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Epigallocatechin-3-gallate induces cell death in acute myeloid leukaemia cells and supports all-*trans* retinoic acid-induced neutrophil differentiation via death-associated protein kinase 2

Adrian Britschgi,[1] Hans-Uwe Simon,[2] Andreas Tobler,[1,3] Martin F. Fey[1,4] and Mario P. Tschan[1,4]

[1]Department of Clinical Research, Experimental Oncology/Haematology, [2]Institute of Pharmacology, University of Bern, [3]Department of Haematology, and [4]Department of Medical Oncology, Inselspital, Bern University Hospital, Bern, Switzerland

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Correspondence: Mario P. Tschan, PhD, Experimental Oncology/Haematology (MEM E829), Department of Clinical Research, University of Bern, Murtenstrasse 35, CH-3010 Bern, Switzerland.
E-mail: mtschan@dkf.unibe.ch

Summary

Acute promyelocytic leukaemia (APL) patients are successfully treated with all-*trans* retinoic acid (ATRA). However, concurrent chemotherapy is still necessary and less toxic therapeutic approaches are needed. Earlier studies suggested that in haematopoietic neoplasms, the green tea polyphenol epigallocatechin-3-gallate (EGCG) induces cell death without adversely affecting healthy cells. We aimed at deciphering the molecular mechanism of EGCG-induced cell death in acute myeloid leukaemia (AML). A significant increase of death-associated protein kinase 2 (*DAPK2*) levels was found in AML cells upon EGCG treatment paralleled by increased cell death that was significantly reduced upon silencing of *DAPK2*. Moreover, combined ATRA and EGCG treatment resulted in cooperative *DAPK2* induction and potentiated differentiation. EGCG toxicity of primary AML blasts correlated with 67 kDa laminin receptor (67LR) expression. Pretreatment of AML cells with ATRA, causing downregulation of 67LR, rendered these cells resistant to EGCG-mediated cell death. In summary, it was found that (i) *DAPK2* is essential for EGCG-induced cell death in AML cells, (ii) ATRA and EGCG cotreatment significantly boosted neutrophil differentiation, and 67LR expression correlates with susceptibility of AML cells to EGCG. We thus suggest that EGCG, by selectively targeting leukaemic cells, may improve differentiation therapies for APL and chemotherapy for other AML subtypes.

Keywords: death-associated protein kinase 2 gene, epigallocatechin-3-gallate, all-*trans* retinoic acid, 67-kDa laminin receptor, acute myeloid leukaemias.

Acute promyelocytic leukaemia (APL) is a subtype of acute myeloid leukaemias (AML) characterised by the *t*(15;17) translocation that fuses the *PML* to the *RARA* gene. APL cells fail to undergo terminal differentiation, but differentiation can be induced by all-*trans* retinoic acid (ATRA) treatment. In APL patients, ATRA therapy alone can induce remission, but because ATRA does not eradicate the malignant myeloid clone in APL, relapse is common (Zheng *et al*, 2007; Nasr *et al*, 2008). To improve long-term survival, ATRA is used in combination with chemotherapeutic agents such as daunorubicin, cytarabine (araC) or arsenic trioxide (As_2O_3). Other shortcomings of ATRA therapy include development of drug resistance and toxicity, particularly the ATRA syndrome (Tallman, 2007; Wang & Chen, 2008). This clearly underscores the need to identify new compounds that can enhance the efficacy of ATRA therapy.

The major constituent of green tea, the polyphenol epigallocatechin-3-gallate (EGCG), possesses remarkable cancer chemopreventive and therapeutic potential against various cancer types. Thirty are phase I and phase II clinical trials are currently running or in preparation in the USA which underpin its potential value as chemotherapeutic agent (Kawasaki *et al*, 2008). EGCG targets multiple signalling pathways resulting in induction of apoptosis and cell cycle arrest; it inhibits proteasomal activity, induces reactive oxygen species and has demethylating activities (for reviews see (Khan *et al*, 2006; Khan & Mukhtar, 2008)). Most interestingly, EGCG specifically causes death in tumour cells

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but spares normal cells (Ahmad *et al*, 1997, 2000; Chen *et al*, 1998). Moreover, EGCG has been extensively evaluated for its safety (Chow *et al*, 2003) and recently, green tea extract (Veregen®, PharmaDerm, Florham Park, NJ, USA) has been approved by the US Federal Drug Administration as the first botanical drug in the USA (Wu *et al*, 2008). EGCG inhibits proliferation of AML cell lines and primary AML blast cells but does not affect the colony-forming capacity of normal haematopoietic progenitor cells (Otsuka *et al*, 1998). Moreover, EGCG-induced apoptosis of myeloid leukaemic cells *in vivo* was shown in a non-obese diabetic severe combined immunodeficient mouse model (Nakazato *et al*, 2005). To date, the molecular mechanisms of EGCG-induced apoptosis of leukaemic cells are not fully understood. The identification of molecular targets and biomarkers of EGCG-treatment are essential for improving the design of clinical trials and would greatly assist in better understanding of the mechanisms underlying its anti-cancer activity. Recently, it was found that EGCG strongly induced the death-associated protein kinase 2 gene (*DAPK2*) in multiple myeloma cells (Shammas *et al*, 2006). We previously reported that expression of *DAPK2* enhances ATRA-induced neutrophil differentiation of myeloid leukaemic cells (Rizzi *et al*, 2007) and have demonstrated its role in tumour-suppressive cell death responses (Britschgi *et al*, 2008). Furthermore, EGCG concentrations used to kill multiple myeloma cells had no effect on survival of normal fibroblasts and peripheral blood mononuclear cells (PBMC) from healthy donors. The authors further suggested that specific killing of multiple myeloma cells may be linked to a high expression of the 67-kDa laminin receptor (67LR) (Shammas *et al*, 2006), which has been shown to be a cell surface receptor for EGCG binding (Tachibana *et al*, 2004). The 67LR is a non-integrin laminin receptor and known to be overexpressed on the cell surface of various tumour cells. The expression level of this protein strongly correlates with the risk of tumour invasion and metastasis (Sylvie Ménard *et al*, 1997). Interestingly, 67LR protein expression was also found in 40% of *de novo* AMLs but not in chronic lymphocytic leukaemia (CLL), chronic myeloid leukaemia or in normal bone marrow haematopoietic cells indicating that EGCG might be effective in killing AML blast cells (Montuori *et al*, 1999).

These findings prompted us to investigate whether EGCG induces cell death in AML cells via *DAPK2*. We found that EGCG significantly induced *DAPK2* mRNA and protein in myeloid leukaemic cells paralleled by enhanced apoptosis. Moreover, we found significantly higher expression of several myeloid differentiation markers upon combination of ATRA with EGCG as compared to ATRA treatment alone. We further provide evidence that this enhancement of ATRA-induced neutrophil differentiation is dependent on *DAPK2* and that it is due to preferred killing of leukaemic blast cells expressing 67LR. Lastly, we investigated possible correlations between 67LR expression on primary AML cells and sensitivity to EGCG-induced cell death.

Materials and methods

Cell culture and drug treatment

The human AML cell lines, HL60 and NB4, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The retinoic-acid resistant NB4-R2 and HL60-R411 cell lines were kindly provided by B. E. Torbett (The Scripps Research Institute, La Jolla, CA, USA). All cell lines were maintained in RPMI-1640 medium with 10% foetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin in a 5% CO_2-95% air humified atmosphere at 37°C.

Isolation of PBMCs, granulocytes and fresh leukaemic blast cells from untreated AML patients has been described (Tschan *et al*, 2001). For short-term cultures, primary cells were maintained in RPMI-1640 medium with 20% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin in a 5% CO_2-95% air humified atmosphere at 37°C. Protocols and the use of all human samples were approved by the Cantonal Ethical Committee at the Inselspital.

For differentiation experiments, NB4 and HL60 cells were seeded at a density of $0{\cdot}1 \times 10^6$/ml and treated with 1 μmol/l ATRA. Successful neutrophil differentiation was assessed morphologically at day 4 and 6 using May-Grünwald-Giemsa staining (Merck, Darmstadt, Germany). Morphological phenotypes were classified into three differentiation classes: promyelocytes/myelocytes, metamyelocytes and banded/segmented neutrophils. At least 100 cells per slide and treatment were analysed. In addition, expression of the differentiation markers CD11b and CD15 was determined by flow cytometric analysis (Becton Dickinson, Basel, Switzerland).

(−)-Epigallocatechin-3-gallate (E4143; Sigma-Aldrich, Buchs, Switzerland) was dissolved in phosphate-buffered saline (PBS; 2 μg/μl) and added to the culture medium in single doses as indicated. For the combined treatment with ATRA, EGCG was added 6 and 24 h after seeding of HL60 and NB4 cells, respectively.

Real-time quantitative reverse transcription-PCR (RQ-PCR)

Total RNA was extracted using the RNeasy Mini Kit and the RNase-Free DNase Set according to the manufacturer's protocol (Qiagen, Hombrechtikon, Switzerland). Total RNA was reverse transcribed using random primers (Roche Diagnostics) and Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA). PCR and fluorescence detection were performed using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland).

For quantification of *DAPK2*, *CEBPE* (CCAAT/enhancer binding protein ε) and *CSF3R* (G-CSFR) mRNA Taqman® Gene Expression Assays Hs00204888_m1, Hs00357657_m1 and Hs00167918_m1, (Applied Biosystems) were used, respectively. *HMBS* primers and probes have been described previously

(Britschgi *et al*, 2005). N-fold changes were calculated using the ΔΔCt method of relative quantification (Livak & Schmittgen, 2001).

Cell lysate preparation and western blotting

Whole cell extracts were prepared using radioimmunoprecipitation assay lysis buffer supplemented with 8 mol/l UREA as described earlier (Britschgi *et al*, 2008). Primary antibodies used were anti-DAPK2 (2323; ProSci, Poway, CA, USA), anti-actin (Sigma-Aldrich) and anti-67LR (MLuC5; Labvision/Thermo Fisher Scientific, Fremont, CA, USA). Secondary antibodies used were donkey anti-rabbit and sheep anti-mouse horseradish peroxidase (HRP)-conjugated IgG (Amersham, Zurich, Switzerland). For 67LR, anti-mouse Ig Biotin-conjugated and a Streptavidin-HRP-conjugate were used as a secondary detection system (Amersham, Zurich, Switzerland).

Assays for cell viability, cell cycle and apoptosis

Leukaemic cells were seeded at a density of $0{\cdot}1 \times 10^6$/ml, then treated with single doses of EGCG and assessed for cell viability 24–48 h later. For synchronisation cells were seeded at a density of $0{\cdot}5 \times 10^6$/ml and serum-starved for 6 h to induce cell cycle arrest prior to EGCG treatment. For XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assays (Biological Industries, Kibbutz Beit Haemek, Israel) cells were seeded in 96-wells in triplicate, incubated as indicated by the manufacturer, and absorbance was measured at 500 nm. For Annexin V staining, $0{\cdot}5 \times 10^6$ cells were washed with cold PBS/5% bovine serum albumin, resuspended in 70 μl binding buffer and labelled with phycoerythrin (PE)-labelled antibody against Annexin V according to the manufacturer's protocol (Bio-Vision, Mountain View, CA, USA). For cell cycle analysis, 1×10^6 cells were washed in PBS, fixed in 70% Ethanol for 60 min at 4°C, washed twice and resuspended in propidium iodide buffer (PBS supplemented with 50 μg/ml propidium iodide, 10 μg/ml RNAse A, 0·1% sodium citrate and 0·1% Triton X-100). At least 10^4 cells per sample were analysed with a FACScan flow cytometer (Becton Dickinson, Basel, Switzerland). Caspase 3/7 activation was measured using Caspase-Glo™ 3/7 Assay according to the manufacturer's protocol (Promega Corporation).

Lentiviral knockdown vectors

Lentiviral vectors expressing shRNAs targeting *DAPK2* were purchased from Sigma-Aldrich (SHGLY-NM_014326). Lentivirus production, transduction and selection were done as described (Britschgi *et al*, 2008).

67LR antibody inhibition experiments and flow cytometry

Cells were pre-incubated for 1 h with 0·1, 1·0, 5·0 or 10 μg/ml of the monoclonal mouse anti-67LR (MLuC5; Labvision/Thermo Fisher Scientific, Fremont, CA,USA) or the same amounts of the control antibody mouse anti-IgM (Amersham, Zurich, Switzerland). Single doses of EGCG were added (0–30 μmol/l) and survival was measured 36 h later using the XTT assay. To detect 67LR protein expression on ATRA-differentiated NB4 cells, $0{\cdot}5 \times 10^6$ cells were washed with cold PBS/2%FCS, incubated with 2 μg/ml of anti-67LR for 30 min and stained with 1 μg/ml mouse anti IgM-PE conjugated for 20 min. At least 10^4 cells per sample were analysed with a FACScan flow cytometer (Becton Dickinson, Basel, Switzerland).

Statistical analysis

Each value reported represents the mean ± standard deviation (SD) of at least four measurements of at least two independent experiments. Nonparametric Mann–Whitney*U*-tests were applied to compare the difference between two groups using the programme JMP4 (SAS, Cary, NC, USA). *P*-values <0·05 were considered to be statistically significant.

Results

EGCG induces cell death in myeloid leukaemic cells paralleled by upregulation of DAPK2

We first investigated the effects of EGCG on cell survival and *DAPK2* expression in HL60 and NB4 acute myeloid leukaemic (AML) cell lines. EGCG reduced viability in both cell lines in a dose-dependent manner with HL60 cells being more sensitive [50% inhibitory concentration (IC_{50}) = 21·5 μmol/l] to EGCG treatment than NB4 cells (IC_{50} = 30·5 μmol/l) (Fig 1A). Reduced viability measured by XTT assays was not due to increased G_0/G_1 or G_2 cell cycle arrest, but EGCG was found to induce DNA-fragmentation as evidenced by cell cycle analysis. The Sub-G_0 DNA content increased from 0·3 to 26·9% in HL60 and from 0·5 to 18·4% in NB4 cells treated with 20 μmol/l (Figure S1). A dose-dependent activation of apoptosis as measured by Annexin V staining and effector caspase 3/7 activity was seen (Fig 1B–C). In parallel, both cell lines showed a dose-dependent increase of *DAPK2* mRNA and DAPK2 protein (Fig 1D–E). These results showed that EGCG-induced cell death in HL60 and NB4 cells is accompanied by an increase of *DAPK2* message and DAPK2 protein.

DAPK2 knockdown markedly rescued myeloid leukaemic cells from EGCG-induced cell death

To examine whether *DAPK2* is essential for EGCG-induced cell death in myeloid leukaemic cells, we used lentiviral vectors to deliver short hairpin (sh) RNAs targeting *DAPK2* in HL60 and NB4 cells. Knockdown efficiency upon EGCG treatment was assessed by Western blotting (Fig 2A). Silencing of *DAPK2* significantly increased viability of two different HL60 and NB4 *DAPK2* knockdown cell lines compared to non-targeting shRNA

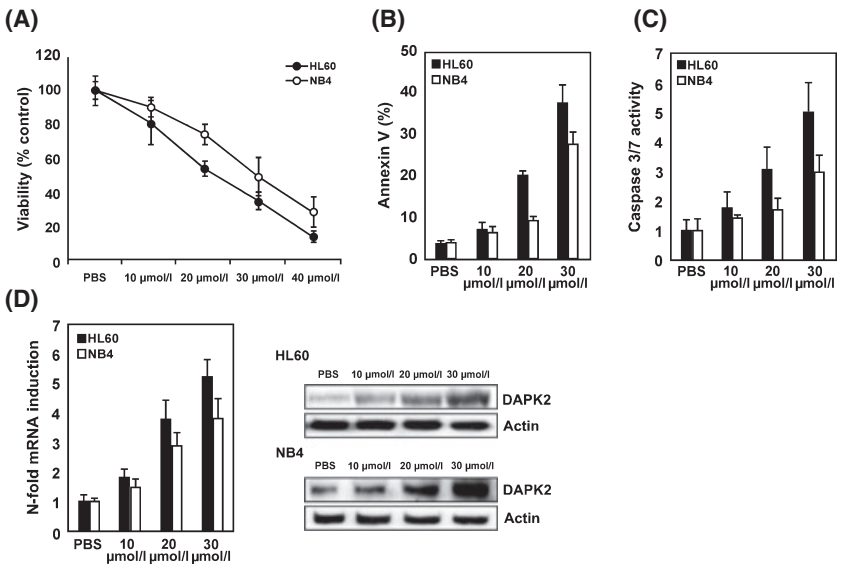

Fig 1. EGCG-induced cell death is paralleled by DAPK2 upregulation in myeloid leukaemic cells. (A) 36 h of EGCG treatment (0–40 µmol/l) dose-dependently decreased survival of HL60 (IC_{50} = 21·5 µmol/l) and NB4 (IC_{50} = 30·5 µmol/l) acute myeloid leukaemia (AML) cells as measured by XTT assay. Mean values of three independent experiments ±SD are given relative to solvent-treated controls. (B) Fractions of Annexin V-positive NB4 and HL60 cells 24 h after EGCG treatment. Mean values of two independent experiments ±SD are given. (C) 24 h of 0–30 µmol/l EGCG treatment dose–dependently increased activity of caspase 3 and 7 in NB4 and HL60 cells as measured by a luminescence-based assay. Mean values of three independent experiments ±SD are given relative to solvent-treated controls. (D) Dose-dependent upregulation of *DAPK2* mRNA after 24 h of EGCG-treatment in NB4 and HL60 cells. Mean values of three independent experiments ± SD are given relative to the untreated control. (E) Upregulation of DAPK2 protein in EGCG-treated NB4 and HL60 cells. Proteins were extracted 24 h after treatment. The experiment was repeated three times; a representative Western blot is shown. Actin was used as a loading control.

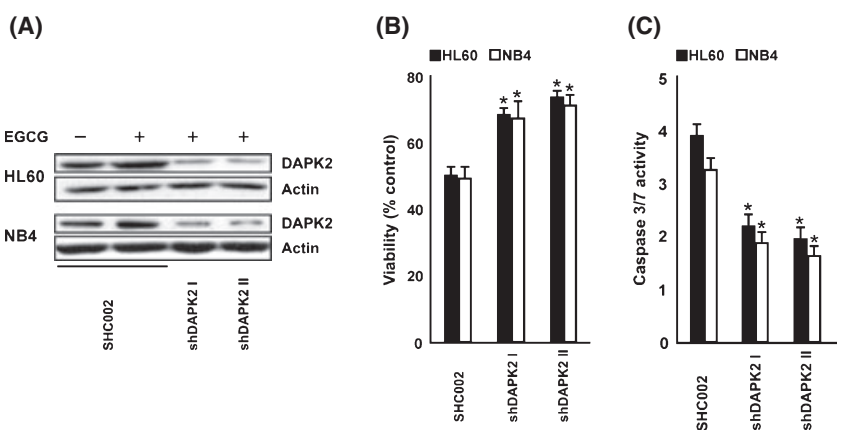

Fig 2. DAPK2 knock down in myeloid leukaemic cells attenuates EGCG-induced cell death. (A) Western blots of NB4 and HL60 scramble control (SHC002) and *DAPK2* knockdown (shDAPK I and shDAPK2 II) cell lines showing efficient *DAPK2* inhibition in EGCG-treated cells. Proteins were extracted 24 h after treatment. The experiment was repeated three times; a representative Western blot is shown. Actin was used as a loading control. (B) XTT assay measuring survival of control and two *DAPK2* knock down NB4 and HL60 cell lines upon EGCG-treatment, respectively. HL60 cells were treated with 20 µmol/l, NB4 cells with 30 µmol/l of EGCG. Viability was assessed 36 h after treatment. Mean values of three independent experiments ±SD are given relative to solvent-treated controls (*$P < 0{\cdot}05$). (C) Caspase 3 and 7 activities of control (SHC002) and two *DAPK2* knockdown (shDAPK I and shDAPK2 II) NB4 and HL60 cell lines 24 h after EGCG treatment were measured using a luminescence-based assay. Mean values of three independent experiments ±SD are given relative to solvent-treated controls (*$P < 0{\cdot}05$).

expressing control cells as measured by XTT assay. Viability of HL60 cells upon EGCG treatment significantly increased from 50·5% to 68·7% and 73·7% in the two HL60 *DAPK2* knockdown lines, respectively. Silencing of *DAPK2* in NB4 cells increased cell viability of EGCG treated cells from 49·5% to 67·6% and 71·4% in the two knockdown lines, respectively (Fig 2B). In addition, apoptosis as measured by caspase 3/7 activity was significantly reduced in *DAPK2* knockdown HL60 and NB4 cells upon EGCG treatment (Fig 2C). To conclude, our knockdown experiments

identified *DAPK2* as a crucial effector of EGCG-induced cell death.

Combined treatment of myeloid leukaemic cells with ATRA and EGCG enhances neutrophil differentiation

Given the role of *DAPK2* in EGCG-induced cell death and in enhancing neutrophil differentiation (Rizzi *et al*, 2007), we wondered whether a combination of ATRA and EGCG would boost neutrophil differentiation. In a first experiment, we tested if the combined addition of ATRA and EGCG to HL60 cells would lead to higher induction of DAPK2 protein than ATRA addition alone. We found that EGCG synergised with ATRA to induce *DAPK2* mRNA and DAPK2 protein in a dose-dependent manner (Fig 3A). Next, we investigated the effect of combined ATRA and EGCG treatment on neutrophil differentiation measuring CD11b and CD15 surface expression by flow cytometry. Treatment of HL60 cells with ATRA led to a 9·9-fold ± 0·9 increase in CD11b expression compared to non-treated cells after 4 d while treatment with EGCG resulted in an 5·7 ± 1·4-fold increase (Fig 3B and data not shown). The combination of ATRA and EGCG, however, yielded a significantly higher 54·1 ± 1·5-fold increase in CD11b-positivity. Similarly, the combination of ATRA and EGCG resulted in a 7·6 ± 0·6-fold induction of CD15 as compared to a 4·5 ± 0·1-fold induction with ATRA alone or a 2·1 ± 0·5-fold induction with EGCG alone (Fig 3B and data not shown). Similar results were obtained when NB4 cells were cotreated with ATRA and EGCG (data not shown). Enhanced ATRA-induced granulocytic differentiation of HL60 cells upon ATRA/EGCG combination treatment was further shown by significantly increased mRNA levels of key regulator genes for terminal neutrophil differentiation, such as *CEBPE* and the *CSF3R*, compared to ATRA only treated cells (Fig 3C). Lastly, morphological analysis confirmed the previous findings. The combination treatment reduced numbers of undifferentiated promyelocytes and myelocytes while increasing the proportion of differentiated cells (Fig 3D). Taken together, our results demonstrate that EGCG potentiated ATRA-induced activation of *DAPK2* paralleled by enhanced granulocytic maturation.

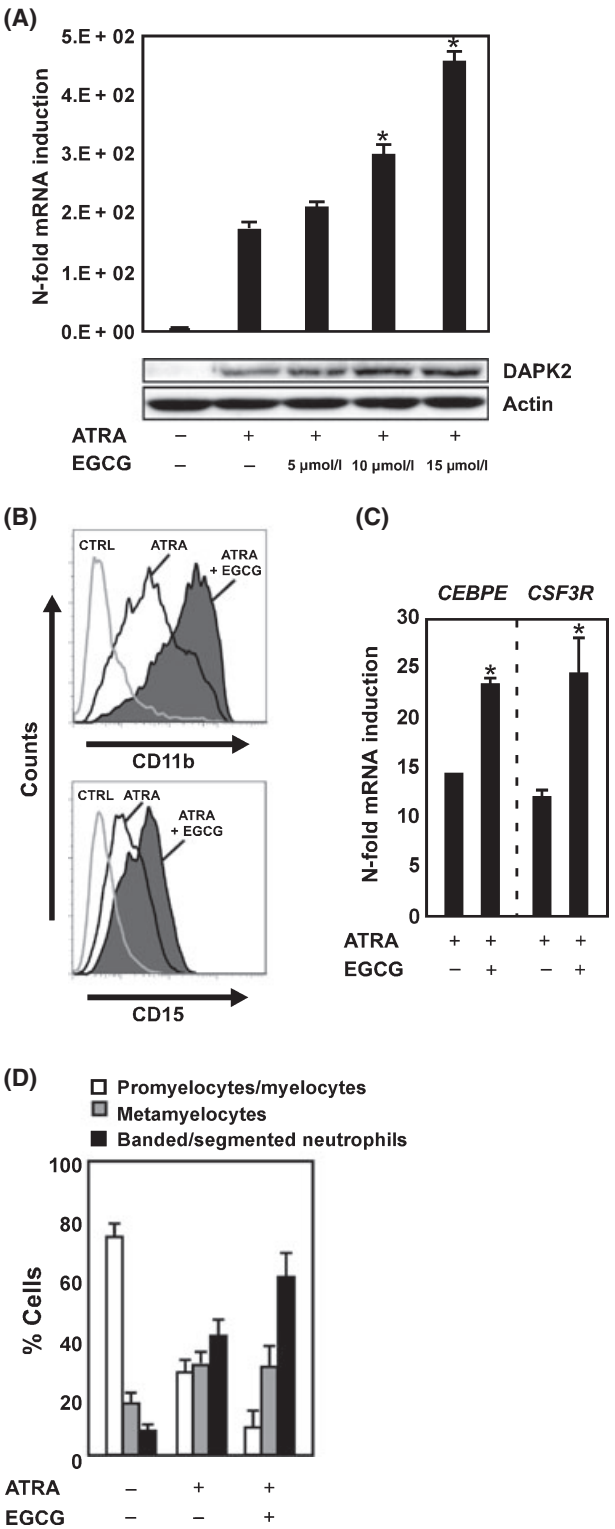

Fig 3. Combined EGCG and ATRA treatment synergistically induces DAPK2 expression and enhances neutrophil differentiation. (A) Synergistic upregulation of *DAPK2* mRNA (upper panel) and DAPK2 protein (lower panel) in HL60 cells after 4 d of 1 µmol/l ATRA-treatment in combination with 0–15 µmol/l of EGCG. Mean values of three independent experiments ±SD are given relative to solvent-treated controls (*$P < 0{\cdot}05$). A representative Western blot is shown. Actin was used as a loading control. (B) Flow cytometry showing upregulation of the lineage-specific differentiation markers CD11b and CD15 in HL60 cells treated with 1 µmol/l ATRA alone or the combination of 1 µmol/l ATRA and 15 µmol/l EGCG (*$P < 0{\cdot}05$). (C) Quantitative RT-PCR of two neutrophil differentiation markers (*CEBPE* and *CSF3R*) after 4 d of 1 µmol/l ATRA alone or the combination of 1 µmol/l ATRA and 15 µmol/l EGCG. Mean values of three independent experiments ±SD are given relative to solvent-treated controls (*$P < 0{\cdot}05$). (D) Morphological analysis of ATRA- and ATRA/EGCG-treated HL60 cells after 6 d of differentiation. Mean values of two independent experiments ±SD are given relative to solvent-treated controls.

DAPK2 knockdown abrogates enhanced neutrophil differentiation upon combined ATRA and EGCG treatment

In order to analyse whether *DAPK2* is functionally involved in the ability of EGCG to potentiate ATRA-induced neutrophil differentiation, we treated two HL60 DAPK2 knockdown lines with ATRA alone or in combination with EGCG and measured myeloid differentiation. We first measured *DAPK2* knockdown efficiency upon ATRA and ATRA/EGCG treatment by Western blotting. Both shRNAs targeting *DAPK2* were able to inhibit DAPK2 protein during ATRA-induced neutrophil differentiation (Fig 4A). Silencing of *DAPK2* resulted in significantly decreased surface expression of the myeloid differentiation markers CD11b and CD15 upon EGCG/ATRA treatment, from 56·4- to 15·7/20·7-fold and from 9·3- to 4·6/4·7-fold in control *versus DAPK2* knockdown HL60 cells, respectively (Fig 4B). In support of these findings, induction of the neutrophil marker *CEBPE* was reduced from 22·8- to 12·6- and 12·0-fold in two *DAPK2* HL60 knockdown lines compared to control cells, respectively. Similarly, induction of *CSF3R* mRNA was reduced from 28·5- to 12·0- and 8·7-fold, respectively (Fig 4C). Thus, DAPK2 is needed for the synergistic effect of EGCG and ATRA treatment on neutrophil differentiation.

EGCG targets ATRA-resistant, cycling and undifferentiated leukaemic cells expressing 67LR

We next investigated whether EGCG efficiently killed primary AML blast cells, and if the cell death response would correlate with expression of the 67-kDa laminin receptor (67LR), as 67LR was recently identified as a surface receptor for EGCG (Tachibana *et al*, 2004; Umeda *et al*, 2008). As shown in Fig 5A (upper panel), granulocytes and PBMC from healthy donors were not affected by EGCG while primary AML patient blasts showed varying degrees of cell death. Moreover, susceptibility of AML patient samples, normal granulocytes and PBMCs was associated with 67LR expression (Fig 5A, lower panel). To further show an association of 67LR protein expression and susceptibility to EGCG, HL60 and NB4 cells were pre-treated with ATRA before adding EGCG, because it was previously reported that 67LR levels decrease during ATRA-induced neutrophil differentiation of HL60 cells (Montuori *et al*, 1999). Indeed, ATRA-pre-treatment rendered these cells more resistant to EGCG (Fig 5B) indicating that differentiated cells were protected from EGCG-induced cell death. Accordingly, we found downregulation of 67LR in NB4 and HL60 cells upon ATRA-treatment, but not in the ATRA-resistant sublines HL60-R and NB4-R (Fig 5C and Figure S2). EGCG treatment reduced survival of these cells similarly to the parental cell lines (Fig 5D). Further, proliferating leukaemic cells were more susceptible to EGCG treatment (IC_{50} value for HL60 and NB4 was 21·5 and 30·5 μmol/l respectively) than cells arrested in the G_1/G_0 phase of the cell cycle (IC_{50} values

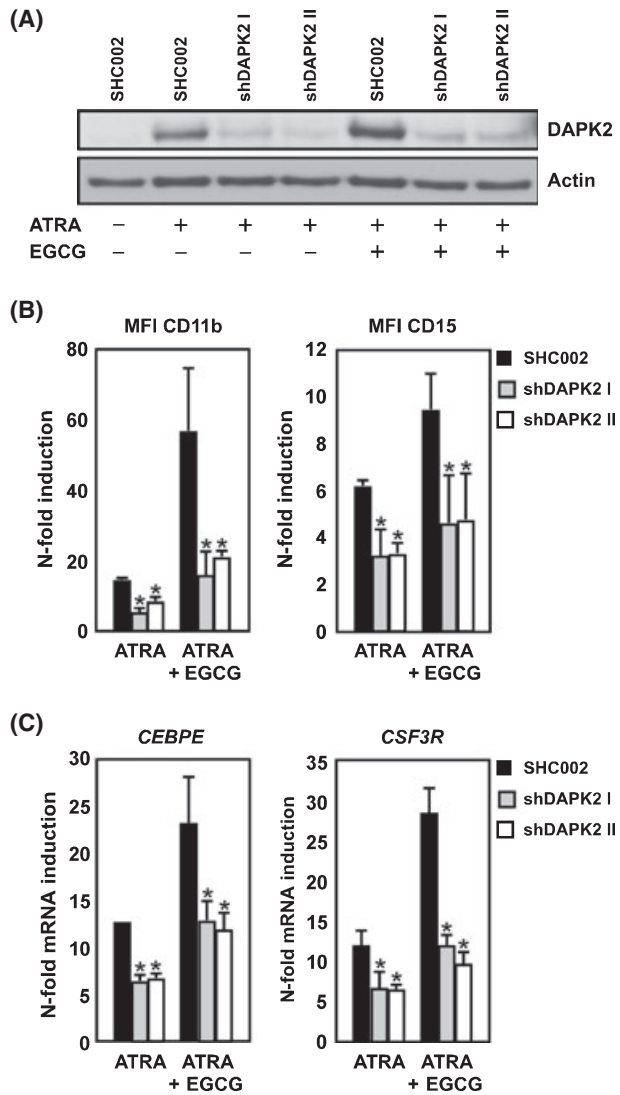

Fig 4. Knocking down DAPK2 abrogates enhanced differentiation upon combined EGCG/ATRA treatment. (A) Western blot analysis showing synergistic upregulation and knockdown efficiency of *DAPK2* in HL60 SHC002 control and two *DAPK2* knockdown cell lines respectively, after 4 d of 1 μmol/l ATRA in combination with solvent or 15 μmol/l EGCG treatment. A representative Western blot of three experiments is shown. Actin was used as a loading control. (B) Flow cytometry of the lineage-specific differentiation markers CD11b and CD15 in HL60 control and *DAPK2* knockdown cells treated as in (A). Means of median fluorescence intensities of three independent experiments ±SD are given relative to solvent-treated controls (*$P < 0{\cdot}05$). (C) Quantitative RT-PCR of two neutrophil differentiation markers, *CEBPE* and *CSF3R*, measured in HL60 control and two *DAPK2* knockdown cell lines treated as in (A). Mean values of three independent experiments ±SD are given relative to undifferentiated, DMSO-treated controls (*$P < 0{\cdot}05$).

68·8 and 78·1 μmol/l) (Figure S3). To confirm a role for 67LR in EGCG killing of AML cells, we performed antibody blocking experiments. As shown in Fig 5E, both HL60 and NB4 cells were significantly protected from EGCG-induced cell death when 67LR was blocked by a monoclonal antibody compared

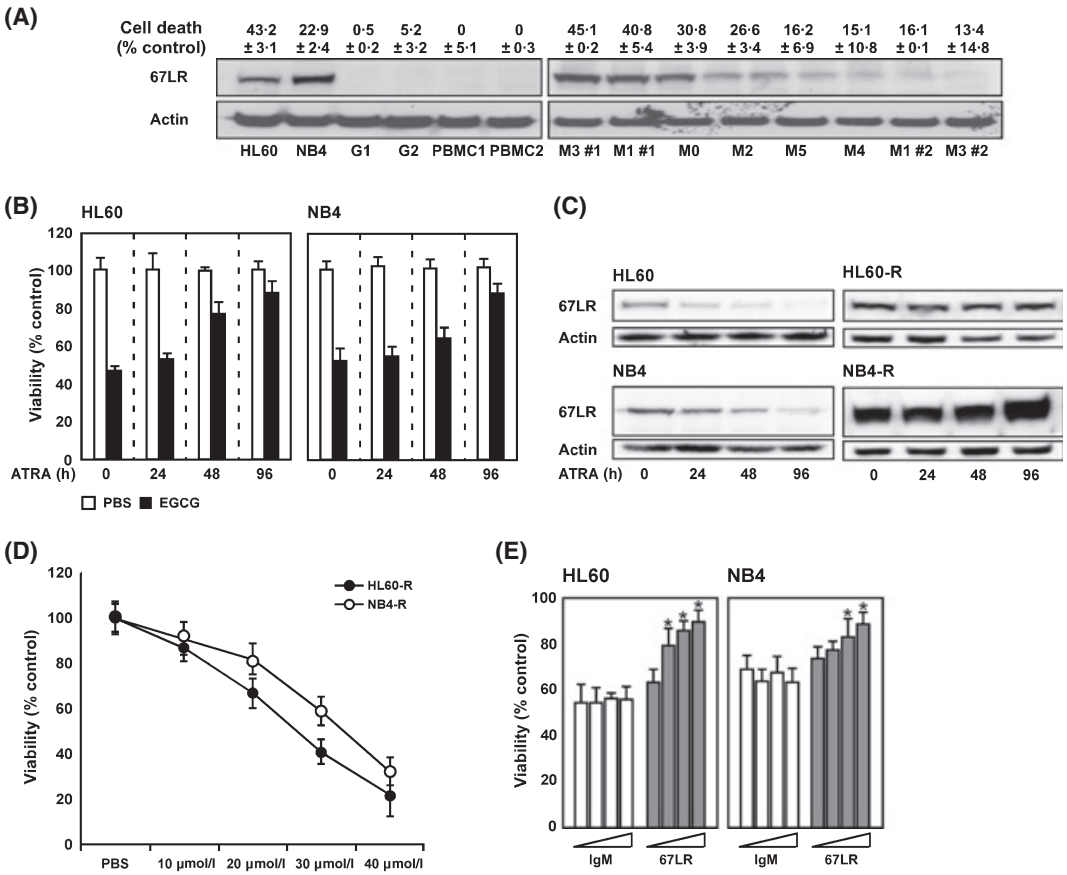

Fig 5. EGCG-induced cell death in ATRA-resistant and undifferentiated myeloid leukaemic cells expressing 67 kD laminin receptor (67LR). (A) Primary acute myeloid leukaemia (AML) blast cells, granulocytes and PBMCs from healthy donors were treated with 20 µmol/l of EGCG. 24 h later, viability relative to the PBS-treated controls was measured with XTT assay. The French-American-British-subtypes of patient samples are indicated. Means ± SD of two independent cell death measurements are given. Western blots were applied to assess the 67LR protein expression in AML samples and normal controls. Actin is shown as a loading control. (B) HL60 and NB4 cells were pre-treated with 1 µmol/l ATRA for the number of hours indicated, then single doses of 20 µmol/l (HL60) or 30 µmol/l (NB4) of EGCG were added and viability was measured 24 h later by XTT assay. Mean values of two independent experiments ±SD are given relative to the PBS-treated controls. (C) Western blot analysis of 67LR expression in HL60 and NB4 cells as well as in their ATRA-resistant counterparts treated with 1 µmol/l ATRA for hours indicated. The experiment was repeated three times; a representative Western blot is shown. Actin was used as a loading control. (D) HL60 and NB4 cells were pre-incubated with 5 µg/ml of 67LR blocking antibody or an IgM control antibody, then treated with 20 µmol/l of EGCG. Viability was assessed by XTT assay 24 h later. Mean values ± SD of three independent experiments are given relative to PBS-treated controls (*$P < 0{\cdot}05$). (E) ATRA-resistant NB4-R and HL60-R sublines were treated with single doses of EGCG as indicated and their survival was measured 36 h later by XTT assay. Mean values of two independent experiments ±SD are given relative to PBS-treated controls.

to the IgM antibody controls. Thus, 67LR expression was clearly associated with sensitivity to EGCG-induced cell death in AML cells.

Discussion

Epigallocatechin-3-gallate (EGCG), the most abundant and biologically most active polyphenol in green tea (Khan *et al*, 2006; Khan & Mukhtar, 2008), is appreciated for its chemopreventive effects (Lambert & Yang, 2003; Surh, 2003). As a chemotherapeutic agent, it has been shown to be a potent inducer of cell death and cell cycle arrest in various cancer types *in vitro* and *in vivo* via different signalling pathways and mechanisms (Fang *et al*, 2003; Khan *et al*, 2006; Landis-Piwowar *et al*, 2008). In view of a therapeutic application, the tumoursuppressive functions of EGCG need further investigations. In leukaemia, EGCG has been shown to inhibit vascular endothelial growth factor signalling in CLL B cells leading to apoptosis (Lee *et al*, 2004). In T-cell acute lymphoblastic leukaemia Jurkat cells, EGCG induced apoptosis via production of hydrogen peroxide (Nakagawa *et al*, 2004) and by binding to and inhibiting the Zap70 kinase (Shim *et al*, 2008). The present study established a role for the Death-associated protein kinase 2 gene (*DAPK2*) in EGCG-induced cell death of AML cells. *DAPK2* has been shown to be a positive mediator of cell death in various solid cancers and also in non-transformed cells (Bialik & Kimchi, 2006; Gozuacik & Kimchi, 2006;

Britschgi *et al*, 2008). We now showed that upregulation of endogenous *DAPK2* through EGCG causes apoptosis in myeloid leukaemic cells. Inhibition of *DAPK2* led to a significant decrease in cell death, but did not completely rescue the leukaemic cells from EGCG-induced apoptosis, suggesting the presence of other signalling pathways contributing to overall cell death. As an example, Nakazato *et al* (2005, 2007) established a role for reactive oxygen species (ROS) and myeloperoxidase (*MPO*) in EGCG-induced apoptosis of myeloid leukaemic cells.

There is increasing awareness of the fact that cancer preventive agents could enhance the efficacy of cancer therapeutics during treatment (Sarkar & Li, 2006). Resveratrol, a polyphenol found in grapes, was shown to enhance ATRA differentiation in APL cell lines and patient samples (Asou *et al*, 2002). We found that co-treatment of AML cells with ATRA and EGCG greatly increased neutrophil differentiation via *DAPK2*. In a previous study, we established a role for *DAPK2* in neutrophilic differentiation, but it should be pointed out that *DAPK2* overexpression neither increased cell death nor cell cycle arrest and the precise role of DAPK2 during differentiation remains unknown (Rizzi *et al*, 2007). We have now shown that *DAPK2* is capable of inducing cell death in leukaemic blasts in response to EGCG treatment. Thus it is tempting to assume a dual function for *DAPK2*: as a mediator of cell death in immature blasts and as an enhancer of differentiation in maturing AML cells. This assumption begs the question of how and under which circumstances *DAPK2* could function in cell death response or differentiation. Different binding partners might explain its different functions, and thus we suggest that identification of interaction partners of *DAPK2* is a critical issue to be addressed in future studies.

EGCG treatment alone upregulated the expression of some differentiation markers and differentiation-inducing genes (e.g. 8·5-fold increase in CD11b, 2·1-fold in CD15, 1·3-fold in *CEBPE* and *CSF3R* expression levels), but the enhancing effects of co-treatment suggested additional mechanisms. We found that ATRA-resistant cells were killed by EGCG and that cycling cells and undifferentiated cells were preferentially targeted compared to differentiated cells, which are arrested at G_1 and G_2 phases of the cell cycle. These observations provide a possible explanation for the more differentiated phenotype of the co-treated cell population by EGCG-induced, selective killing of immature blasts. Additionally, EGCG killing was closely associated with expression of the 67 kDa laminin receptor (67LR) in AML patient samples and differentiating AML cells, while normal controls did not express the receptor. 67LR is a critical cell-surface mediator for EGCG killing (Tachibana *et al*, 2004; Shammas *et al*, 2006; Umeda *et al*, 2008). Moreover, 67LR is overexpressed in 40% of *de novo* AML but is undetectable in normal bone marrow haematopoietic cells (Montuori *et al*, 1999), which only upregulate the receptor in order to egress from the bone marrow (Selleri *et al*, 2006). By blocking the receptor, we found that AML cells were significantly protected from EGCG-induced cell death. Thus, low 67LR expression in normal cells and its overexpression in transformed cells could not only be of use as a predictive marker for efficacy of EGCG treatment, but moreover provide a molecular explanation for the specific activity of EGCG on cancer cells. Interestingly, recent studies found high 67LR expression on $CD34^+$ AML cells compared to normal $CD34^+$ cells and could show that high 67LR expression was related to high granulocyte-macrophage colony-stimulating factor receptor expression and phosphorylated-STAT5 activities, possibly contributing to a poor prognosis of these AML patients (Koji *et al*, 2008). One might speculate that these patients with high 67LR expression – suffering from a highly proliferative and apoptosis-resistant blast cell population – would most efficiently benefit from EGCG treatment.

It remains to be determined whether the observed mechanisms and effects of EGCG on AML blasts can be confirmed *in vivo*. Limited bioavailability of EGCG will certainly pose a problem (Henning *et al*, 2005; Feng, 2006) and high doses might be needed for treatment. Nevertheless, high doses of concentrated EGCG have been tolerated in different animal models without adverse effects (Lu *et al*, 2002; Isbrucker *et al*, 2006). Of note, the latter studies used a regimen of multiple high dosage administration of EGCG over several weeks, while single high dose treatments, presumably less detrimental, have not been assessed for their toxicological effects so far. Because of the great general interest in EGCG as a potential therapeutic not only in cancer treatment, it has also been well evaluated for its safe administration in humans (Chow *et al*, 2003). Furthermore, green tea polyphenol derivates of improved stability and bioavailability have been synthesised (Huo *et al*, 2008) and EGCG encapsulated in nanoparticles has been shown to have a 10-fold lower IC_{50} value than non-encapsulated EGCG (Siddiqui *et al*, 2008).

To summarise, we demonstrated that EGCG significantly enhances ATRA-induced differentiation of myeloid cell lines most likely by targeting resistant, undifferentiated and cycling cells. High expression of the cell-surface 67LR can partly explain the selectivity of EGCG for immature blasts. Additionally, we found that *DAPK2* is a key player in both cell death response and enhancement of neutrophil differentiation via EGCG. We propose that EGCG represents a novel, effective and selective drug in leukaemia treatment. EGCG has the potential to improve ATRA-based differentiation therapy of APL patients, especially in refractory APL and patients developing the ATRA-syndrome. Moreover, 67LR-positive AML patients of any subtype shown to have a poor clinical outcome could benefit from a combined chemotherapy and EGCG regimen specifically targeting this cell surface receptor. Thus, besides providing the proof of principle for the use of EGCG in the treatment of AML, our data also suggests that the 67LR could represent a cell surface marker predicting responsiveness to treatment.

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Authors' contributions

AB designed and performed the experimental research, interpreted the data and drafted the article. HUS, AT and MFF instigated the experimental design and revised the drafted article. MPT designed the project, analysed data, wrote the manuscript and gave final approval of the submitted manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig S1. EGCG induces cell death, but not cell cycle arrest in myeloid leukemic cells.

Fig S2. ATRA-induced 67LR downregulation in parental but not ATRA-resistant NB4 cells.

Fig S3. Proliferating AML cells are preferentially targeted by EGCG.

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