

Extended treatment with physiological concentrations of dietary phytochemicals results in altered gene expression, reduced growth and apoptosis of cancer cells.

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Abbreviations: 5-Aza-dC, 5-Aza-2'-deoxycytidine; DIM, 3,3'-diindolylmethane; EGCG, epigallocatechin gallate (EGCG); EMT, epithelial-mesenchymal transition; ER, estrogen receptor; IL-6, interleukin-6; I3C, indole-3-carbinol; TSA, trichostatin A; uPA, urokinase plasminogen activator.

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Abstract

Dietary phytochemicals exhibit chemopreventive potential *in vivo* through persistent low dose exposures, whereas mechanistic *in vitro* studies with these agents generally employ a high dose single treatment. Since the latter approach is not representative of an *in vivo* steady state, we investigated anti-tumor activity of curcumin, 3,3'-diindolylmethane (DIM), epigallocatechin gallate (EGCG), genistein or indole-3-carbinol (I3C) in breast cancer MDA-MB-231 cells, exposed in long-term culture to low concentrations, achievable *in vivo*. Curcumin and EGCG increased cell doubling time. Curcumin, EGCG and I3C inhibited clonogenic growth by 55-60% and increased 1.5-2 fold higher levels of the basal caspase-3/7 activity. No changes in expression of cell-cycle-related proteins or survivin were found; however, I3C reduced EGFR expression, contributing to apoptosis. Since some phytochemicals are shown to inhibit DNA and histone modification, modulation of expression by the agents in a set of genes (cadherin-11, p21Cip1, uPA, IL-6), was compared with changes induced by inhibitors of DNA methylation or histone deacetylation. The phytochemicals modified protein and/or RNA expression of these genes, with EGCG eliciting the least and DIM the most changes in gene expression. DIM and curcumin decreased cadherin-11 and increased uPA levels correlated with increased cell motility. Curcumin, DIM, EGCG and genistein reduced cell sensitivity to radiation-induced DNA damage without affecting DNA repair. This model has revealed that the basal line apoptosis is likely to be responsible for growth inhibition. It also implicated new molecular targets and activities of the agents, under conditions relevant to human exposure.

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Introduction

Epidemiological studies indicate that consumption of vegetables, containing dietary phytochemicals, reduces cancer risk [1]. Many dietary phytochemicals not only block development of tumors, but also inhibit metastatic growth in animal models (Supplementary Table 1). Potential use of dietary agents in combination therapies has been considered amongst novel treatment approaches, since combining phytochemicals with radio- and chemo-therapy improves outcome in animal models (Supplementary Table 1).

The majority of studies on these agents in cell culture employ short exposure times to high concentrations, often orders of magnitude greater than those achievable *in vivo*. Moreover, treatment with a single dose provides data on an acute induction response, whereas *in vivo* anti-cancer activity arises from a steady-state response to the continued presence of dietary agents. Therefore, many reported effects obtained in cell culture studies may be irrelevant for *in vivo* activity. All the chemopreventive phytochemicals induce cell cycle arrest and apoptosis in a variety of cancer cells, albeit often at non-physiological doses [2, 3]. Therefore, we investigated whether any positive anti-tumourigenic effects, e.g. growth, apoptosis and expression of selected biomarkers, could be detected following extended treatments with low doses. Furthermore, some of the agents inhibit DNA topoisomerases, DNA methyltransferase and histone-modifying enzymes (Supplement Table 2) thereby causing alterations in chromatin packaging and gene expression, which may also modulate response to DNA damage. Hence, we also investigated effect of the phytochemicals on cell response to radiation-induced DNA damage.

We used long-term culturing conditions with concentrations of agents which do not affect viability following a single treatment and are comparable with serum or tissue concentrations achievable *in vivo*. Concentrations of phytochemicals were 3 μ M

curcumin, 10 μ M DIM, 8 μ M EGCG, 2.5 μ M genistein and 30 μ M I3C. Comparable serum concentrations for curcumin (1.77 \pm 1.87 μ M), DIM (20 μ M), EGCG (0.29-7.7 μ M), genistein (2.5 \pm 1.6 μ M) and I3C (28 μ M) were detected in humans and/or animal models [4, 5].

Aggressively metastatic breast cancer cells MDA-MB-231 were chosen for this study to investigate whether the phytochemicals had any anti-cancer therapeutic potential in this model. These cells express ER β , known for particularly high binding affinity to genistein [6], and exhibit a basal-like subtype with epithelial-mesenchymal transition (EMT) to invasive mesenchymal phenotype [7]: epithelial genes, e.g. E-cadherin, are significantly downregulated by DNA methylation in these cells, whereas mesenchymal biomarkers, e.g. vimentin and cadherin-11, or prognostic metastatic biomarker uPA are markedly upregulated.

Methods

Cell culture

The human breast cancer cell line MDA-MB-231 was cultured in DMEM with 10% fetal bovine serum (FBS) as previously described [8]. Cells were grown in the presence of DMSO (0.1%) or phytochemicals with fresh medium replenished every 3-4 days. In some experiments MDA-MB-231 cells were treated with 5-Aza-2'-deoxycytidine (5-Aza-dC, Calbiochem) or trichostatin A (TSA, Sigma-Aldrich) or PD153035 (Calbiochem).

Cell growth and apoptosis

Growth at the end of the long-term treatments was estimated by plating $N_0=10^5$ cells and growing in the medium replenished every 3 days. The number of cells from each treatment was estimated as a percentage of the number in the DMSO control. The

number of cell generations (g) was counted according to the formula $g = \ln_2(N/N_0)$, where N is the final number of cells. The time of growth comprises the sum of the time for initial adhesion (considered here to be 5 hours), the time of complete division cycles (pxg) and a part of the unfinished last cycle ($=p/2$ on average), where p is one cell cycle duration. Hence, the time of growth is calculated according to the equation $T = 5 + pg + p/2$. Therefore, cell cycle duration (p) was estimated as $p = (T - 5)/(g + 0.5)$.

For the clonogenic assay, 300 cells were grown for further 14 days in the presence of each phytochemical with replenished medium. Colonies were washed, fixed and stained with crystal violet.

Background apoptotic activity of caspase3/7 was measured using Caspase-Glo-3/7 kit (Promega), as previously described [9].

Comet assay

Cells (2×10^5 /vial) in growth medium were irradiated with X-Ray doses of 5 and 10 Gy for the DNA damage study. For the repair study, cells were irradiated with 10 Gy, followed by incubation in medium at 37 °C with 5 % CO₂ for 0, 5, 15 and 30 min. Non-irradiated samples were also analyzed. The induction of DNA damage and the comet assay were performed as previously described [10]. Percentage of tail DNA was measured in two independent cell cultures and data from 200 cells per variant were pooled and presented as mean \pm SE.

Quantification of mRNA and protein expression

Total RNA isolation, cDNA synthesis and real-time PCR were performed as previously described [8]. Assays-on-demand gene expression kits (Applied Biosystems) were used to quantify mRNA levels for caspase-1, p21Cip1, Bcl-xL, E-cadherin, cadherin-11, uPA and IL-6 and 18S RNA. Non-muscle beta actin was quantified using Cybergreen master mix with the oligonucleotides (available on request) in parallel to 18S

RNA. The levels of gene expression were calculated by $\Delta\Delta\text{CT}$ method using 18S RNA as a reference.

Analysis of protein expression was performed using secondary HRP-conjugated antibody (Dako), detected with ECL (Amersham) or ECLplus (E-cadherin only), as previously described [8]. Alternatively, the secondary antibodies, labeled with fluorescent IR800 and IR680 (Li-Cor) dyes were detected using with the Odyssey system (Li-Cor). Antibodies used in this study were against: EGFR (Biosource), β -actin (polyclonal, Sigma-Aldrich), p21Cip1 (Dako Cytomation), CDK6 (Santa Cruz Biotechnology), cyclin B1 (Novocastra), cyclin D1 (Dako Cytomation); Bcl-xL (BD Biosciences), survivin (Novius Biological Inc), ER β (Santa Cruz Biotechnology), E-cadherin (Calbiochem), cadherin-11 (Zymed laboratories Inc), vimentin (BD Pharminogen).

Scratch-wound assay

Monolayers of cells, seeded in 12-well plates, were scratched with 20 μ l pipette tips and allowed to fill the scratched area for 10 hours. Afterwards, cells were fixed and stained. The width of the gap on images was measured using AxioVision software (Carl Zeiss Ltd).

Data analysis

Differences among the groups were analyzed using a one-way ANOVA in Statistical Package for the Social Sciences, followed by Dunnett's test to determine whether the treatment groups were different from a control group. $P < 0.05$ was selected as the level of statistical significance. All data are presented as the means \pm SE.

Results and Discussion

Anti-tumourigenic activity

In regard to viability of MDA-MB-231 cells, IC₅₀ concentrations for curcumin, DIM, EGCG and I3C were 30, 100, 80 and 300 μM (Supplementary Fig. 1), respectively, and are comparable with reported IC₅₀ values in this cell line [9, 11-13]. In contrast, genistein reproducibly increased growth in the concentration range 15-25 μM. Likewise, 10 μM genistein enhanced cell growth by 20% in culture and dietary soy supplementation increased breast cell proliferation in patients [14, 15]. Published data on genistein are inconsistent, with the IC₅₀ in MDA-MB-231 cells ranging from 10 μM [16] to 120-131 μM [17, 18].

Concentrations equal to 10% of the IC₅₀s, namely 3 μM curcumin, 10 μM DIM, 8 μM EGCG and 30 μM I3C, were used for long-term treatment of cells. The concentration of genistein (2.5 μM) was chosen as the maximal dose which did not affect cell number. These concentrations did not change cell viability after 48 hours (not shown), in agreement with other reports [2, 14, 19]. Cells in passages 10-16 growing in the presence of phytochemicals or DMSO, as control, were used for analyses.

Cell growth was estimated after culturing for sufficient time to allow several divisions, to facilitate detection of fairly small changes. Curcumin, DIM and EGCG delayed cell growth by 25-30% (Fig. 1A). Curcumin and EGCG increased the doubling time to 36.0 and 35.8 h, respectively, compared to a doubling time of 29.5 hours in control cells. The clonogenic assay was used to take account of any influence of autocrine growth factors, which should be significantly decreased by low cell density. Curcumin, EGCG and I3C decreased the number of colonies by about 2.5-3 fold, with markedly smaller size of colonies in the curcumin and I3C groups (Fig. 1B).

Since tumor growth can be hindered by cell death, we investigated whether any of

the phytochemicals might affect the basal level of apoptosis. The background caspase-3/7 activity levels were increased in curcumin-treated cells (1.5 fold) and EGCG- and I3C-treated cells (2 fold) (Fig. 1C). Interestingly, the basal caspase3/7 activity levels were inversely related to the colony formation rates in the clonogenic assay (compare Fig. 1B and 1C).

Modulation of RNA and protein expression

Treatment with DIM increased mRNA levels of cyclin-dependent kinase inhibitor 1A (p21Cip1) by 11 fold and anti-apoptotic Bcl-xL by 3 fold, whereas I3C increased only p21Cip1 mRNA by 3 fold (Fig. 2A). Analysis of protein expression indicated that expression of ER β , cell-cycle-related biomarkers (p21Cip1, CDK6, cyclins B1, D1) and survival-related proteins (survivin, Bcl-xL) was not changed by treatments, except for Bcl-xL, which was increased up to 145% in DIM-treated cells (Fig. 2B). p21Cip1 protein was barely detectable. I3C reduced EGFR levels by 48% (Fig. 2B) and vimentin by 35% (Fig. 3A).

EMT-related genes, encoding E-cadherin, vimentin, cadherin-11, IL-6 and uPA, were investigated. E-cadherin protein and mRNA levels were very low. Although E-cadherin protein appeared to be upregulated by curcumin in 4 out of 5 experiments, no statistically significant increase was found (Fig. 3A, 3B). Cadherin-11 protein levels were reduced to 78% and 74% by curcumin and DIM, respectively; similar decreases in cadherin-11 RNA levels were detected, although they did not reach statistical significance (Fig. 3A-3B). All agents increased IL-6 mRNA levels and all, but EGCG, increased uPA mRNA levels (Fig. 3C). **Decreased cadherin-11 and increased uPA expression may diminish cell-cell adhesion and attachment to the extracellular matrix, which would result in increased cell motility. Hence, cell motility was investigated using the wound assay. Curcumin, DIM, genistein and EGCG decreased clear areas in the**

scratch assay by 61, 72, 85 and 92%, respectively (Fig. 3D). Thus, increased cell motility correlated with the increased uPA and decreased cadherin-11 levels in curcumin, DIM and genistein groups. The cause of increased motility in the EGCG group has not been established.

To investigate regulation of these genes by DNA or histone modification, inhibitors of DNA methylation and histone deacetylase were used. 5-aza-dC significantly increased mRNA expression of IL-6, uPA, p21Cip1 and E-cadherin, whereas cadherin-11 mRNA was decreased; TSA affected gene expression to much lesser extent (Fig. 4A-4C). 5-aza-dC also increased protein levels of E-cadherin and vimentin, and decreased cadherin-11. TSA similarly affected expression of cadherin-11 and E-cadherin. Upregulation of p21Cip1, IL-6 and uPA by 5-aza-dC, has been shown previously [20-22]. Despite a significant increase in p21Cip1 mRNA in 5-aza-dC-treated cells, protein levels were too low to allow reliable detection.

Collectively, these data do not support a proposed role for EGCG in the reactivation of gene expression by inhibiting DNA methylation [23], but are in agreement with the findings of Stresemann et al [24]. Neither did we find significant changes in the histone-3 acetylation levels in the curcumin group (Supplementary Fig. 2), which would be expected if curcumin inhibited histone acetyltransferase p300 at physiological concentrations [25-27]. Most of the phytochemicals modulated levels of cadherin-11, metastatic biomarkers IL-6 and uPA similarly to the inhibitors. This on its own would be an undesirable effect of dietary agents or inhibitors, if it were not counter-balanced in the chemopreventive net effect. Further studies elucidating roles of these agents in metastatic invasion are required. Compared to the other agents, DIM induced most changes in expression of genes, which can be regulated epigenetically. No evidence of DIM involvement in epigenetic regulation has been reported so far and further investigation is required.

It was encouraging to note that EGFR was downregulated by I3C in the present study, as observed in response to single high doses [8, 9]. I3C-induced reduction in vimentin was not related to decreased EGFR signaling (Fig. 4D).

Response to radiation

Radiation-induced DNA damage, detected using the comet assay, was investigated. Some protection against radiation-induced damage ($\leq 16\%$) was observed in curcumin, DIM, EGCG and genistein-treated cells (Fig. 5A). DNA repair was not significantly affected (Fig. 5B). Protection from DNA damage was not altered by inhibition of DNA methylation or histone deacetylation (Fig. 5C). DNA damage by X-rays is caused by a burst of free radicals and is likely to be reduced by antioxidant properties of these phytochemicals. Several studies have demonstrated a protective role of dietary phytochemicals in response to ionizing radiation. For example, curcumin protected DNA from chromatid breaks induced by radiation and from chromosome aberrations, DNA damage and consequent mammary tumors in animal models [28-30]. Furthermore, a large body of evidence supports a protective role of dietary phytochemicals in response to UV and oxidative effects [31-35]. Moreover, clinical studies show that green tea protects against DNA damage, decreases generation of free radicals and increases antioxidant activity in healthy volunteers [36].

It is important to note that a protective effect against induced DNA damage can be detected at low concentrations of phytochemicals, which do not induce massive DNA laddering and apoptosis. It has been reported that higher concentrations of curcumin (15-50 μ M) [37, 38] or EGCG (100 μ M) [39] induce DNA damage, which is likely to be a result of ongoing apoptosis. Our data suggest that phytochemicals *in vivo* may protect cells from DNA damage induced by environmental factors and, thereby, slow down their transformation into increasingly malignant phenotypes. However, the data also suggest

that dietary phytochemicals may, to a very limited extent, protect cancer cells against radiotherapy.

Comparison of long-term treatment with physiological doses with higher dose single treatments and in vivo studies

This *in vitro* model of long-term exposure to dietary molecules allows the analysis of important activities, summarized in Table 1, at physiologically relevant concentrations. As discussed above, use of higher doses of agents may produce misleading results in regard to DNA damage and in a number of other respects. Our previous studies showed that treatment of MDA-MB-231 cells with 250 μ M I3C decreased protein levels of EGFR, cyclin D1 and CDK6, whereas p21Cip1, p27Kip1 and cyclin E were increased [8]. We also showed that I3C-induced apoptosis is related to downregulation of EGFR in these cells [8, 9]. The latter is consistent with the data obtained in this model, since reduced EGFR signaling in combination with diluted autocrine TGF-alpha is bound to increase apoptosis and reduce growth in clonogenic assay. Also in agreement with previous data, I3C increased p21Cip1 RNA [8, 40]. However, no changes in cell-cycle-related proteins, including p21Cip1, were found in this study. Therefore, data presented here suggest that the main *in vivo* mechanism of anti-cancer activity by I3C is likely to be EGFR-related apoptosis, rather than cell-cycle-related events. This conclusion is in agreement with the increased apoptosis in tumors of I3C-treated animal models (Supplementary Table 1).

Similarly, modulation of caspase activity and expression of cell-cycle-related proteins, such as p21Cip1, CDK6, cyclin B1, cyclin D1, and anti-apoptotic Bcl-xL, have been related to the action of curcumin, EGCG and DIM, investigated in much higher doses [3]: e.g. p21Cip1 is increased by high concentrations of genistein (30-74 μ M) or EGCG (90 μ M) [2, 41, 42]. Our data support involvement of apoptosis, rather than cell cycle arrest in the action of curcumin and EGCG at physiological doses in metastatic

breast cancer cells. Downregulation of survivin, proposed as a target in DIM-induced cell death in MDA-MB-231 cells [12], was not observed in our study, nor was there any change in caspase activity. Upregulation of p21Cip1 mRNA by DIM, observed in several studies [43, 44], was also detected in our model. However, mRNA levels did not correlate with the protein levels, possibly due to fast modification and degradation of the protein. Importantly, increased apoptosis, induced by curcumin and EGCG, is detected in tumors in animal models (Supplementary Table 1), including the MDA-MB-231 xenograft model.

Altogether, our data show that some phytochemicals had significant anti-tumourigenic effect on MDA-MB-231 cells, with curcumin and EGCG having the greatest effect on cell viability. The effects of I3C and DIM, a major *in vivo* acid-catalyzed condensation product of I3C, were different, implying distinct mechanisms of action. Unfortunately, no anti-cancer effects of genistein were found in this study. Similarly, animal studies did not show inhibition of tumor growth in the MDA-MB-231 cell xenograft by genistein in a serum concentration around 1 μ M [45]. This correlates with the absence of a protective effect of genistein in ER α -negative tumors [46].

This model has revealed important physiological activities and new molecular targets and effects, e.g. cell motility or DNA damage protection, of several chemopreventive phytochemicals. It reinforces the view that these agents can exert anti-cancer activity at physiologically relevant doses and may also be useful for analysis of the effects of dietary phytochemicals on cancer therapies. Future research strategies may include investigation into modulation of gene expression and cell motility by dietary agents.

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Table 1. Physiological activities of dietary phytochemicals in breast cancer MDA-MB-231 cells

Agent Effect	Curcumin	DIM	EGCG	Genistein	I3C	TSA	5-Aza-dC
Doubling time	↑	≅	↑	≅	≅	ND	ND
Colony formation	↓, smaller size	≅	↓	≅	↓, smaller size	ND	ND
Apoptosis	↑	≅	↑	≅	↑	ND	ND
DNA damage	↓	↓	↓	↓			
Motility	↑	↑	↑	↑	≅	ND	ND
RNA levels	↑ IL-6 , ↑ uPA	↑ p21Cip1 , ↑ Bcl-xL , ↑ IL-6 , ↑ uPA	↑ IL-6	↑ IL-6 , ↑ uPA	↑ p21Cip1 , ↑ IL-6 , ↑ uPA	↓ cadherin-11	↑ p21Cip1 , ↑ IL-6 , ↑ uPA ↑ E-cadherin , ↓ cadherin-11 ,
Protein levels	↓ cadherin-11	↑ Bcl-xL , ↓ cadherin-11			↓ EGFR , ↓ vimentin	↑ E-cadherin , ↓ cadherin-11	↑ E-cadherin , ↓ cadherin-11 , ↑ vimentin

ND, not determined.

Figure legends

Fig. 1. The effect of long term treatment on cell growth and death.

A. Cell growth following incubation of 1×10^5 for 167 and 194 h after plating is shown as number of cells and cell doubling time. * $P < 0.05$, $n = 6$. **B.** Results of a clonogenic assay are shown. * $P < 0.05$, $n = 6$. **C.** Caspase 3/7 activity was measured 30 hours after plating the cells. *, $P < 0.05$, $n = 16$.

Fig. 2. Expression of biomarkers related to cell viability and cell cycle regulation.

A. The mRNA levels are shown. * $P < 0.05$, $n = 4$, analyzed in duplicates. **B.** Protein levels were analyzed by immunoblotting with the antibodies indicated. Protein quantification is shown as the ratio to actin. Lanes 1-6 are DMSO, curcumin, DIM, EGCG, genistein and I3C, respectively * $P < 0.05$, $n = 5$.

Fig. 3. Expression of EMT biomarkers.

A. Protein levels were analyzed as in Fig. 2. * $P < 0.05$, $n = 5$. The levels of mRNA for E-cadherin and cadherin-11 (**B**) and IL-6 and uPA (**C**) are shown. * $P < 0.05$, $n = 4$, analyzed in duplicates. **D.** Results of scratch assay are shown with the width measured in μm . * $P < 0.05$, $n = 12$, in triplicate.

Fig. 4. Expression of biomarkers in the presence of inhibitors.

Cells were treated with $7.5 \mu\text{M}$ 5-Aza-dC or 300 nM TSA for 72 hours (**A-C**) or $2.5 \mu\text{M}$ PD153035 for 48 hours (**D**). Protein levels were analyzed by immunoblotting with the antibodies indicated. Protein quantification is shown on the graphs, as the ratio to actin. * $P < 0.05$, $n = 3$. The levels of mRNA are shown as a fold increase versus DMSO control. * $P < 0.05$, $n = 3$, analyzed in triplicates.

Fig. 5. Effect of phytochemicals on DNA damage.

A. Comet assay was used to detect DNA damage caused X-ray irradiation (0.5 and 10 Gy). Damage is expressed as a percentage of DNA in the comet tail. *P<0.05 versus DMSO control; n=200. **B.** Comet assay was also used to detect DNA repair following a 10 Gy X-Ray irradiation dose. Time for repair was 5, 15 or 30 min. The repair capacity is expressed as the percentage of the initial damage that is repaired at each incubation time. * P<0.05 versus 0 min. n=200 cells. **C.** DNA damage in cells treated with 5-Aza-dC or TSA, was detected as in **A.**

Fig. 1

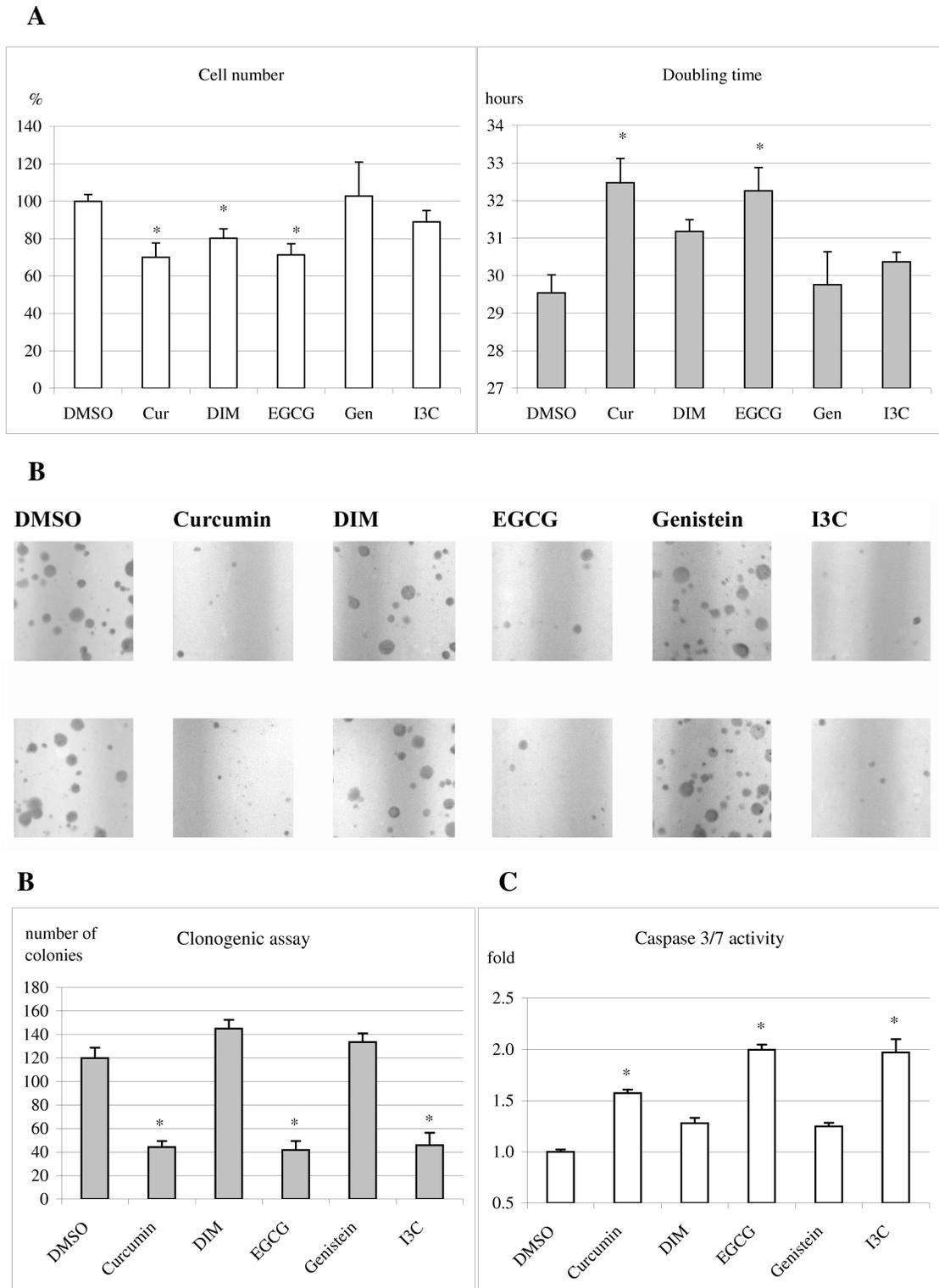


Fig. 2

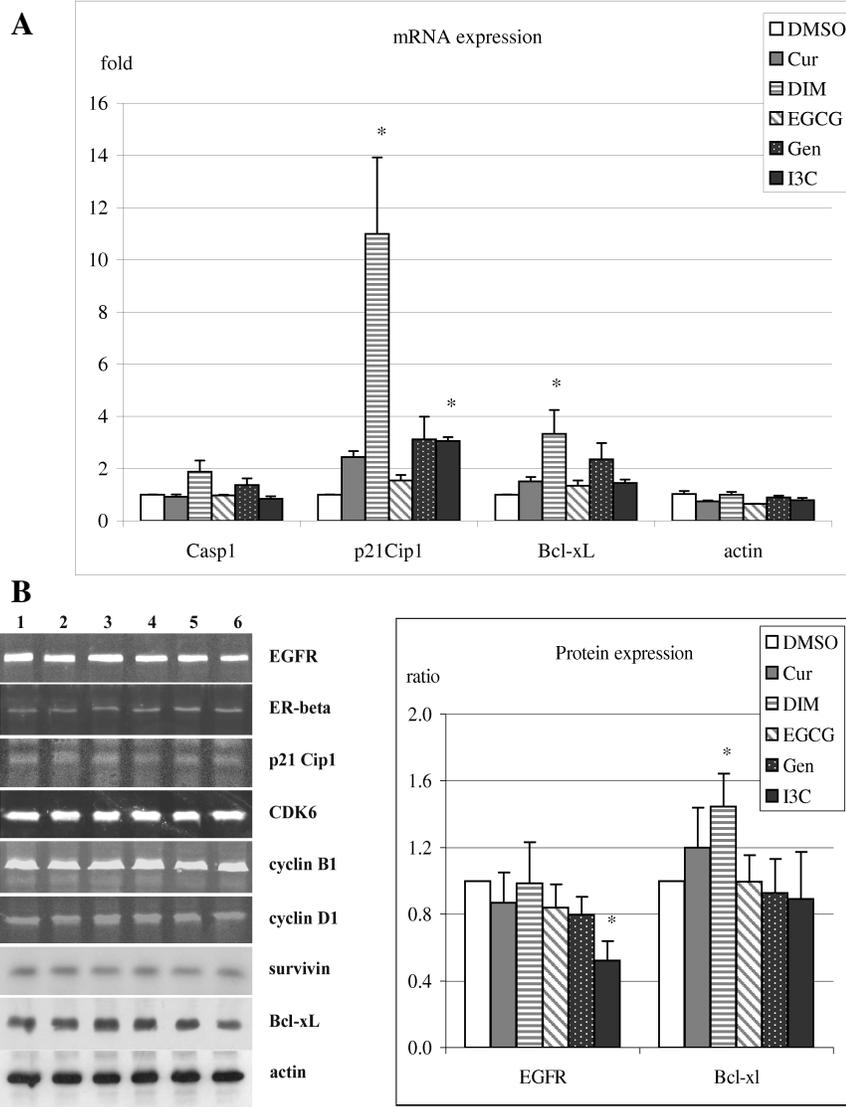


Fig. 3

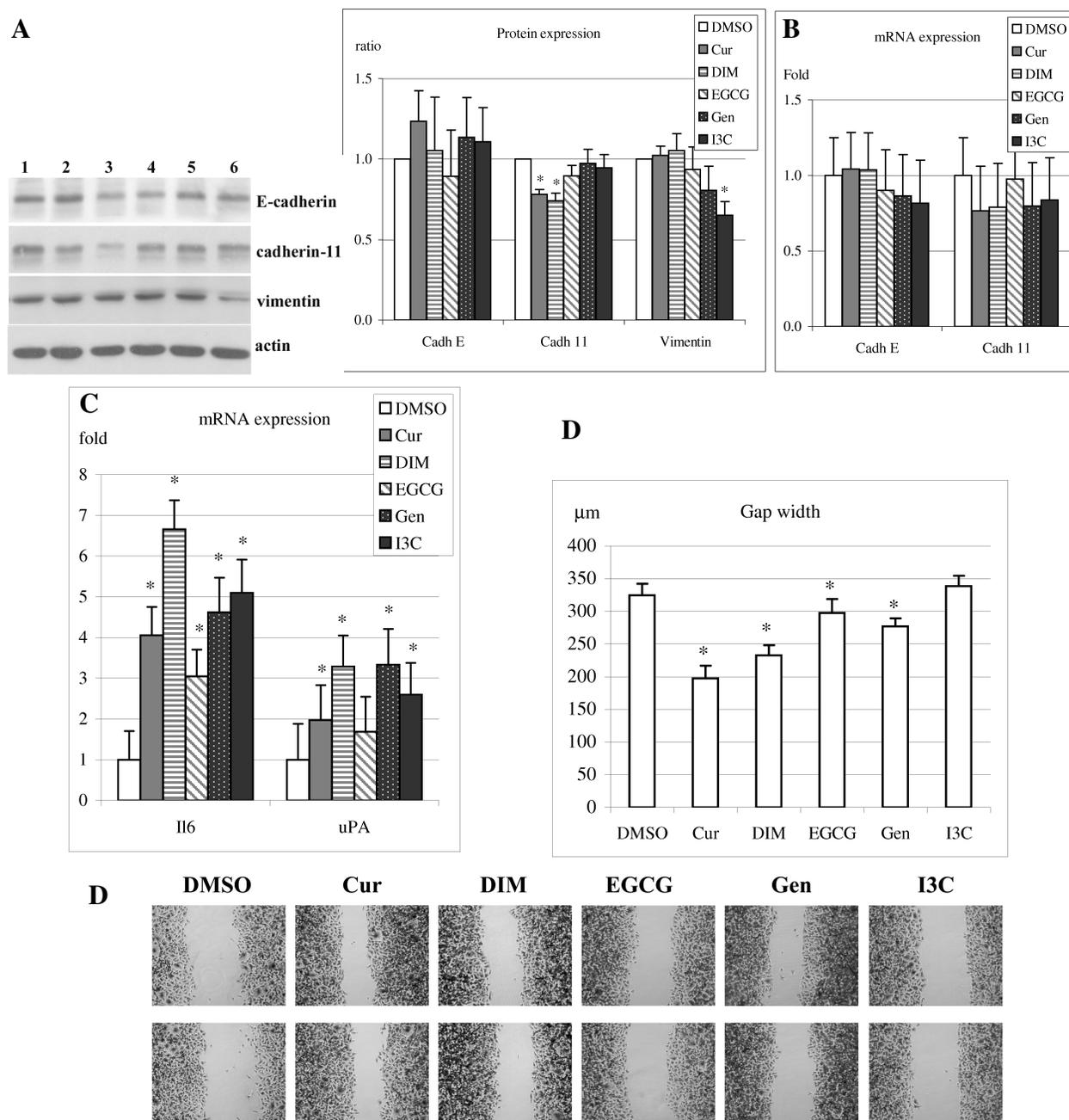


Fig. 4

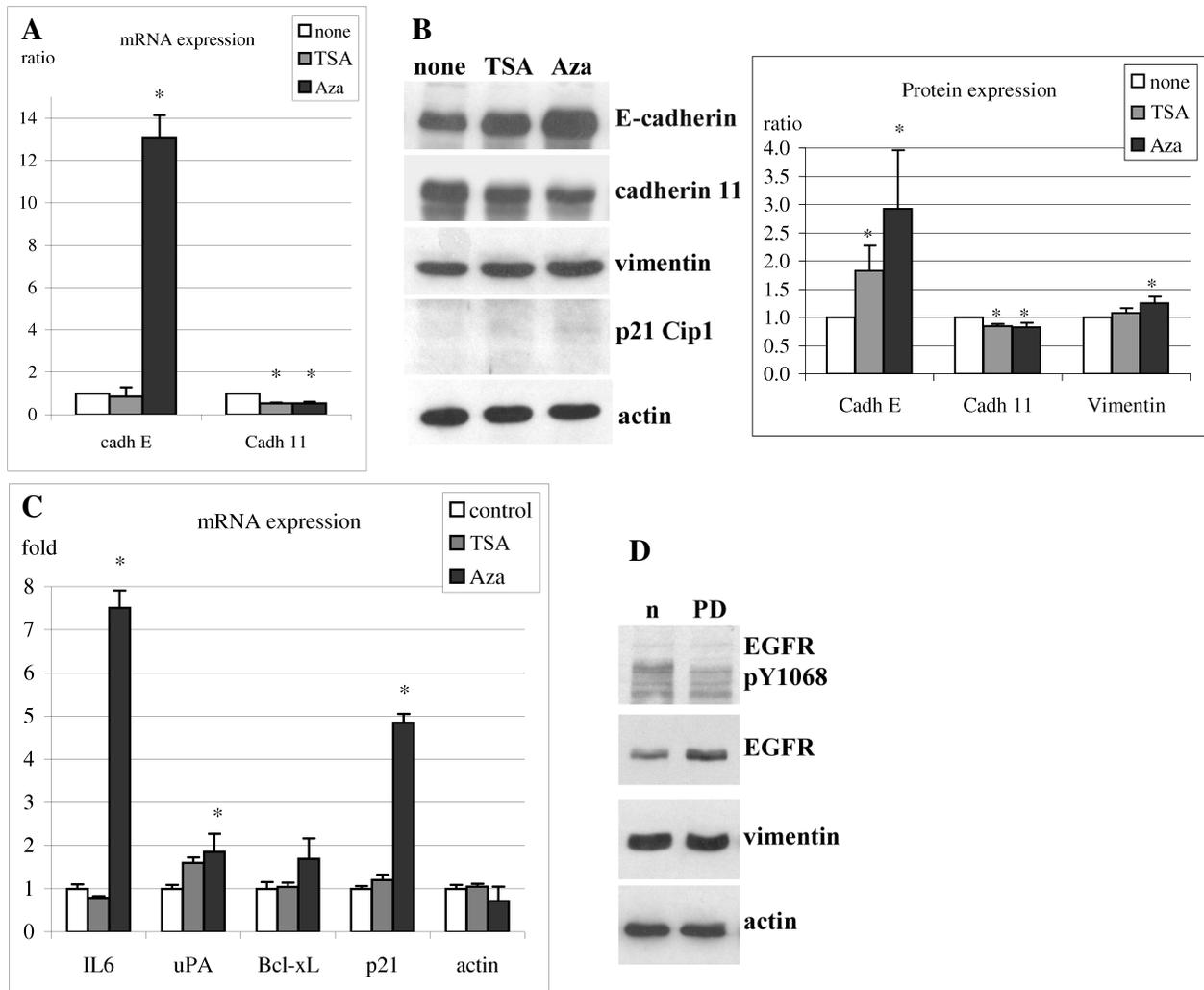


Fig. 5

