Research article

Binding of (-)-epigallocatechin-3-gallate to the Hsp70 ATPase domain may promote apoptosis in colorectal cancer

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Colorectal cancer (CRC) patients frequently have a poor prognosis because of metastases and drug resistance. Heat-shock protein 70 (Hsp70) over-expression in cancer may exacerbate these factors by inhibiting apoptosis, thus providing a potential therapeutic target. The green tea flavonoid (-)-epigallocatechin-3-gallate (EGCG) induces apoptosis in numerous cancer cell lines by unclear mechanisms. This study proposed and investigated a novel mechanism by which EGCG might inhibit the anti-apoptotic activity of Hsp70, namely, by competing with adenosine triphosphate (ATP) for binding to the Hsp70 ATPase domain. It also examined the impact of EGCG on the CRC cell line HT-29, which is known to over-express Hsp70.

HT-29 cells were treated with 10–300 μM EGCG for 8, 24 and/or 48 h, then analysed using an MTS proliferation-assay or flow cytometry with Annexin V-FITC and propidium iodide. The competitive binding of EGCG to the Hsp70 ATPase domain was assessed using ATP-agarose, a dot blot and chemiluminescence techniques.

EGCG significantly (P < 0.001) and dose-dependently inhibited HT-29 cell viability. Viability was inhibited by 50% (IC50) at 89 μM and 74 μM after 24- and 48-h treatments, respectively, also suggesting a time-dependent effect. Apoptosis was induced both dose- and time-dependently, commencing at 50 μM after 8- and 24-h treatments. Further, apoptosis correlated significantly (P < 0.01) with reduced viability as measured by MTS at 24 h, indicating 98% causality. The dot blot suggested 200 μM EGCG competed with ATP for binding to Hsp70, presumably by binding its ATPase domain, theoretically implying this flavonoid could inhibit the anti-apoptotic effect of Hsp70.

Key words: colorectal cancer, (-)-epigallocatechin-3-gallate, Hsp70, apoptosis.

Introduction

Cancer of the colon and/or rectum, collectively termed colorectal cancer (CRC), is the third most common cancer in the UK, and the second commonest cause of mortality.1 The disease arises as a result of multi-step genetic derangements (Fig. 1) which disrupt molecular homeostatic regulation.2 Possibly as a consequence of molecular disruption,3 CRC cells commonly over-express heat shock protein 70 (Hsp70).4–6 This highly conserved protein, which is normally expressed constitutively at low levels,7 allows cells to survive lethal environments by assisting the folding of stress-induced non-native proteins.8 Over-expressed Hsp70 further carcinogenesis, in part, by inhibiting apoptosis of cancer cells;8 its inhibition therefore, may prove useful in chemo-prevention and/or therapy;8 however, there are presently no known selective molecular inhibitors of Hsp70 accessible to humans.8

The green tea catechin (-)-epigallocatechin-3-gallate (EGCG) (Fig. 2) is known to selectively induce apoptosis in various cancer cell-lines,9 although its mechanisms of action remain unclear.10 From the perspective of CRC, despite the low bioavailability of EGCG, heightened exposure is enabled by intestinal excretion;12 further, 4-week daily ingestion of 800 mg EGCG has been found safe and tolerable,13 collectively implying therapeutic potential.10,14

Recently, EGCG was found to bind the HSP70 family protein ‘glucose regulated protein 78’ (Grp78) thus inhibiting its anti-apoptotic action.16 Notably, Hsp70 activity is

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dependent on the co-operative functioning of its COOH-(C-)-terminal substrate binding domain (SBD) and its NH₂-(N-)terminal ATPase domain (Fig. 3). Research suggests the SBD is essential to inhibiting apoptosis and that many apoptotic events are inhibited by Hsp70 in an ATPase domain-dependent manner (Fig. 4). Ermakova et al. found that EGCG directly interacted with the ATPase domain of Grp78, thereby inhibiting function by competing for ATP binding. Given the highly conserved nature of these family proteins, it is conceivable that EGCG has similar effects on Hsp70. This suggestion is supported: human Grp78 shares 60% sequence homology with human Hsp70, and its endoplasmic reticulum functions are parallel to those of cytosolic Hsp70. The N-terminal of HSP70 family members are particularly conserved, and although the precise molecular mechanisms regulating Hsp70 activity remain unclear, increasingly, the essential regulatory components of the ATPase domain are being identified. As these factors appear conserved among all Hsp70 proteins, this suggests a universal mechanism of ATPase domain regulation.

Accordingly, this study seeks to determine whether EGCG reduces viability and induces apoptosis in the CRC cell line HT-29, which is known to over-express Hsp70, and, whether EGCG competes with ATP for binding to Hsp70, thus explaining, at least partially, the pro-apoptotic effect of EGCG.

**Materials and methods**

Cell-culture materials were purchased from Lonza Wokingham Ltd, (Wokingham, Berkshire); unless otherwise specified, reagents were from Fisher Scientific UK, (Loughborough, Leicestershire).

**Cell culture and passage**

Adherent HT-29 cells (European Collection of Cell Cultures (ECACC), Porton Down, Salisbury, Wiltshire), (passage 141 on purchase), were cultured in a humidified 5% CO₂ atmosphere at 37°C, using McCoy’s medium (suppl. 10% foetal bovine serum, 100 U/ml penicillin/100 mg/ml streptomycin). Cells were passaged 18 times in accordance with ECACC instructions: at 70–80% confluence cells were trypsinized for 10 min, then sub-cultured at 3–4 × 10⁴ cells/ml in fresh medium.

**Effect of EGCG on cell viability: MTS-assay**

Cell viability was assayed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Southampton), in accordance with the Promega protocol. Prior to treatments, 50 µl of 1 × 10⁵ cells/ml cells were incubated in 96-well plates for 24 h to exponential growth. Cells were then incubated with 10–300 µM EGCG (Sigma-Aldrich, Dorset) (from 10 mM stock in McCoy’s) in triplicate; (100 µl total liquid/well). Controls included cells or media-only without treatment (media-only blank), and EGCG dilutions with dead cells or media only (treatment blank). After 24 or 48 h, cells were re-incubated with 20 µl

**Figure 1.** Gene changes during colorectal carcinogenesis. For reviews see²⁻⁹.

**Figure 2.** (-)-Epigallocatechin-3-gallate. The catechin epigallocatechin-3-gallate (EGCG) is one of a subgroup of polyphenolic flavonoids found in tea. For detail see.¹⁵

**Figure 3.** ATP/ADP dependent substrate binding of Hsp70. Adenosine triphosphate (ATP) binding (left) to the Hsp70 ATPase domain decreases the substrate binding domain’s (SBD’s) substrate affinity, leading to rapid binding and release of substrates; when adenosine diphosphate (ADP)-bound (right), substrate binding is sustained. Substrate binding coupled with for example Hsp40 activity, stimulates ATP hydrolysis, returning the SBD to its high affinity conformation.
of reagent (comprising a tetrazolium compound [3-(4, 5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and the electron coupling reagent phenazine ethosulfate), for 1.5 h. Absorbance was recorded at 490 nm with an MRX Microplate Reader (Dynatech, West Sussex). Each experiment was repeated three times independently.

EGCG-induced cell death: flow cytometric analysis

Cell death was analysed using the Annexin V-FITC Apoptosis Detection Kit I (product 556547) (BD Biosciences Pharmingen, Oxford). Protocol adapted from van England et al.\(^1\) First, 1 x 10^5 HT-29 cells were incubated in a 6-well plate for 24 h. Cells were incubated with 0–200 μM EGCG for 8 or 24 h; non-adherent cells were dislodged to supernatant, which was subsequently centrifuged (400g, 10 min). Cells were washed in cold Dulbecco’s phosphate buffered saline (DPBS), then re-suspended in 100 μl binding buffer (BB) (0.1 M Hepes/NaOH (pH 7.4) 1.4 M NaCl, 25 mM CaCl\(_2\), diluted x10 in distilled H\(_2\)O (dH\(_2\)O)). Next, 5 μl Annexin V-FITC (AV-FITC) and 5 μl propidium iodide (PI) were added; tubes were vortexed and incubated in the dark (RT, 15 min); samples were added to 400 μl BB in BD Falcon flow cytometry tubes.

![Figure 4. Suggested mechanisms by which Hsp70 inhibits apoptosis. Shown, are points in the intrinsic and extrinsic apoptotic pathways inhibited by Hsp70. For reviews see.\(^5\).\(^1\) Hsp70 shown with an ATP ‘star’ indicate ATPase domain-dependent mechanisms.\(^2\)\(^4\) – \(^3\)\(^0\) Hsp70 with an ATP? star are those whose ATPase-dependent anti-apoptotic activity have been suggested because of the direct interaction of Hsp70.\(^3\)\(^1\), \(^3\)\(^2\) ”](https://academic.oup.com/biohorizons/article-abstract/1/1/9/232983)
Adherent cells were washed twice with cold DPBS; 2 ml BB was added, plus 5 μl AV-FITC/5 μl PI. Plates were gently vortexed, incubated in the dark (RT, 15 min), then washed twice with BB. Cells were harvested and re-suspended in 500 μl BB.

Within 1 h of staining, 10 000 cell events from each sample were analysed using a Becton Dickinson FACScanto Flow Cytometer and BD FACSDiva software (BD Biosciences Pharmingen); fluorescence was compensated via unstained, PI only and AV-FITC only controls; (further control: AV-FITC and PI). Excitation was at 488 nm; the emission filters used were 500–560 band-pass (BP) (green; FITC), 543-627 BP (orange; PE), and 670 longpass (red PI).

Analysing competitive binding of EGCG to the Hsp70 ATPase domain: ATP agarose beads and dot blot

Protocol adapted from Ireland et al. ATP-agarose beads (Sigma-Aldrich) were equilibrated with TEDM (26 mM Tris (pH 7.4), 0.1 mM EDTA, 3 mM DL-Dithiothreitol and 1 M MgCl2). Run-throughs were conducted using 100 μg of the beads in micro-centrifuge tubes. Using 50 μl total liquid, the first run-through comprised 5 μg/ml bovine recombinant Hsp70 (Sigma-Aldrich) in TEDM. The beads were vortexed briefly, incubated (RT, 10 min), then centrifuged (400g, 1 min). The eluate was removed and the beads washed three times in TEDM. Bound Hsp70 was released with 3 mM ATP in TEDM, using the same process of vortexing, incubation, centrifugation, eluate removal and washing. To evaluate the competitive binding of EGCG to the Hsp70 ATPase domain, the second run-through comprised 200 μM EGCG in TEDM and 5 μg/ml bovine Hsp70. Again, bound Hsp70 was released using 3 mM ATP.

Nitrocellulose was soaked in Tris-buffered Saline (TBS) (30 mM Tris, (pH 7.5), 0.5 M NaCl2 in dH2O) and placed over a BioRad dot-blot apparatus (BioRad Laboratories Ltd, Hemel Hempstead) under vacuum. Next, 50 μl of eluted Hsp70 samples were pipetted onto the dots, plus a control comprising 5 μg/ml bovine Hsp70 in TEDM; this membrane was washed in TBS. The nitrocellulose was then blocked (RT, 1 h) with 10% bovine serum albumin (BSA) in TBS, and given three 5-min washes in Tween-20/Tris-buffered saline (TTBS) (5% Tween-20). It was probed (1 h) on an orbital shaker with 1 mg/ml stock Hsp70 biotin-conjugate primary antibody (mouse monoclonal) (Cambridge Bioscience Ltd, Cambridge) diluted 1:5000 in antibody buffer (AB) (1% BSA in TTBS) and given three 5-min washes with TTBS. Finally, the membrane was incubated (1 h) on an orbital shaker with Extravidin Peroxidase-conjugated secondary antibody (Sigma-Aldrich) diluted 1:2500 in AB, then given three 5-min washes with TTBS.

The immunostained nitrocellulose was immersed in a 1:1 mixture of luminal/peroxide (Supersignal West Pico Chemiluminescent Substrate (product 34080), Pierce, Cramlington, Northumberland) for 5 min then sandwiched in plastic sheeting (Pierce protocol). Kodak Developer and Fixer (Sigma-Aldrich) was prepared 20% in dH2O; film was exposed to the blot for 1 min, developed (5 min), washed in water (30 s), fixed (5 min) and washed in water (5 min).

Statistical analysis

Using the SPSS statistics software, normalized MTS 24- and 48-h data were compared using a Wilcoxon rank sum test; the minimum significant inhibitory concentration (MSIC) was determined using Kruskal–Wallis and post hoc Mann–Whitney U. Correlations between reduced viability and apoptosis were determined using Spearman’s Rank (50 μM and 75 μM concentrations used in the flow cytometry experiment were correlated with the mean 40 μM and 60 μM concentrations, and the 80 μM concentration used in the MTS-assay).

Results

Effect of EGCG on cell viability

This study found that 24- and 48-h EGCG treatments significantly (Hidgrees of freedom(df)10)= 87.5, P < 0.001; H(df 10)= 89.9, P < 0.001) reduced HT-29 viability in a dose-dependent manner; total inhibition was achieved by 200–250 μM after both treatment times (example Fig. 5). The mean concentration at which EGCG inhibited viability by 50% (IC50) was 89 μM and 74 μM after 24- and 48-h treatments, respectively (not shown), intimating a time-dependent effect. The minimum statistically significant growth inhibitory concentration (MSCG) of EGCG after 24- and 48-h EGCG treatment was 60 μM and 40 μM, respectively, also suggesting a time-dependent effect. Nevertheless, 24- and 48-h treatments were determined to be statistically comparable.

Effect of EGCG on apoptosis

Given the similarity between 24- and 48-h samples, flow cytometry experiments utilized 8- and 24-h treatments. Changes in apoptosis were seen in adherent samples, which were therefore used in analysis. Figure 6 (24-h data) demonstrates apoptosis clearly in the adherent sample (top left) at maximal EGCG treatment. Necrosis seen in the non-adherent sample (bottom left), but not the 8-h sample (not shown), could be explained by prolonged exposure to the toxic micro-environment. As delineated in Figs 7 and 8, apoptosis commenced at 50 μM after both treatment times, increasing dose-dependently in parallel with reducing viability. The relationship between decreasing cell viability as measured by MTS, and apoptosis as measured by flow cytometry over 24 h,
was highly significant \( (r = -0.99, P < 0.01) \), and the correlation coefficient \( (r^2 \times 100) \) suggested 98% causality.

**Competitive effect of EGCG on ATP for binding to Hsp70**

Figure 9 illustrates that S3 (200 μM EGCG and 5 μg/ml Hsp70) is more visible than S1 (5 μg/ml Hsp70) and S4 (bound Hsp70 released using 3 mM ATP). This suggests that 200 μM EGCG reduced the affinity of Hsp70 for the ATP-agarose beads, presumably by competing with ATP for the Hsp70 ATPase binding domain.

**Discussion**

Given the potential of EGCG as an anti-cancer agent, revealing its pro-apoptotic mechanisms is considered vital. This study suggests that EGCG competes with ATP for binding to Hsp70, thus potentially inhibiting its activity. The following discussion first evaluates the study-findings and then proposes mechanisms by which EGCG may exert its pro-apoptotic effects by inhibiting Hsp70.

**Evaluation of the effect of EGCG on cell viability and apoptosis**

This study determined that 24- and 48-h EGCG treatments resulted in IC\(_{50}\) of 89 μM and 74 μM, respectively; comparably, Chen et al.\(^{36}\) found that EGCG treatment in HT-29 cells resulted in an IC\(_{50}\) of 100 μM after 36 h, while Shimizu et al.\(^{37}\) and Yang et al.\(^{38}\) found IC\(_{50}\) of 50 μM or 65 μM after 48 h, respectively. Chen et al.\(^{36}\) also found that viability reduction commenced at 50 μM after 36-h treatment, which compares to the MSIC of 60 μM and 40 μM at 24 and 48 h, respectively, found here.

Also like this study after 8- and 24-h EGCG treatments (Figs 7 and 8), Chen et al.\(^{36}\) found that 36-h treatment induced apoptosis commencing at 50 μM in HT-29 cells. Shimizu et al.\(^{37}\) noted that apoptosis commenced at approximately 30 μM after 48 h in the same cell-line, which as per the MTS data, indicates increased potency over time. Both authors demonstrated a comparable dose-dependent increase in apoptosis up to 200 μM EGCG.

**Binding of EGCG to the Hsp70 ATPase domain**

A primary intention of this study was to show qualitatively that EGCG reduces the affinity of Hsp70 for ATP in a cell-free system, presumably by binding to the Hsp70 ATPase domain. As seen clearly in Fig. 9, the preliminary dot-blot results support this hypothesis. While the non-specific nature of the dot blot could be criticized, the antibody used here is utilized extensively in the Chester Centre for Stress Research laboratory and has been found to be specific for Hsp70.\(^{39}\) Furthermore, because only one pure protein was loaded, and great care was taken not to contaminate, it is unlikely that the result was compromised by these factors.

Although this data does not allow for quantitative analysis, Ermakova et al.\(^{16}\) found that binding of 5 μM and 10 μM EGCG to the Grp78 ATPase domain reduced ATPase activity by 56% and 61%, respectively, suggesting a considerable effect. This offers an opportunity to propose mechanisms.
Figure 6. HT-29 cell-death characteristics after 24-h EGCG treatment analysed using flow cytometry. Viable = no stain; Apoptotic = AV-FITC only; Necrotic = PI uptake.

Figure 7. Flow cytometric analysis. HT-29 cells treated with 0.0–200 μM EGCG for 8 h.
which may contribute, at least partially, to explaining the well-recognized anti-apoptotic effect of EGCG in cancer cells.

**The intrinsic pathway: EGCG activation of ASK1 and JNK/p38 via reactive-oxygen-species production**

Saeki et al. found that 4-h 200/400 μM EGCG treatments in leukaemic cell lines stimulated reactive-oxygen-species (ROS)-induced apoptosis via activation of apoptosis-signal-regulating kinase 1 (ASK1), with subsequent stimulation of the c-Jun N-terminal kinase (JNK)/p38 pathway. Various studies have demonstrated that EGCG initiates ROS-induced apoptosis in assorted cancer cells, and that ROS triggers apoptosis via ASK1, so this finding has support.

EGCG could have a dual role here. As explained, Hsp70 can inactivate various pro-apoptotic molecules during carcinogenesis (Fig. 4); moreover, when treated with various toxic substances, Hsp70 is specifically directed to preventing apoptosis by binding, for example, to pro-apoptotic Bcl2-associated X protein (Bax), and death receptors 4 and 5 (DR4/DR5), while its inactivation enables apoptosis. So, while it is intuitive to assume that Hsp70 may increase in response to toxic ROS initiated by EGCG (such protection against oxidative stress by Hsp70 is well recognized), it could be that the abrogation of Hsp70 by EGCG enables ROS to trigger the apoptotic pathway. More specifically, given that Hsp70 can bind, thus inactivating ASK1 in an ATPase domain-dependent manner, EGCG may inactivate bound Hsp70 by competing with ATP for binding to its ATPase domain, thus allowing ASK1 to commence the phosphorylation cascade leading to stimulation of JNK/p38. As the Hsp70 ATPase-independent inhibition of JNK is thought insufficient to block apoptosis, this should allow apoptotic signalling to progress. EGCG may then counteract the ATPase domain-dependent anti-apoptotic influence of Hsp70 at later stages of the apoptotic cascade.

This latter possibility is given weight by research which indicates that EGCG enables various stages of the apoptotic pathway downstream of JNK/p38, where the ATPase-dependent anti-apoptotic activity of Hsp70 is involved. First, Saeki et al. found the apoptotic pathway triggered by EGCG comprised: ASK1 activation of JNK/p38, then downstream primary activation of caspase-9, and cleaved, active caspase-3. The authors theorize the caspase-cascade resulted directly from JNK/p38 signalling via the...
mitochondria, leading to cytochrome c/ APAF-1 (apoptosis-activating-factor-1)/caspase-9 (Fig. 10). While an intervening molecule between JNK/p38 and the mitochondria remains to be determined in EGCG-induced apoptosis, translocation of Bax is considered likely. 40, 44 Qanungo et al. 41 found that 100–200 μM EGCG induced ROS-dependent apoptosis in pancreatic cancer cells, also via activation of JNK; apoptosis was pre-empted by Bax translocation, with subsequent mitochondrial depolarization and cytochrome c release. This supports the role of Bax in the ASK1 pathway stimulated by EGCG.

The actual mechanism by which EGCG triggers Bax remains unknown.45 Beltz et al. 14 noted that 20–25 μM EGCG inhibited anti-apoptotic B-cell lymphoma 2 (Bcl-2) and Bcl-2 family member Bcl-XL with subsequent Bax up-regulation in ovarian carcinoma and melanoma cell lines. In CRC cell lines, 40–100 μM EGCG also inhibited Bcl-2 and Bcl-XL and induced apoptosis.14, 37 These studies did not review Bax activation, though interestingly, suppression of Bcl-2 in CRC has been shown to induce p53-mediated apoptosis via Bax, thus promoting the ratio towards apoptosis even without toxicity,46 indicating perhaps that Bcl-2 inhibition is key. Given that both p53 and Bax activity may be compromised by the ATPase-dependent binding of Hsp70 (Fig. 4), EGCG may facilitate its own activity by inhibiting Hsp70, thus permitting p53 (if active) and/or Bax to respond to pro-apoptotic signalling (Fig. 10). Once these signals have collected at the mitochondria, the Hsp70 ATPase domain-dependent prevention of mitochondrial permeabilization, cytochrome c release, apoptosis formation and caspase-3 activation (Fig. 4) could be inactivated by EGCG (Fig. 10). Thus, the anti-Hsp70 activity of EGCG may reinforce its own pro-apoptotic function at many stages.

**The extrinsic-stimulated pathway: EGCG and TRAIL-induced apoptosis**

Recently, Nishikawa et al. 47 determined that concurrent 24-h administration of 100–200 μM EGCG with 100 ng/ml TNF-related apoptosis-inducing-factor ligand (TRAIL) in a hepatocellular carcinoma cell line, dose-dependently induced significantly greater apoptosis than either treatment alone. Previously, Nishikawa et al. 47 had learned that 24-h 100–200 μM EGCG treatment alone inactivated nuclear-factor-kappa B (NF-κB), with subsequent down-regulation of Bcl-2 and Bcl-XL and activation of Bid, caspases-8, -9 and -3; they therefore suggested the synergistic effect was a consequence of this activity. Given that NF-κB is a transcription factor that partly regulates these proteins, 45 and that Bcl-2 and Bcl-XL have been shown to delay TRAIL-induced apoptosis, 48 this theory is logical.

There could be a further explanation for the combined effect. TRAIL induces apoptosis by binding receptors DR4 and DR5, however, Hsp70 can directly bind these receptors in response to TRAIL therapy 43 in a potentially ATPase domain-dependent manner (Fig. 4). It is possible that synergism was promoted by the binding of EGCG to Hsp70, thus allowing TRAIL to effectively bind its receptors. Given that increased expression of DR4 and DR5 could improve TRAIL efficacy in CRC cells, 48 it can be suggested by corollary that increased availability might have a comparable effect.

NF-κB inactivation by EGCG remains unexplained, 47 nevertheless, it is known that inactivation of NF-κB with concurrent activation of p53 induces apoptosis. 49 Hastak et al. 50 reported that EGCG indeed induced concurrent inactivation of NF-κB and activation of p53 in human prostate carcinoma cells, causing an apoptosis-inducing ratio of Bax/Bcl-2; like Nishikawa et al. 47 they found that apoptosis was mediated by activation of caspases-8, -9 and -3. So, NF-κB inactivation/p53 up-regulation may explain the Bcl-2 down-regulation/Bax up-regulation described earlier.

In conclusion, this study has shown not only that EGCG is capable of inducing apoptosis in a CRC known to over-express Hsp70, but further, has described a novel mechanism by which EGCG could promote apoptosis: by binding the Hsp70 ATPase domain, thus potentially inhibiting its anti-apoptotic function. Despite the limited bioavailability of EGCG, it is an easily available nutrient which could potentially change the outcome of CRC patients at both early and late stages of disease, either consumed alone, or as an adjunct to therapy. Collectively, this suggests that EGCG
remains an interesting research focus, and that further investigation into its intracellular effect on Hsp70 is justified.

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