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Impact of Quercetin and EGCG on Key Elements of the Wnt Pathway in Human Colon Carcinoma Cells

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The flavonoids quercetin (QUE) and (−)-epigallocatechin-3-gallate (EGCG) are discussed as potential chemopreventive food constituents. Both compounds have been shown to affect a spectrum of different cellular signaling pathways. Glycogen synthase kinase-3β (GSK3β) is one of the key elements of the Wnt pathway, governing β-catenin homeostasis. The inhibition of GSK3 kinase activity might lead to the onset of β-catenin/TCF/LEF-mediated gene transcription, representing a potentially mitogenic stimulus. The aim of the study was to elucidate whether QUE and EGCG possibly mediate undesired proliferative stimuli in human colon carcinoma cells by interference with the Wnt pathway. In HT29 cells QUE did not inhibit the activity of GSK3α and -β, measured as phosphorylation at Ser21 and Ser9, respectively. In accordance, QUE did not substantially affect β-catenin homeostasis. In a reporter gene assay QUE was found to act as a weak inductor of T-cell factor/lymphoid enhancer factor (TCF/LEF) mediated luciferase expression, which was, however, not associated with a stimulation of cell growth. Treatment of HT29 cells with EGCG led to a potent inhibition of GSK3α and -β activity. Subsequently, the amount of phosphorylated β-catenin was diminished in a concentration-dependent manner. Concomitantly, the overall amount of β-catenin was decreased to a similar extent, which might result from a downregulation of β-catenin neogenesis, indicated by reduced levels of β-catenin mRNA. In accordance, no induction of TCF/LEF-mediated luciferase expression was observed. In conclusion, the results allow the assumption that QUE and EGCG do not mediate proliferative stimuli in HT29 cells by interference with key elements of the Wnt pathway.

KEYWORDS: Quercetin; (−)-epigallocatechin-3-gallate; glycogen synthase kinase; β-catenin; Wnt pathway

INTRODUCTION

Diet and lifestyle are considered to play an important role in the genesis of human colorectal cancer, one of the leading causes of cancer mortality in Western countries. Several epidemiological studies have shown an inverse correlation between a diet rich in polyphenols/flavonoids and the risk to develop chronic diseases such as coronary heart disease, stroke, type-2 diabetes, and cancer (*1*−*3*). The flavonol quercetin (QUE), the most abundant flavonoid in the human diet, and the biologically most active constituent of green tea, (−)-epigallocatechin-3-gallate (EGCG), are proposed to possess cancer-preventive activities (*4*−*6*). Malignant transformation of cells is often associated with changes in cellular signaling cascades, steering cell proliferation, differentiation, and apoptosis induction. The Wnt pathway (**Scheme 1**) appears to play an important role particularly in colon carcinogenesis (*7*−*10*). In the absence of pathway stimulation, β-catenin is destabilized by a cytoplasmatic multiprotein complex consisting of the scaffolding proteins, axin, adenomatous polyposis coli (APC), and the protein kinase, glycogen synthase kinase-3 (GSK3). In quiescent cells, β-catenin is marked for proteasomal degradation by phosphorylation through active GSK3. The maintenance of low intracellular levels of β-catenin enables the repression of Wnt target genes via the association of transcriptional corepressors with transcription factors of the TCF/LEF family. Activation of the Wnt receptor complex, consisting of the seven-transmembrane receptor frizzled (Fz) and coreceptors leads to inhibition of GSK3 mediated by dishevelled (Dsh), consequently preventing β-catenin phosphorylation and degradation. Free β-catenin accumulates in the cytoplasm and subsequently translocates into the nucleus, stimulating TCF/LEF-driven gene transcription. As a consequence cell proliferation is induced or apoptosis is blocked. The serine/threonine kinase GSK3 plays a central role in Wnt signaling by steering β-catenin homeostasis. Furthermore, GSK3 represents one of the key mediators of insulin signaling, by controlling the activity of glycogen synthase, the rate-limiting enzyme of glycogen deposition (*11*, *12*). QUE and EGCG have

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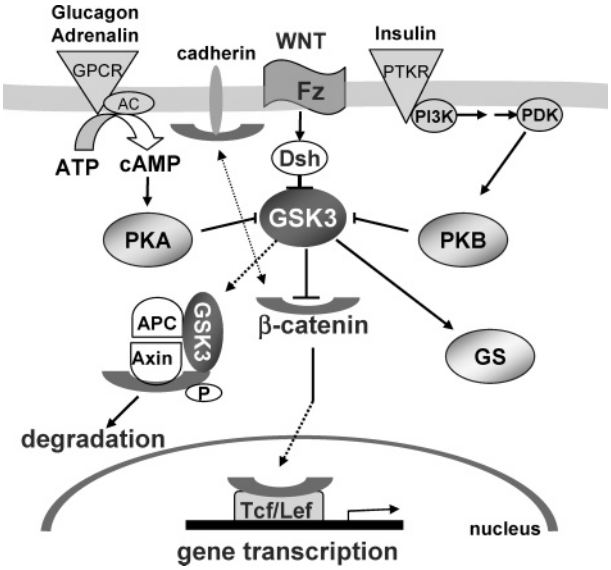
Scheme 1. Scheme of the Wnt Signaling Pathway and Cross Talk between Insulin/PI3K and cAMP Signaling Cascade[a]



[a] GSK3, glycogen synthase kinase 3; APC, adenomatous polyposis coli; Dsh, dishevelled; TCF-LEF, T-cell factor-lymphoid enhancer factor, GPCR, G-protein-coupled receptor; AC, adenylate cyclase; PKA, protein kinase A; PTKR, protein tyrosine kinase receptor; PI3K, phosphoinositol-3-kinase; PDK, 3-phosphoinositide-dependent protein kinase; PKB, protein kinase B; GS, glycogen synthase.

been shown *in vivo* to possess hypoglycemic/insulin-like properties, suggesting a potential usefulness of these compounds as antidiabetics. In this case the inhibition of GSK3 might be desirable and potentially beneficial. Yet, with regard to colon carcinogenesis, interference with GSK3 activity might imply an undesired mitogenic signal. Thus, the modulation of GSK3 activity by food constituents such as QUE and EGCG may lead to a spectrum of downstream consequences depending on the cellular/tissue context as well as on the respective concentration.

Antiproliferative effects of QUE and EGCG appear to depend on the respective model and concentration range. QUE has been reported to stimulate cell proliferation in several human tumor cell lines at low concentrations (*13*). *In vivo* studies on the impact of green tea catechins on carcinogenesis did not consistently indicate chemopreventive properties. In F344 rats green tea catechins have been reported to enhance tumor development in the colon (*14*). Little is known so far about the impact of these flavonoids on the regulation of colon tumor cell growth mediated by the Wnt pathway. The aim of the study was to address the question whether QUE and EGCG mediate proliferative stimuli in human colon carcinoma cells by interference with key elements of the Wnt pathway. We investigated the effect of both food constituents on GSK3 and β-catenin and studied their impact on the stabilization of intracellular β-catenin with emphasis on TCF/LEF-mediated transcriptional activity.

MATERIALS AND METHODS

Chemicals. (−)-Epigallocatechin-3-gallate (EGCG) was a kind gift from Nestlé (Vevey, Switzerland), and quercetin (QUE) was obtained from Sigma Aldrich, Taufkirchen, Germany. SB-216763 was purchased from Tocris, U.K. For all assays, compounds were freshly prepared by being dissolved in DMSO shortly prior to the experiment. The final concentration of DMSO in the different test systems did not exceed 1%.

Cell Culture. The human colon carcinoma cell line HT29 and the human embryonic kidney cells Hek293 were purchased from the German Collection of Microorganisms and Cell Cultures in Braunschweig, Germany. Both cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) with 4500 mg/L glucose, L-glutamine, without sodium-pyruvate, supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS) in a humidified atmosphere at 37 °C and 5% CO_2. Cell culture medium and the supplements were obtained from Invitrogen Life Technologies, Karlsruhe, Germany.

Sulforhodamine B Assay. To determine growth inhibitory effects on HT29 cells, the sulforhodamine B assay (SRB assay) was performed according to a modified method of Skehan *et al.* (*15*) as reported previously (*16*). Cells were incubated with the respective compound for 72 h in serum-containing medium (10% FCS). Analogous to the studies on GSK3 and β-catenin expression, HT29 cells were grown for 24 h in cell culture medium with 1% FCS prior to serum-free incubation with QUE and EGCG for 24 h. Cell growth inhibition was determined as percent survival and plotted as the number of treated over control cells × 100 (% T/C).

Preparation of Cellular and Nuclear Extracts. HT29 cells were seeded in Petri dishes (145 cm^2), at 2×10^6 cells/dish, and allowed to grow for 72 h. Thereafter, the FCS content of the culture medium was reduced to 1% for 24 h. The cells were incubated for 24 h (serum-free) with test compounds dissolved in DMSO (final DMSO concentration of 1%). Before cell lysis, the cell culture medium was removed and cells were washed twice with ice cold phosphate buffered saline (PBS). *Cellular Extract.* HT29 cells were harvested on ice in 0.5 mL of lysis buffer (25 mM Tris-HCl, pH 7.4, 3 mM EDTA, 3 mM EGTA, 50 mM NaF, 0.27 M sucrose, 10 mM Na-β-glycerophosphate, 5 mM Na-pyrophosphate, 0.5% (v/v) Triton-X 100, 2 mM Na_3VO_4; 2% (v/v) protease inhibitor cocktail (Roche Diagnostics, Germany), 0.1% (v/v) β-mercaptoethanol, and 0.1% (v/v) microcystin LR were added just before lysis). Thereafter, the lysate was homogenized thoroughly and subsequently centrifuged for 30 min (100000*g*, 4 °C). *Nuclear Extract.* Cells were homogenized in 1 mL of PBS and centrifuged for 5 min at 15000*g* and 4 °C. The pellet was consecutively resuspended in 200 μL of lysis buffer A (10 mM HEPES−KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5% (v/v) Igepal CA-630, and 2% (v/v) protease-inhibitor cocktail), incubated on ice for 20 min, and centrifuged for 5 min at 15000*g* and 4 °C. The precipitated nuclei were resuspended in 200 μL lysis of buffer B (20 mM HEPES−KOH, pH 7.9, 1.5 mM $MgCl_2$, 25% (v/v) glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 2% (v/v) protease-inhibitor cocktail), incubated on ice for 20 min, and centrifuged for 5 min at 15000*g* and 4 °C. The pellet was discarded and the supernatant (nuclear extract) stored at −80 °C until use.

The colorimetric procedure of Bradford (*17*) was applied to determine the protein content using bovine serum albumin as a standard.

Western Blot Analysis. Forty micrograms of protein of cellular or nuclear extract was separated by SDS−PAGE (10% acrylamide gel), and proteins were transferred onto a nitrocellulose membrane. Blocking the membrane for nonspecific protein binding was carried out overnight in 5% (w/v) dry milk (Roth, Karlsruhe, Germany) in Tris buffered saline (TBS)/Tween-20 (0.1% v/v). Western blot was performed using antibodies against human GSK3α/β (Santa Cruz Biotechnology, Santa Cruz, CA) and human β-catenin (Cell Signaling Technology, MA) as well as against phosphorylated GSK3α/β (Ser21/9) and phosphorylated β-catenin (Ser33/Ser37/Thr41) (Cell Signaling Technology, MA) following the manufacturer's protocol. Peroxidase conjugated immunoglobulin G (IgG) against rabbit (Cell Signaling Technology, MA) and mouse (Santa Cruz, Biotechnology, Santa Cruz, CA) were used as secondary antibodies. The respective chemoluminescent signals (LumiGLO, Cell Signaling Technology, MA) were analyzed using the Lumi-Imager with the LumiAnalyst 3.0 software for quantification (Roche Diagnostics, Mannheim, Germany). Arbitrary light units were plotted as test over control (%) with the control representing cells treated with 1% DMSO (solvent control).

TCF/LEF Reporter Gene Assay. Hek293 cells (0.5×10^6) were seeded per Petri dish (57 cm^2) and grown for 72 h. Thereafter, cells were transiently transfected with 5 μg of the luciferase reporter constructs (TOPflash or FOPflash, Upstate Biotechnology, VA) using 15 μL of FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany). Twenty-four hours after transfection, cells were detached and reseeded into a 12-well dish at a density of 200 000 cells

per well and grown for 24 h. Cells were incubated at 37 °C for 24 h with test compounds or 1% DMSO (solvent control) and lysed in cell culture lysis buffer (500 μL per well, Promega, Madison, WI). Luciferase activity was measured as arbitrary light units using a luminometer and was normalized for cell number variation with respect to protein concentration.

Quantitative Real Time TaqMan PCR. HT29 cells were seeded in Petri dishes (57 cm^2) at a density of 1.2×10^6 cells. After 48 h, the serum concentration of the medium was reduced from 10% to 1%. After a further 24 h, cells were incubated at 37 °C for 24 h with substances in serum-free medium adopted to a final DMSO concentration of 1%. Total RNA was isolated with the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Reverse transcription of RNA was performed using random hexamers and the High Capacity cDNA Archive Kit (Applied Biosystems, CA). Transcripts for β-catenin and for the housekeeping gene β-actin were quantified using gene specific primers and TaqMan probes (*β-catenin*, Pre-Developed Assay Reagent, part number 4318286F, RefSeq NM_001904; *β-actin*, human ACTB endogenous control, VIC/MBG probe, primer limited; Applied Biosystems, CA), as well as TaqMan Universal PCR Master Mix (with AmpErase UNG and passive reference ROX) and the ABI PRISM 7900 HT Sequence Detection System from Applied Biosystems.

RESULTS

Effect on GSK3α and -β Activity. The phosphorylation status of GSK3α (Ser21) and GSK3β (Ser9) was determined by Western blot analysis as a measure for the inhibition of enzyme activity. HT29 cells were incubated with the respective flavonoid for 24 h under serum-free conditions. QUE did not significantly affect the intracellular level of phosphorylated GSK3α (**Figure 1A,C**) and GSK3β (**Figure 1B,C**) in HT29 cells up to 75 μM, indicating no substantial inhibitory effect of QUE on GSK3α and GSK3β enzyme activity. However, a slight yet significant decrease in the protein level of both GSK3 isoforms was observed. For both isoforms, the effect was not concentration-dependent but remained at an approximately constant level. In the case of GSK3α a recurrence to the level of the solvent-treated control was observed at 75 μM QUE.

In contrast, incubation of HT29 cells with EGCG for 24 h resulted in increased levels of phosphorylated GSK3α at concentrations $\geq$0.5 μM, reaching an apparent maximum at 25 μM (**Figure 2A,C**). A significant increase of phosphorylated GSK3β was observed at concentrations $\geq$0.5 μM of EGCG, albeit less pronounced than for the GSK3α isoform (**Figure 2B,C**). Concomitantly, the protein level of total GSK3α and -β was not affected by EGCG (**Figure 2**).

Effect on Intracellular β-Catenin Levels. We further addressed the question whether the observed effects on the GSK3 phosphorylation status at Ser21/9 (EGCG) or on total GSK3 protein levels (QUE) are of relevance for the intracellular β-catenin level in human colon tumor cells. Inhibition of GSK3 within the multiprotein complex (APC complex) of the canonical Wnt signaling pathway (**Scheme 1**) is expected to diminish the amount of phosphorylated β-catenin accompanied by an increase of the total intracellular β-catenin level. Western blot analysis using antibodies against total β-catenin and β-catenin phosphorylated at Ser33, Ser37, and Thr41 was employed to investigate the impact of both test compounds on β-catenin homeostasis in HT29 cells. Incubation of HT29 cells with 10 μM SB216763, a GSK3 inhibitor, for 24 h led to a strong reduction in phosphorylated β-catenin, accompanied by a significant increase in total β-catenin (**Figure 3**), supporting the hypothesis of effective GSK3 inhibition within the APC complex.

The level of phosphorylated β-catenin was not significantly affected by QUE at concentrations ranging from 10 up to 75 μM (**Figure 3A,B**). The overall β-catenin level in HT29 cells remained unaffected as well (**Figure 3A,B**). These results are supported by data from quantitative real time TaqMan PCR, showing no significant change in the level of β-catenin transcripts in HT29 cells after incubation with QUE up to 80 μM (data not shown).

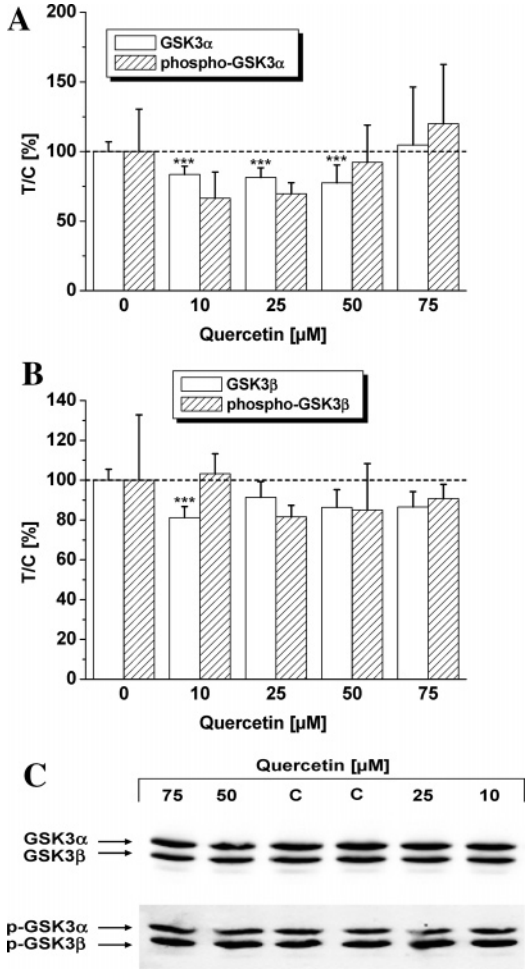


Figure 1. Western blot analysis of (**A**) GSK3α and (**B**) GSK3β in HT29 cells after 24 h treatment with QUE. The data are presented as test over control (%) with the control being cells treated with 1% DMSO. The data are the mean ± SD of at least 3 independent experiments with similar outcome with panel **C** showing a representative Western blot with C as control (solvent-treated cells). The significances indicated are calculated compared to the solvent control using Student's *t* test (*** = $p < 0.001$).

In contrast, treatment with EGCG effectively decreased the amount of phosphorylated β-catenin with an IC_{50} value of 24 ± 7 μM (**Figure 4A,B**). However, in parallel with the effective drop of phosphorylated β-catenin, the amount of total β-catenin was found to be diminished as well and stands in sharp contrast to the effects observed for SB216763 that effectively abrogated phosphorylation of β-catenin, resulting in a concomitant increase in total β-catenin.

Quantitative real time PCR showed an EGCG induced decrease of the messenger RNA levels for β-catenin in a concentration-dependent manner (**Figure 4C**). Already at a concentration of 0.1 μM EGCG a slight decrease of β-catenin transcripts was observed, significant at 25 μM with a reduction of about 40%.

As a crucial parameter for transcriptional response to an interference with β-catenin homeostasis, the impact of QUE and EGCG on the level of β-catenin in the nucleus of HT29 cells was determined. As expected, the GSK3 inhibitor SB216763

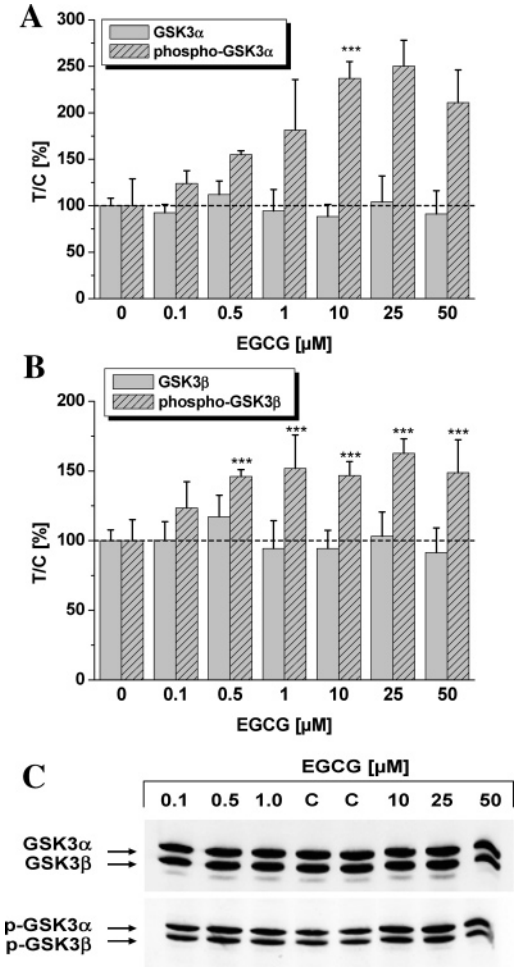


Figure 2. Western blot analysis of (**A**) GSK3α and (**B**) GSK3β in HT29 cells after 24 h treatment with EGCG. The data are presented as test over control (%) with the control being cells treated with 1% DMSO. The data are the mean ± SD of at least 3 independent experiments with similar outcome, with panel **C** showing a representative Western blot with C as control (solvent-treated cells). The significances indicated are calculated compared to the solvent control using Student's *t* test (*** = $p < 0.001$).

caused a strong increase of β-catenin in HT29 nuclei (**Figure 5**), pointing to a possible transcriptional activation of Wnt target genes. Treatment with QUE led to a slight yet not significant decrease of the nuclear β-catenin level at 25 μM of 19 ± 11% (**Figure 5**). However, the effect apparently was not concentration-dependent, remaining within the same range at 10 to 50 μM QUE. In contrast, EGCG concentration-dependently diminished nuclear β-catenin achieving about 50% decrease at 50 μM EGCG (**Figure 5**), suggesting a suppression of TCF/LEF driven gene transcription.

Impact on TCF/LEF Mediated Gene Transcription. We addressed the question whether changes in cellular and, particularly, in nuclear β-catenin by QUE or EGCG are of relevance for β-catenin/TCF/LEF-mediated gene transcription using a reporter gene approach (**Scheme 2**). Human embryonic kidney cells (Hek293), known to express an intact and mutation-free APC complex, were transiently transfected with the reporter gene construct (TOPflash), containing two sets of three copies of the TCF-binding site (wild type) upstream of the thymidine kinase (TK) minimal promotor and the luciferase open reading frame. FOPflash, containing mutated TCF binding sites, was used as a control for leakage of luciferase expression. Treatment of TOPflash transfected Hek293 cells with the GSK3 inhibitor SB216763 potently enhanced luciferase activity up to 24-fold

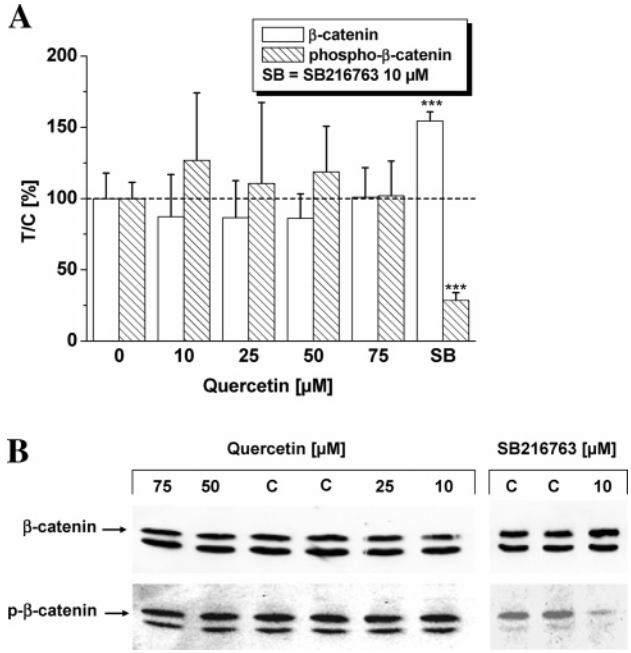


Figure 3. (**A**) Western blot analysis of β-catenin protein and phosphorylated β-catenin in HT29 cells treated with QUE for 24 h. The GSK3 selective inhibitor SB216763 served as positive control. The data are plotted as test over control (%) with the control being cells treated with 1% DMSO. The data are the mean ± SD of at least 3 independent experiments with similar outcome, with panel **B** showing a respective representative Western blot. The significances indicated are calculated compared to the solvent control using Student's *t* test (*** = $p < 0.001$).

(**Figure 6A,B**) compared to cells incubated with the solvent control.

Treatment with QUE slightly, yet significantly, increased luciferase activity at concentrations ranging from 10 to 50 μM (**Figure 6A**), suggesting an inducing effect of the flavonol on β-catenin/TCF/LEF-driven reporter activity. EGCG did not affect TCF/LEF-mediated luciferase expression up to 50 μM (**Figure 6B**).

Effect on Tumor Cell Growth. The effect of QUE and EGCG on the growth of HT29 cells was determined using the SRB assay according to the method of Skehan *et al.* (*15*). After 24 h incubation with QUE or EGCG under serum-free conditions the growth of HT29 cells was found to be inhibited to a similar extend by both compounds with IC_{50} values of 124 ± 8 μM and 147 ± 18 μM, respectively (**Figure 7A**). Prolonged incubation (72 h) in the presence of serum substantially enhanced the growth inhibitory effect of QUE and EGCG (IC_{50} value of 52 ± 11 μM and 40 ± 6 μM, respectively) (**Figure 7B**). A stimulatory effect on cell growth was not observed under the chosen experimental conditions.

DISCUSSION

The Wnt pathway represents one of the major signaling cascades involved in colon carcinogenesis. However, information on effects of flavonoids on key elements of the canonical Wnt signaling pathway is scarce. In the present study, we investigated the effect of QUE and EGCG on the activity of GSK3 as one of the key elements of the Wnt pathway, crucial for the regulation of β-catenin homeostasis. As a measure for the phosphorylation-dependent inhibition of GSK3α and -β kinase activity the respective phosphorylation status was determined by Western blot analysis (*18*). The arylindolemaleimide SB216763 represents a selective, potent, and cell

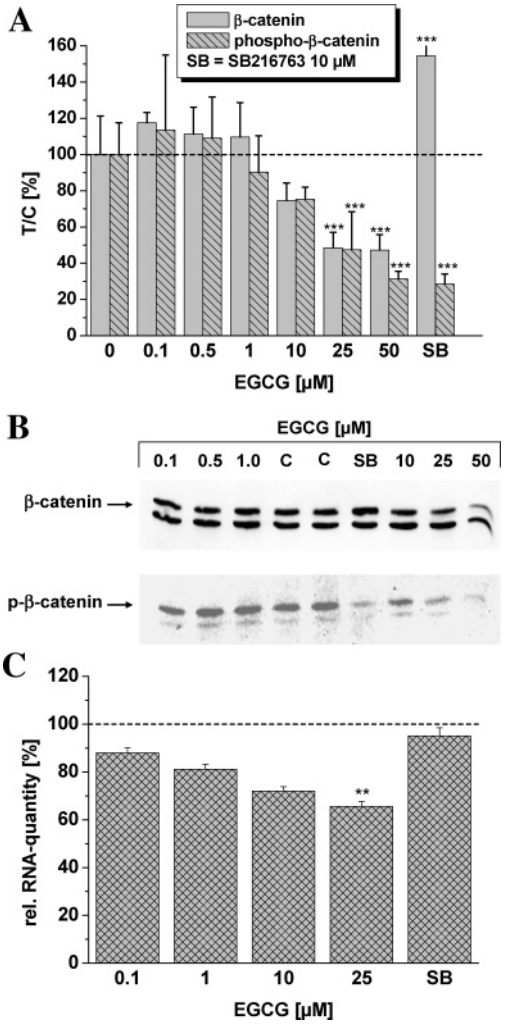


Figure 4. (**A**) Western blot analysis of β-catenin protein and phosphorylated β-catenin in HT29 cells treated with EGCG for 24 h. The GSK3 selective inhibitor SB216763 serves as positive control. The data are plotted as test over control (%) with the control being cells treated with 1% DMSO. The data are the mean ± SD of at least 3 independent experiments with similar outcome with panel **B** showing a respective representative Western blot. (**C**) Reduction of β-catenin transcripts in HT29 cells incubated with EGCG for 24 h. Data are normalized by β-actin expression and presented as % of the solvent control. The data are the mean ± SD of at least three independent experiments performed in triplicate. The significances indicated are calculated compared to the solvent control using Student's *t* test (** = $p < 0.01$; *** = $p < 0.001$).

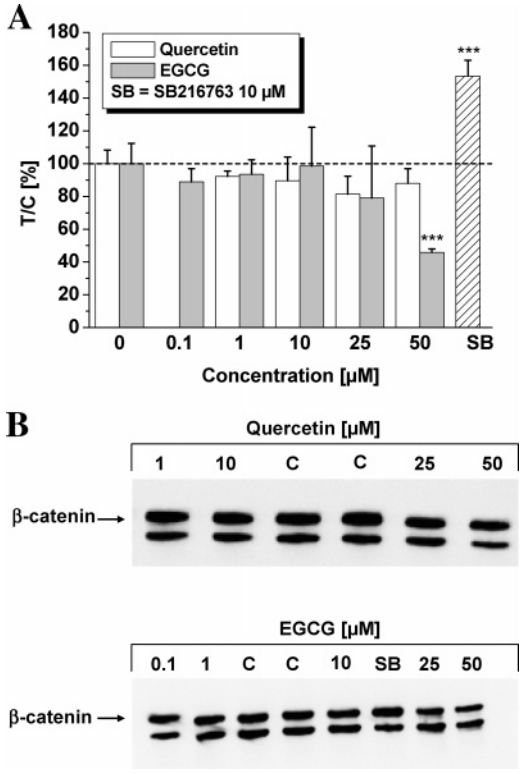
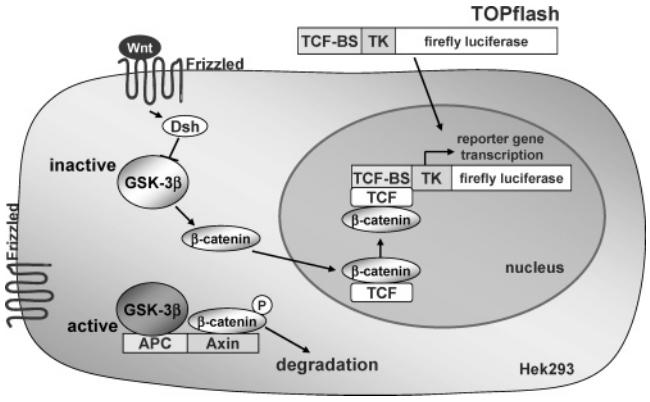


Figure 5. (**A**) Western blot analysis of nuclear β-catenin content in HT29 cells treated with QUE and EGCG for 24 h. The GSK3 selective inhibitor SB216763 serves as positive control. The data are plotted as test over control (%) with the control being cells treated with 1% DMSO. The data are the mean ± SD of at least three independent experiments with panel **B** showing respective representative Western blots. The significances indicated are calculated compared to the solvent control using Student's *t* test (*** = $p < 0.001$).

permeable inhibitor of both isoforms of GSK3, causing activation of glycogen synthase and β-catenin/TCF/LEF-driven reporter gene transcription in Hek293 cells (*19*). Therefore SB216763 served as a positive control in the experiments. Yet, due to its ATP competitive inhibitory properties on GSK3, SB216763 was not included as a control for phosphorylation-mediated GSK3 inhibition.

QUE did not significantly affect the level of phosphorylated GSK3α and -β in HT29 cells (**Figure 1**), thus indicating that the activity of both isoforms remained largely unaffected, merely the protein levels were found to be slightly but significantly decreased for both proteins. The lack of concentration dependency and the rather limited effects observed argue for some unspecific mechanisms, rather than a specific interference with the respective target enzyme. It might be considered that these apparently unspecific effects result from the impact of QUE on protein synthesis (*20*). Taken together, on the level of GSK3α and -β, no substantial inhibition of the kinase activity is to be expected by treatment with QUE in HT29 cells.

Scheme 2. Scheme of the Reporter Gene Assay for the Detection of TCF/LEF-Mediated Gene Transcription Using Firefly Luciferase as a Reporter[a]



[a] APC, adenomatous polyposis coli; Dsh, dishevelled; frizzled receptor; GSK3β, glycogen synthase kinase 3β; TCF, T-cell factor; TCF-BS, TCF binding site (wild type); TK, thymidine kinase minimal promoter; TOPflash, TCF reporter plasmid.

Cellular levels of β-catenin and phosphorylated β-catenin in HT29 cells were not significantly affected by QUE up to 75 μM (**Figure 3**), indicating no β-catenin stabilizing effect of QUE. Also the level of β-catenin in the nucleus remained unaffected (**Figure 5**). Taken together, QUE did not affect substantially β-catenin homeostasis in HT29 cells. These results

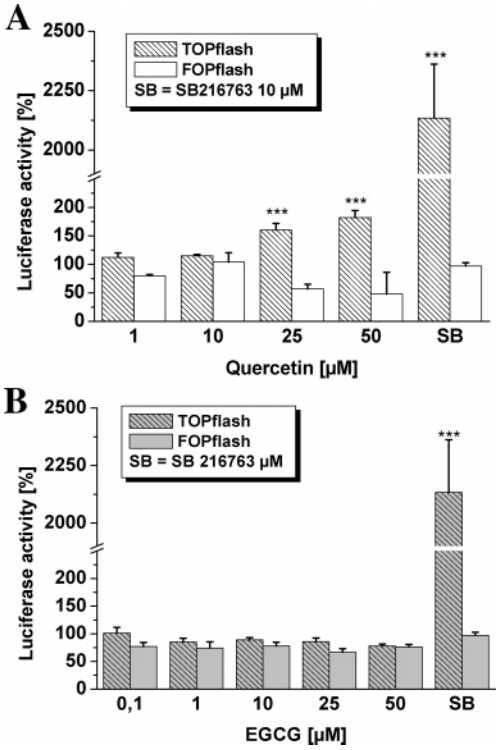


Figure 6. Induction of luciferase expression as a measure for β-catenin stabilization by (**A**) QUE and (**B**) EGCG. Hek293 human embryonal kidney cells were transiently transfected with the reporter gene constructs TOPflash (containing wt TCF binding sites) and FOPflash (negative control vector containing mutated TCF binding sites). Cells were reseeded 24 h posttransfection and prior to the incubation with QUE (**A**) and EGCG (**B**) for 24 h. Luciferase activity was determined 72 h after transfection and normalized with respect to the protein concentration. Data are presented as percent of the solvent control and are the mean ± SD of at least three independent experiments. The significances indicated are calculated compared to the control using Student's *t* test (*** = $p < 0.001$).

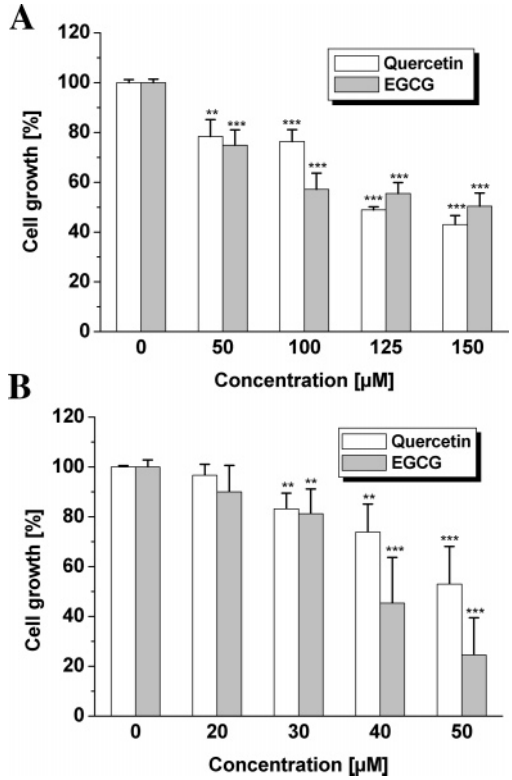


Figure 7. Growth inhibitory properties of QUE and EGCG on HT29 cells after incubation (**A**) for 24 h (serum-free) and (**B**) for 72 h (with 10% FCS) using the SRB assay. The data are the mean ± SD of at least 3 independent experiments. The significances indicated are calculated compared to the solvent control using Student's *t* test (** = $p < 0.01$; *** = $p < 0.001$).

allow the assumption that β-catenin/TCF/LEF-dependent gene transcription will not be influenced by QUE. However in the respective reporter gene model (**Scheme 2**), treatment with QUE mediated a slightly enhanced and concentration-dependent TCF/LEF-driven reporter gene expression. The induction of luciferase expression by QUE appeared marginal in comparison to the effect of the specific GSK3 inhibitor SB216763. QUE induced small increases in luciferase expression (15% at 10 μM up to 82% at 50 μM) whereas 10 μM SB216763 enhanced luciferase expression by more than 2000% (**Figure 6A**). In principle, an increase in TCF/LEF-mediated gene expression is expected to represent a proliferative stimulus. The limited effect of QUE in the reporter gene assay raised the question whether this is of relevance for the regulation of cell growth. Indeed, in the SRB assay no growth stimulatory effect of QUE on HT29 cells was detectable. In contrast, QUE was found to inhibit the growth of HT29 cells in a concentration-dependent manner (**Figure 7**).

Of note, in SW480 cells a reduction in nuclear β-catenin and unchanged cytosolic β-catenin levels as a result of QUE treatment have been reported (*21*). Such differential effects on β-catenin levels might be a reflection of different cellular context and experimental conditions, leading to differential responses. Incubation with QUE starting 3 h after transfection was reported to result in an effective decrease of TCF/LEF-mediated reporter gene expression (*21*). In our study, the cells were allowed to recover and stabilize after transfection for 48 h, in order to minimize additional stress factors, prior to the incubation with the test compound. Under these experimental conditions, QUE was found to act as a weak inductor of TCF/LEF-mediated gene transcription, an effect, however, which was not associated with a stimulation of cell growth (**Figure 7**).

EGCG has been shown to be taken up by HT29 cells and to be predominantly localized in the cytosol (*22*). Recently, it has been speculated that under cell culture conditions the presence of EGCG might result in the formation of H_2O_2, which is discussed at least to contribute to the cellular effects observed by EGCG treatment (*23*). Hydrogen peroxide itself (100 μM and 500 μM) has been reported to activate GSK3β in Hek293 and in other cell lines (*24*). In contrast to the reported effect of H_2O_2, we found an effective inhibition of GSK3α and GSK3β in HT29 cells as a result of treatment with EGCG. Furthermore, a significant increase in GSK3β phosphorylation, indicative for the inhibition of enzyme activity, was already observed at 0.5 μM EGCG (**Figure 2B**), far below the reported concentrations of EGCG expected to generate substantial amounts of H_2O_2 (*23*, *25*). Thus, it is not likely that H_2O_2 production by EGCG is of substantial relevance for the biological effects observed in our studies on key elements of the Wnt pathway.

In contrast to QUE, the green tea catechin EGCG was identified as a potent inhibitor of GSK3 activity in HT29 human colon carcinoma cells. Already at low micromolar concentrations EGCG potently inhibited the activity of GSK3α and GSK3β in HT29 cells after 24 h incubation as indicated by increased phosphorylation at Ser21 and Ser9 (**Figure 2**). However, EGCG did not affect the activity of isolated GSK3β up to 100 μM (data not shown). Therefore it is conceivable that the inhibitory effect of EGCG on the cellular GSK3 isoforms results from the interference with upstream signaling cascades rather than

from direct targeting of the kinase. Signaling cascades such as the insulin/phosphoinositol-3-kinase (PI3K) cascade and/or the cAMP pathway (**Scheme 1**) might be involved in mediating inhibitory effects of EGCG on GSK3 isoenzymes. Ser21 in GSK3α and Ser9 in GSK3β represent physiological substrates of several kinases, such as protein kinase B, protein kinase C, and cAMP-dependent protein kinase A (*26*−*28*).

In accordance with the inhibition of the activity of GSK3α and -β, treatment of HT29 cells with EGCG resulted in a concentration-dependent decrease of phosphorylated β-catenin (**Figure 4A,B**). However, unexpectedly, the decrease of β-catenin phosphorylation was not associated with a stabilization and accumulation of intracellular β-catenin. These results are supported by the finding that not only the amount of total cellular β-catenin was diminished (**Figure 4A,B**) but also the amount of β-catenin in the nucleus (**Figure 5**). The decrease of the cellular β-catenin level, despite reduced phosphorylation rate, might result from the suppression of β-catenin expression, indicated by a decrease of β-catenin mRNA (**Figure 4C**). Thus, the interfering effect of EGCG with β-catenin homeostasis might primarily be governed by its effect on β-catenin expression.

In accordance with these results, EGCG did not induce TCF/LEF-mediated gene expression in the reporter gene assay (**Figure 6B**). The experimental design of the employed reporter gene system was optimized to detect stimulating effects on TCF/LEF-dependent gene expression, as shown for the GSK3 inhibitor SB216763 (**Figure 6B**). Noteworthy are the findings of Dashwood et al. (*25*), who described inhibitory properties of EGCG on β-catenin/TCF-4 reporter activity. In support of the results observed with the reporter gene assay, EGCG was found to inhibit the growth of HT29 cells in the SRB assay (**Figure 7**).

Taken together, treatment with EGCG was found to result in substantial inhibition of GSK3α and GSK3β activity in HT29 cells presumably mediated by the interference of the compound with upstream signaling elements. The resulting decrease in β-catenin phosphorylation is presumably counterbalanced by the suppression of β-catenin neogenesis, preventing the activation of TCF/LEF-mediated proliferative signals. Reduced levels of cellular and in particular of nuclear β-catenin even indicate a suppression of TCF/LEF-mediated gene transcription, which might contribute to the growth inhibitory properties of EGCG (*24*).

In summary, the results allow us to conclude that QUE and EGCG do not mediate proliferative stimuli in human colon carcinoma cells by interference with key elements of the Wnt pathway.

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