 2A,B) but not for 24 h (data not shown). The percentage of cells arrested in G0/G1 when incubated with different concentrations of EGCG for 48 or 72 h was 77–91.6%; in the control only 59.8–65.2% of cells were in G0/G1. This increase in the G0/G1 cell population was accompanied by an increase of the sub-G0/G1 cell population (apoptotic cells) in EGCG treated cells.

No DNA fragmentation was detected at any of the four doses of EGCG when the NBT-II cells were incubated with EGCG for 24 h (data not shown). Apoptosis was evident at ≥ 10 μmol/L when the cells were incubated with EGCG for 48 h (Fig. 3).

On RT-PCR there was less expression of CCND1 in cells incubated with 20 μmol/L EGCG bur no obvious change in the expression of CDK4, CDK6 or GAPDH when the cells were treated with EGCG under the same conditions (Fig. 4).

There was a dose- and time-dependent decrease in the protein level of cyclin D1,

cdk4, cdk6 and pp-Rb when NBT-II cells were treated with 20 or 40 μmol/L EGCG for 48 or 72 h (Fig. 5A,B) but no obvious change for the expression of β-actin when cells were treated with EGCG under the same conditions.

DISCUSSION

Superficial bladder tumour is a common disease in urological practice, with a recurrence rate of up to half even after transurethral resection of bladder tumour with intravesical administration of chemotherapy and immunotherapeutic drugs [15]. In animal studies, green tea given after carcinogens has been found to decrease bladder tumour formation. Tea catechins, particularly EGCG in green tea, are thought to contribute to this anticancer effect [16]. In the present study a bladder tumour cell line, NBT-II, derived from induced bladder tumours in rats, was used to evaluate the anticancer effects of EGCG.

EGCG at 10–40 μmol/L significantly inhibited the growth of cancer cells after 48 or 72 h of incubation, while there was no significant difference at 24 h. The effects of EGCG were both time- and dose-dependent. Flow cytometry of cells incubated with the same concentrations of EGCG also confirmed a G0/G1 cell-cycle arrest after 48 and 72 h of incubation, but not at 24 h. The G0/G1 arrest was accompanied by an increase in apoptotic cells, with DNA fragmentation in cells incubated with EGCG at 10, 20 and 40 μmol/L for 48 h. This study confirms for the first time a growth inhibition and cell-cycle arrest effect of EGCG in NBT-II bladder tumour cells.

One of the fundamental abnormalities in cancer development is dysregulation of the cell cycle; cdk-Rb protein machinery was thought to control the cell cycle checkpoints. In mammalian cells, the D-type cyclins, through the interaction with cdks, are mainly responsible for driving the cell cycle from the G1 to S phase. The cyclin D1-cdk4/6 complexes, under the regulation of cdk inhibitors, determine the phosphorylation status of the Rb protein and mediate transcription. Rb proteins exist in hypophosphorylated form in quiescent cells and can be phosphorylated by cyclin D1-cdk4/6 complexes during the mid- to late G1 phase. The ability of Rb to bind transcriptional factor E2F can be abolished after phosphorylation,

*FIG. 1. Growth inhibition effect of EGCG in NBT-II bladder tumour cells incubated with 0 (control, green) 10 (red), 20 (light green) or 40 μmol/L (open bars) EGCG for 24, 48 or 72 h; the number of viable cells was detected using the MTT assay. Data are the mean (SEM) of six independent experiments. *P <0.01 vs corresponding controls.*

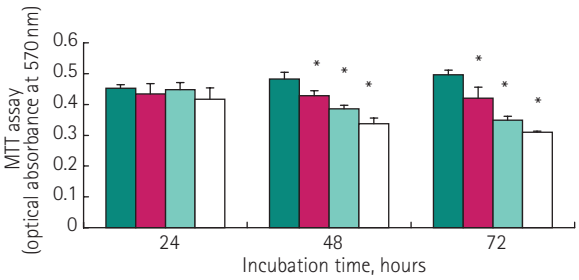


*FIG. 2. Cell cycle arrest and apoptosis induced by EGCG in NBT-II bladder tumour cells treated with vehicle or EGCG (10, 20 or 40 μmol/L) for 48 h **(A)** or 72 h **(B)** and analysed by flow cytometry. The percentage of cells in G0/G1 and sub-G0/G1 phase for each concentration was, respectively: A, 59.8 and 0.16; 85.0 and 10.9; 91.15 and 13.8; 91.6 and 15.1: B, 65.2 and 2.3; 77.0 and 6.44; 87.3 and 29.0; 88.3 and 29.4. The data are from a representative experiment repeated three times with similar results.*

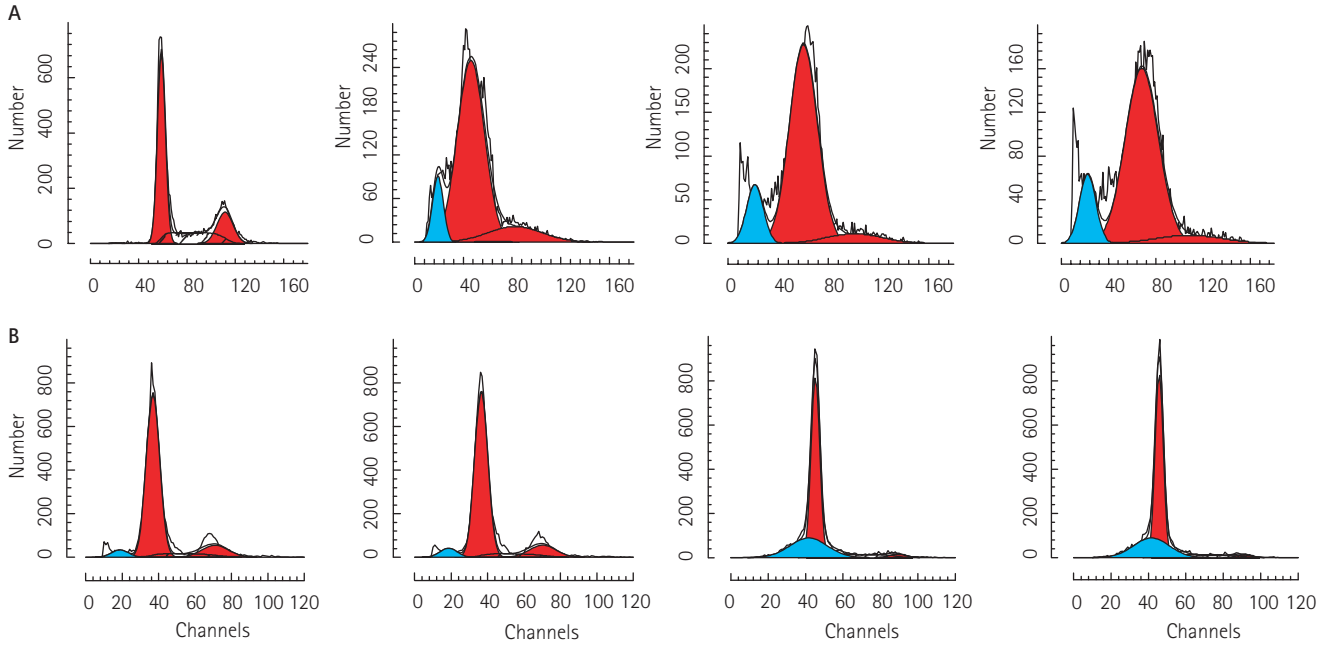


FIG. 3. DNA fragmentation by EGCG in NBT-II bladder tumour cells treated with vehicle and EGCG (5, 10, 20 or 40 μmol/L) for 48 h, respectively (lanes 1–5). After incubation, the cells were collected, and cellular DNA isolated and subjected to agarose-gel electrophoresis followed by visualization of the bands. The data are from a representative experiment repeated three times with similar results.

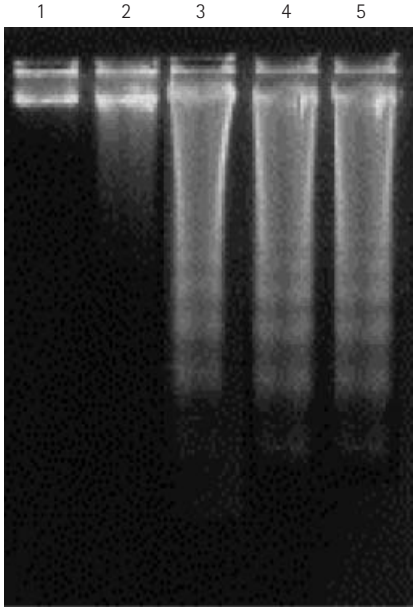
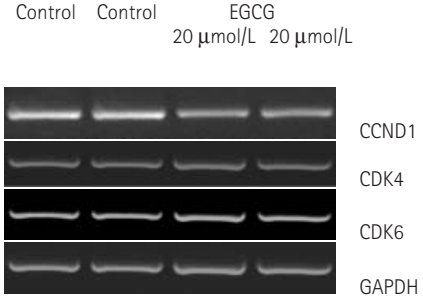
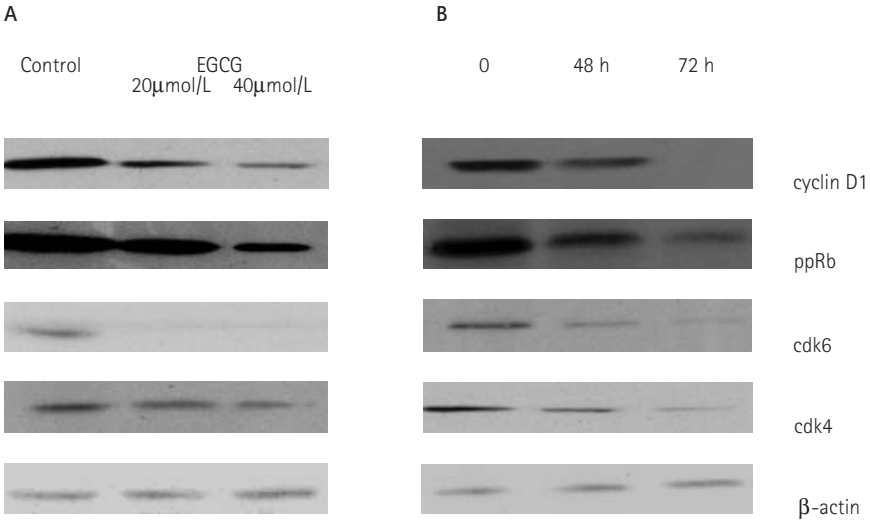


FIG. 4. Effects of EGCG on cell cycle-related oncogene expression. The cells were treated with 20 μmol/L EGCG or vehicle for 48 h. Total RNA was isolated from the cells and then analysed by RT-PCR; the expression of GAPDH served as internal control. The data are from a representative experiment repeated three times with similar results.



while E2F is an essential component of the transcriptional complex required in S-phase entry and DNA replication [17,18]. Also, the kinase activity of the cyclin D1-cdk4/6 complex is subjected to inhibition by the binding of cdk inhibitor such as CIP1/KIP1and Ink4 families of protein, including p21, p27, p16 and p18 [19–21].

FIG. 5. Effects of EGCG on cell cycle-related protein expression. The cells were treated with vehicle or EGCG (20 or 40 μmol/L) for 48 h ***(A)****, and with 20 μmol/L EGCG for 0, 48 or 72 h* ***(B)****. Total cell lysates were prepared and 50 μg protein subjected to SDS-PAGE followed by Western blot analysis, using appropriate primary antibodies and secondary horseradish peroxidase-linked antibodies. The expression of β-actin served as an internal control. The data are from a representative experiment repeated three times with similar results.*



In the present study the cell-cycle oncogene CCND1 was significantly down-regulated when NBT-II cells were incubated with 20 μmol/l EGCG for 48 h, but with no obvious change in the expression of CDK4, CDK6 or GAPDH when the cells were treated with EGCG under the same conditions. Considering the implication of cell-cycle oncogenes in the cell cycle-arrest effect of EGCG, Western blot analysis on cell-cycle regulatory proteins, including the molecules in cyclin D1-cdk4/6-Rb protein axis, was examined; there was a dose- and time-dependent decrease in cyclin D1, cdk4, cdk6 and ppRb expression.

Based on these results a down-regulation of cyclin D1 on both protein and mRNA levels in NBT-II bladder tumour cells incubated with EGCG was confirmed by RT-PCR and Western blot analysis. We suggest that down-regulation of cyclin D1 induced by EGCG could be the key factor blocking the G1 to S transition of bladder cancer cells. Down-regulation of cdk4 and cdk6 proteins may also contribute to the cell cycle arrest effects of EGCG, because the down-regulation of both cyclin D1 and cdk4/6 proteins could lower the kinase activities associated with cyclin Dl-cdk4/6 complexes, which determine the phosphorylation status of Rb protein. Subsequently, Rb phosphorylation and cellular transcriptional factor E2F release will be decreased, leading to cell-cycle arrest. The dose- and time-dependent decrease in ppRb expression in NBT-II cells incubated with EGCG support this hypothesis. However, a reduction in CDK4 and CDK6 mRNA level was not detected in the RT-PCR analysis. Thus EGCG may modulate cdk4/6 at the protein level only. There are studies reporting an up-regulation of p16, p18, p21 and p27 in cells incubated with EGCG, and that may account for the down-regulation of cdk4 and cdk6 proteins [11,12].

As to the signal transduction pathway by which EGCG exerts cell cycle arrest effects and down-regulates cyclin D1-cdk4/6-Rb protein machinery, none seems well established. However, several mechanisms of cell-cycle arrest by EGCG have been postulated. Ahmad *et al.* [22] reported that EGCG -induced caused cell-cycle arrest and apoptosis of cancer cells may be mediated through NFκB inhibition. Several research teams reported that oxidizing agents such as reactive oxygen species affect key transcription factors, some of which help to regulate cell growth. They activate NFκB, which turns on the genes for a variety of transducer molecules leading to cell-cycle progression, inflammation and tumour promotion [23]. As EGCG is a strong antioxidant, and can suppress NFκB by inhibition of reactive oxygen intermediate generation, it may induce growth inhibition and cell-cycle arrest in cancer cells through these mechanisms. Furthermore, Liang *et al.* [24] reported that EGCG blocked epidermal

growth factor binding to its receptor in A431 cells and then inhibited the receptor tyrosine kinase activity both *in vivo* and *in vitro*. Most mitogenic signals initiated by the binding of a growth factor to its cell-surface receptor are transmitted to the cell nucleus, leading to a change in gene expression, DNA synthesis and cell proliferation. Therefore, it is possible that EGCG can suppress the epidermal growth factor receptor-mediated extracellular signals and then inhibit cell-cycle progression [24], but the exact mechanisms of action require further study.

Based on the present results we conclude that the cell cycle arrest and growth inhibition by EGCG in NBT-II cells may be mediated by down-regulation of the cyclin D1-cdk4/6-Rb protein machinery which is responsible for driving cells from the G1 to S phase. Studies are now underway to investigate the signal transduction pathway of EGCG involved in down-regulating cyclin D1-cdk4/6-Rb protein machinery in bladder tumour cells.

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CONFLICT OF INTEREST

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Abbreviations: **EGCG**, epigallocatechin gallate; **cdk/CDK**, cyclin-dependent kinase; **ppRb**, phosphorylated retinoblastoma protein; **MTT**, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide.