Running title: **I3C-induced modulation of NF- κ B signalling is not related to cell death**

Research Article

Title: **Indole-3-carbinol-induced modulation of NF- κ B signalling is breast cancer cell-specific and does not correlate with cell death**

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Abstract

Indole-3-carbinol (I3C), a dietary chemopreventive compound, induces cell death in human breast cancer cells by modulating activities of Src and EGFR. The effect of I3C on NF- κ B, constitutively activated in breast cancer cells, was investigated. Nuclear extracts of MDA-MB-468, MDA-MB-231 and HBL100 cells contained all of the Rel proteins with similar expression patterns in the latter two. The level of NF- κ B-regulated reporter gene expression was in the order HBL100<<MDA-MB-468<<MDA-MB-231. Upstream inhibition, using PI3K, EGFR or IKK β inhibitors, resulted in cell-specific effects on expression of the NF- κ B-regulated reporter gene and endogenous genes Bcl-xL, I κ B α and IL-6, as well as on cell viability. The expression patterns of Rel and several NF- κ B-regulated genes and the response to LY249002 in MDA-MB-468 cells contrasted with those in other cells. I3C induced NF- κ B-regulated reporter gene expression at 12 h in MDA-MB-468 cells. Conversely, it was reduced at 24 h in HBL100 cells. I3C treatment for 6 h alone or in combination with TNF α induced NF- κ B-regulated reporter gene expression, detected 5 h later, in MDA-MB-468, but not HBL100 cells. I3C induced NF- κ B p65/p50 DNA binding at 6.5 h, preceded by association of IKK β with the Src/EGFR complex and increased phospho-I κ B α in MDA-MB468 cells. TNF α increased I3C-induced apoptosis in MDA-MB-468 and MDA-MB-231 cells. It also induced apoptosis, enhanced by I3C, in HBL100 cells. Hence, regulation of constitutive NF- κ B was cell-specific. I3C influenced the NF- κ B pathway in a cell-specific manner, which was not related to apoptosis. However, the combination of I3C and TNF α increased apoptosis in all cell lines.

Words: 250

Abbreviations. EGFR, epidermal growth factor receptor; ER, oestrogen receptor; DIM, 3,3'-diindolylmethane; I3C, indole-3-carbinol.

Introduction

Breast cancer accounts for the highest incidence of cancer and cancer-related deaths in women in both developed and developing countries [1]. Many studies show a connection between diet and breast cancer, with cancer incidence inversely correlated with consumption of fruit and vegetables [2-7]. Consumption of cabbage, in particular, may play an important role in decreasing the cancer risk [8, 9]. Indole-3-carbinol (I3C), a dietary compound found in cruciferous vegetables, such as cabbage, has shown chemopreventive potential in several tissues in animal models. I3C reduces incidence and multiplicity, and increases latency of mammary tumours in animal models [10-15]. It exhibits chemopreventive effects against tumours induced by carcinogens [13, 15] and viruses [10], and is also effective against spontaneous [11, 14] and xenograft tumours [12].

I3C, an FDA-approved nutritional supplement, is effective in treatment of neoplasias and recurrent papillomatosis and is associated with very few side effects [16-18]. It may enhance efficacy of chemotherapeutic drugs and may be used in combination with them, e.g. a combination of I3C and tamoxifen reduced mammary tumourigenesis in a rat model in a cooperative manner [19]. Toxicity of an experimental anti-tumour drug ET-743, with promising activity in breast carcinoma and currently under phase II clinical evaluation, was almost completely abolished by I3C in a rat model [20]. I3C can be converted into 3,3'-diindolylmethane (DIM), a major *in vivo* acid-catalysed condensation product of I3C. The maximal serum concentrations of I3C and DIM were detected in mice at 30 min and 2 hours, respectively, after I3C ingestion [21], whereas only DIM was detected in humans 1h-12h after I3C ingestion, with the peak concentrations at 2 hours [22]. Since maximal DIM concentrations, following I3C administration at similar doses (mg/kg), are comparable in mice and humans, the discrepancy in I3C detection is likely to be caused by sensitivity of methods and selection of time points. Nonetheless, several studies have revealed distinct responses to I3C and DIM *in vivo* [20, 23-26]. Therefore, the *in vivo* activity of dietary I3C cannot be attributed completely to the production of DIM, although partial response due to DIM conversion is feasible.

I3C exerts several effects that may contribute to cancer prevention. It induces phase I and II enzymes [11, 14, 15, 19], leading to increased catabolism and excretion of carcinogens and oestradiol. It also elicits anti-proliferative and pro-apoptotic responses in oestrogen receptor (ER)-alpha-negative breast cancer cells. Several molecular mechanisms have been suggested for I3C-induced cell death. Inhibition of Akt activity was proposed as a pro-apoptotic event [27, 28]. However, later studies showed that downregulation of Akt does not account for I3C-induced apoptosis in several breast cancer lines [29]. EGFR downregulation and modulation of Src were found critical in I3C-induced apoptosis in several subtypes of breast cancer cells [30, 31]. Inhibition of NF- κ B pathway by I3C has been found in several types of cancer cells [28, 32, 33].

The NF- κ B pathway is most commonly involved in suppression of apoptosis by transactivation of expression of anti-apoptotic genes, e.g. Bcl-xL, although it also can promote cell death in response to certain signals in some cell types [34]. NF- κ B regulates expression of multiple genes, including all NF- κ B inhibitors, i.e. I κ B α , I κ B β , I κ B ϵ , p105/ I κ B γ and p100/I κ B δ [35]. It is implicated in cancer development, progression to malignancy and therapy resistance [36]. NF- κ B is constitutively upregulated in breast tumours and is activated in most breast cancer cell lines, regardless of the ER status. Amplification of the RelA/p65 gene and aberrant nuclear expression of multiple subunits, including p50, p52, RelA, RelB and c-Rel, has been shown in human primary breast cancers [36-39]. The causes of constitutive activation of NF- κ B are numerous. Elevated levels of IKK activity in breast tumours and cell lines, as well as decreased levels of I κ B are well documented [36]. CK2, which also phosphorylates I κ B, is implicated in NF- κ B upregulation in the mammary cell lines and tumours [40]. Some breast cancer cells secrete cytokines, which generate autocrine NF- κ B activation, e.g. MDA-MB-231 cells produce high levels of IL-1 and TNF α and HBL100 cells also secrete IL-1 [41, 42]. Increased EGFR signalling is involved in regulation of NF- κ B and increased p50/p105 levels in breast cancer cells [43, 44]. Upregulated PI3K/Akt activity, due to loss of PTEN or upregulated growth factor signalling, also causes activation of NF- κ B in breast cancer cells [45].

Constitutive NF- κ B signalling augments expression of metastatic biomarkers, e.g. IL-6, ICAM1 and uPA [41, 46, 47], and anti-apoptotic Bcl-xL [34]; the latter is also involved in increased metastatic spreading and resistance to TNF α in breast cancer [48, 49]. The causal role of NF- κ B in breast cancer development and progression to malignancy, as well as in chemo- and radio-therapy resistance, makes this pathway an attractive therapeutic target. The role of NF- κ B signalling in I3C-induced inhibition of cancer cell survival has not been elucidated and was the aim of this study.

Materials and methods

Cells

Human breast cell lines MDA-MB-468, MDA-MB-231 and HBL100 originated from the American Type Culture Collection. The cell lines were cultured in DMEM with 10% foetal bovine serum (FBS), as described previously [30]. They all belong to the basal-like breast cancer subtype [50].

Immunoblotting and immune precipitation

Preparation of whole cell lysates, immune precipitation, SDS-PAGE and immunoblotting were performed as described previously [31]. The polyclonal antibodies against IKK β , NF- κ B p65 (C-terminus), NF- κ B p50 (C-terminus), monoclonal antibodies against phospho-I κ B α and lamin A/C were purchased from Santa Cruz. The other polyclonal antibodies against NF- κ B p65 (N-terminus), p50 (N-terminus), p52 (N-terminus), RelB (C-terminus) and cRel (internal) were a kind gift from Dr Nancy Rice [51].

Electromobility shift assay (EMSA)

Standard techniques with addition of phosphatase inhibitors were used to prepare cytoplasmic and nuclear extracts [28, 32]. Cells were extracted with a hypotonic buffer [10 mM Hepes, pH7.8, 10 mM KCl, 2 mM MgCl₂, 1mM DTT, 0.1 mM DTT, 0.4% NP40, inhibitors for proteases and phosphotyrosine and phosphoserine / phosphothreonine phosphatases (all cocktails

from Sigma)] to obtain cytoplasmic extracts. The remaining pellet was extracted with a hypertonic buffer [50 mM Hepes, pH7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM DTT, 10% glycerol, inhibitor cocktails as above] to obtain nuclear extracts. A [³²P]-5' labelled NF-κB consensus oligonucleotide duplex 5'-AGTTGAGGGGACTTTCCCAGGC (Promega) or STAT5 consensus oligonucleotide duplex 5'-AGATTTCTAGGAATTCAATCC (Santa Cruz), separated from non-incorporated [³²P]ATP, were used in EMSA. Normalised extracts containing equal protein levels were incubated with 3.5 pmol of labelled oligonucleotides in the gel shift binding buffer (Promega) for 20 min at room temperature, followed by separation in non-denaturing 7% PAGE. For supershifts, 2.5 µl of antibodies were incubated with nuclear extracts for 20 min at room temperature prior to addition of the 5'-labelled oligonucleotide and an excess of the unlabeled oligonucleotide. Protein-DNA complexes were quantified by autoradiography of dry gels using a phospho-imager (Storm 820, Molecular Dynamics).

Transfection

The NF-κB transcriptional activity was evaluated using transient transfection with a gene-reporter pNF-κBLuc plasmid (Stratagene), containing the firefly luciferase reporter gene regulated by a promoter with multiple NF-κB sites. A plasmid pRL-SV40 (Promega), containing a renilla luciferase reporter gene, was used as an internal control of transfection efficiency. Conditions and reagents were optimised for maximal transfection efficiency in MDA-MB-468 cells. The following protocol was used in all experiments: cells, seeded in Packard Viewplates-96 (2500 cell/well) in 10% FBS in DMEM the previous night, were transfected with 50 ng/well pNF-κB-luc DNA and 8 ng/well pRL-SV40 DNA using 0.3 µl/well of GeneJuice transfection reagent (Novagen) in 100 µl of DMEM for 2 h, followed by the same volume of 20% FBS in DMEM, as previously [31].

After 36-48 h, cells were treated with I3C, TNFα or specific inhibitors for the indicated number of hours. Luciferase activities were measured using a dual luciferase assay kit LucLite (Perkin-Elmer, discontinued) or Dual-Glo luciferase assay system (Promega) according to the

manufacturers' instructions in a FluoStar luminometer (BMG, UK). The data are presented as a percentage of the ratio of activities of firefly luciferase to renilla luciferase.

Quantification of gene expression

Total RNA from cells was isolated using a Qiagen kit. cDNA was synthesised using a Promega reverse transcription kit. The cDNA amounts transcribed from 50 ng of RNA were used in real-time PCR (ABI 7300) to quantify the levels of mRNA for Bcl-xL, ICAM1, I κ B α , uPA and IL-6 and 18S RNA using Taqman probes (gene expression products from Applied Biosystems), as previously described [31]. The levels of gene expression were calculated by Δ CT method, using 18S RNA as a reference.

Cell viability and apoptosis-related studies

Cells were seeded overnight in 10% FBS in DMEM, followed by treatments in the same medium. The number of viable adherent cells was estimated using the ATPlite kit (Perkin-Elmer) and caspase 3/7 activity, as a measure of apoptosis, was determined using a Caspase-Glo 3/7 kit (Promega) as described previously [30]. The percentage of live, apoptotic or necrotic cells in the whole population was measured using annexin V binding to externalised phosphatidylserine with the Annexin V-FITC kit (Bender Medsystems) as described previously [30].

Data analysis

Differences among the groups were analysed using one-way ANOVA in Statistical Package for the Social Sciences, followed by Scheffe's test to determine whether the treatment groups were different, or 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.

Results

NF- κ B constitutive activation and signalling in breast cancer cells

Immunoblotting of nuclear and cytoplasmic extracts showed that all NF- κ B subunits are present in both fractions in various amounts (Fig. 1A). The pattern of expression was similar in HBL100 and MDA-MB-231 cells: RelA (p65), RelB, cRel and p52 were present at similar levels in both extracts. Surprisingly, p105 and p100 were also present in nuclear extracts. The expression pattern in MDA-MB-468 cells was different. NF- κ B p105 was absent, a doublet of p52 was present and lower levels of p65 and cRel were present in the nuclear extract, with cRel expressed at a much higher level in cytoplasmic extract.

NF- κ B DNA binding in cells was represented by two specific bands, UB and LB, with slightly stronger LB in HBL100 (Fig. 1B-1C). Constitutive NF- κ B DNA binding in these cell lines is in agreement with published data [28, 44, 47]. Since the DNA binding in MDA-MB-231 cells was similar to MDA-MB-468 cells (Fig. 5B), only nuclear extracts of HBL100 and MDA-MB-468 cells were analysed. Antibodies against C-termini of p65 and p50, marked SC, prevented NF- κ B DNA binding in the lower band in both cell lines. Other antibodies significantly reduced DNA binding in the upper band. Antibodies against p65-N-terminus and cRel also reduced the LB in HBL100 cells. Hence, the LB is likely to be mostly a p65/p50 heterodimer, whereas the upper band is likely to represent various NF- κ B heavier complexes, possibly with other transcription factors. The lower band was more intense in HBL100 and MDA-MB-231 cells compared to MDA-MB-468, which had less nuclear p65 (compare Fig. 1B-1C, and 1A).

The NF- κ B transcriptional activity, measured as NF- κ B-regulated reporter gene expression, was significantly lower in HBL100 cells compared to the other cells (Fig. 1D). Activity was in the following order MDA-MB-231>>MDA-MB-468>>HBL100. This is in agreement with the expression of an NF- κ B-regulated set of anti-apoptotic genes in these cell lines [52].

NF- κ B-regulated expression of mRNA for IL-6, ICAM-1, uPA, Bcl-xL and I κ B α , regulated by different Rel dimers in various cell types [53, 54], were measured in the cells (Fig. 1E). No

differences in ICAM-1 mRNA levels were found in three cell lines. The mRNA expression levels for uPA, IL-6, Bcl-xL and $\text{I}\kappa\text{B}\alpha$ were in the following order MDA-MB-231>HBL100≥MDA-MB-468, although not all differences were statistically significant. Expression of uPA mRNA correlated with reported levels [47] and invasiveness of the cells [55].

Modulation of constitutive NF- κ B signalling did not correlate with cell survival

Regulation of NF- κ B transcriptional activity in breast cancer cells by PI3K, Src, EGFR and IKK β was investigated using specific inhibitors. Aspirin was used as a specific IKK β inhibitor [56]. Inhibition of PI3K with 10 μ M LY294002, EGFR with 1 μ M PD 153035 or IKK β with 100 μ M aspirin modulated NF- κ B-regulated reporter gene expression in a cell-specific manner (Fig. 2A) with LY294002 affecting all cell lines. A treatment time of 24 h was chosen to examine a steady-state of NF- κ B constitutive activation. Reduced reporter gene expression by LY294002 coincided with increased mRNA expression of NF- κ B inhibitor $\text{I}\kappa\text{B}\alpha$ and Bcl-xL in HBL100 cells, but no changes in mRNA levels were found in MDA-MB-231 cells (Fig. 2B). Similarly, reduced NF- κ B signalling and NF- κ B-regulated reporter gene expression by LY204002 in MDA-MB-231 cells were found previously [57, 58]. Increased reporter gene expression by LY294002 coincided with reduced IL-6 levels in MDA-MB-468 cells (Fig. 2B). Hence, regulation of NF- κ B target genes was also cell-specific.

LY294002 reduced cell number in HBL100 cells, reduced caspase 3/7 activity in HBL100 and MDA-MB-231 cells, but no changes in the percentage of live/apoptotic/necrotic cells were found (Fig. 2C-2E). Hence, PI3K inhibition in HBL100 cells resulted in a decreased cell number due to decreased proliferation, but did not increase cell death, possibly, due to increased transcription of Bcl-xL. Aspirin, EGFR and Src inhibitors induced caspase 3/7 activity in cell line-specific manner (Fig. 2D), although this increase was low (<150%) and did not affect cell number. Altogether, all inhibitors had a cell-specific effect on NF- κ B-regulated reporter gene activity, while LY294002 influenced it in all cell lines; however, alterations in NF- κ B-regulated reporter gene levels did not correlate with cell death.

Effect of I3C on NF- κ B and apoptosis

We showed previously that I3C induced a chain of events initiated by increased tyrosine phosphorylation at 5 hour-treatment, involving Src activation in MDA-MB-468 and HBL100 cells, and proceeded at a different speed to induce apoptosis in MDA-MB-468 (at 16 h), and MDA-MB-231 (at 24 h). No apoptosis was detected in HBL100 despite significantly increased tyrosine phosphorylation [30, 31]. Here we investigated NF- κ B signalling in I3C-treated MDA-MB-468 and HBL100 cells over a period of 6-24 hours (Fig. 3A-3B). NF- κ B-regulated reporter gene expression increased at 12 h-treatment to $134\pm 7\%$ and $130\pm 5\%$ by 100 and 250 μ M I3C, respectively, in MDA-MB-468 cells and gradually decreased from 18 h (not shown) to 24 h of treatment to $64\pm 7\%$ in HBL100 cells. Thus, NF- κ B signalling in these cell lines responded to I3C treatment in a cell-specific manner, as in experiments shown in Fig. 2. Sarkar and co-authors [12] have shown that I3C decreased NF- κ B DNA binding and decreased NF- κ B regulated target genes in MDA-MB-231 cells in cell culture and an animal model.

The changes in the reporter gene expression involve modulation of NF- κ B signalling and translocation, resulting in reporter gene transcription followed by translation, which would take about 4-6 hours. Therefore, changes in NF- κ B signalling would have to take place at about 6 h to account for the increase in the reporter gene activity at 12 h in MDA-MB-468 cells. Hence, we conducted experiments to verify that I3C did indeed induce NF- κ B signalling at 6 hours. Cells were treated for either 1 or 6 h with I3C, followed by incubation in fresh medium for another 5 h to allow the reporter gene transcription and translation to take place. I3C induced NF- κ B signalling following a 6 h treatment in MDA-MB-468 cells ($137\pm 5\%$ versus $100\pm 4\%$), but not HBL100 (Fig. 3C). When the same I3C treatments were combined with a classic NF- κ B inducer TNF α , a significant increase in NF- κ B-regulated reporter gene expression ($311\pm 16\%$ versus $196\pm 6\%$) was observed in MDA-MB-468 cells in a combined treatment with I3C (6 h)+TNF α (the last 30 min), compared with TNF α (30 min) treatment. In contrast, a significant increase in NF- κ B-regulated reporter gene expression was observed in HBL100 cells in response to TNF α only. These

experiments confirmed that I3C induced NF- κ B signalling at 6 h specifically in MDA-MD-468 cells.

Since I3C induces transient activation of Src $\Rightarrow$ EGFR signalling in MDA-MB-468 cells, responsible for I3C-induced apoptosis [31], we examined whether transient Src activation may be involved in I3C-induced NF- κ B signalling. Increased phosphorylation of I κ B α was observed at 4.5-5 hours coinciding with Src activation (Fig. 4A). IKK β , shown to be activated and phosphorylated on Tyr188 and Tyr199 by Src [59], was co-immunoprecipitated with the Src-EGFR complex after 5 hour-treatment with I3C (Fig. 4B). NF- κ B DNA binding in the lower band was induced at 6.5 hours in MDA-MB-468, but not other cells (Fig. 4C-4D). No such changes were found in STAT5 DNA binding in MDA-MB-468 cells, but some increase was observed at 6.5 hours in MDA-MB-231 and HBL100 cells. PP2, a specific Src inhibitor, reduced NF- κ B DNA binding in LB, induced by I3C (Fig. 4D). Similarly, Src-mediated activation of NF- κ B and PP2-caused reduction in induced NF- κ B DNA binding was shown previously [59-61]. Collectively these data indicate that I3C-induced NF- κ B signalling is likely to be related to Src activation in MDA-MB-468 cells, but not HBL100.

Next we investigated I3C-induced apoptosis. I3C induced caspase 3/7 activation in MDA-MB-468 and MDA-MB-231 cells, but not in HBL100 (Fig. 5), as reported previously [30]. Therefore, there was no correlation between I3C-reduced NF- κ B signalling and cell death. TNF α alone induced apoptosis only in HBL100. However, a combination treatment with I3C+TNF α increased apoptosis in all cell lines. This increase was fairly low in MDA-MB-468 cells (22.5 $\pm$ 0.7 fold against 18.7 $\pm$ 1.0 by 250 μ M 3C), but was about two fold in MDA-MB-231 (12.2 $\pm$ 0.5 fold against 5.9 $\pm$ 0.1 by I3C) and HBL100 (3.3 $\pm$ 0.1 fold against 1.8 $\pm$ 0.1 by TNF α) cells. Thus, a combination treatment with I3C+TNF α was efficacious against all cell lines.

Discussion

NF- κ B constitutive activation in breast cancer cells was cell-specific

Analysis of Rel proteins showed similarities between HBL100 and MDA-MB-231 cells and a distinct expression pattern in MDA-MB-468 cells. It is interesting to note that Rel subunits were present together with p105 and/or p100 in nuclear extracts. Despite the general assumption that p100 and p105 should be processed into p50 and p52, respectively, before nuclear translocation, nuclear presence of p105 and p100 was demonstrated previously [62, 63]. The data presented by Saccani et al [63] indicate that p105/p100 processing may be uncoupled from nuclear translocation. Moreover, analysis of p105 heterodimers, e.g. p65/p105 or p50/p105, revealed an exposed nuclear localisation signal, which requires an additional factor to be retained in cytoplasm [64]. The physiological role of p100 or p105 in the nucleus has not been investigated. Altogether, constitutive activation of NF- κ B is likely to be related to nuclear presence of multiple Rel proteins with a cell-specific expression pattern.

NF- κ B DNA binding differed between cell lines: the lower band, which is likely to be a p65/p50 heterodimer, had lower mobility in HBL100 cells, which may be related to modification of NF- κ B subunits [53]. Similarly, strong DNA binding with differential activation of NF- κ B-regulated genes was found in these breast cancer cells previously [47, 52]. It is generally recognised that total NF- κ B DNA binding does not correlate with NF- κ B activity: DNA binding by some Rel dimers is not sufficient for promoter activation; e.g. p50/p50 dimer can be a transcriptional repressor [35].

The order of mRNA levels for uPA, IL-6, Bcl-xL and I κ B α in the cell lines (MDA-MB-231>HBL100 $\geq$ MDA-MB-468) did not correlate with the order of the NF- κ B-regulated reporter gene levels (MDA-MB-231>>MDA-MB-468>>HBL100). Nonetheless, the mRNA levels for these biomarkers correlate with the NF- κ B-regulated reporter gene levels in the cell lines with the similar expression pattern of the Rel proteins, i.e. in MDA-MB-231 and HBL-100 cells. The levels of IL-6 and uPA mRNA correspond to the increased levels of autocrine IL-1 with specific NF- κ B induction

in MDA-MB-231 and HBL100 cells or to the absence of IL-1 in MDA-MB-468 cells [41]. Many other factors **may be** involved in regulation of these genes, e.g. epithelial-mesenchymal transition with modulated DNA methylation and uPA upregulation is reported for MDA-MB-231 and HBL100 cells [65]. **Importantly, the aggressive metastatic cancer MDA-MB-231 cells showed the highest levels of NF- κ B-regulated expression of the reporter gene and metastatic biomarkers Bcl-xL, IL-6 and uPA, consistent with their metastatic potential.**

Modulation of constitutive NF- κ B activity did not correlate with cell survival

The significance of IKK or PI3K/Akt signalling in NF- κ B is well documented, whereas involvement of Src and EGFR is less recognised. Src is linked to induced NF- κ B signalling via physical association and phosphorylation of Tyr188 and Tyr199 of IKK β [59, 66] and also via phosphorylation of I κ B α on Tyr42 in inducible NF- κ B signalling [67, 68]. EGFR influences NF- κ B via association with TNFR-interacting protein RIP [69] and SHP-2/Gab1 proteins, with SHP-2 being a dual regulator of NF- κ B [70]. PI3K modulates NF- κ B via multiple mechanisms. The regulatory subunit p85 α associates with and sequesters phosphotyrosine phosphorylated I κ B α from NF- κ B heterodimers, whereas inhibition of the catalytic subunit p110 results in inhibition of Akt [71]. In turn, Akt induces IKK activity and enables p65 translocation via p65 phosphorylation [53].

Treatment with LY294002, a specific PI3K inhibitor, which also inhibits CK2 [72], modulated the reporter gene expression in all cell lines, whereas treatments with aspirin and the EGFR inhibitor had effect only in MDA-MB-231 cells. Effects of aspirin on NF- κ B may be related directly to IKK β or indirectly to COX2 activity. Our data suggest that regulation of constitutive NF- κ B activity, measured as NF- κ B-regulated reporter gene activity, and expression of genes regulated by this pathway is breast cancer cell-specific. For example, LY294002 also modulated expression of NF- κ B target genes in HBL100 (Bcl-xL and I κ B α) and MDA-MB-468 (IL-6) cells, but not in MDA-MB-231.

Moreover, modulation of constitutive NF- κ B did not affect survival nor caused cell death in any cell line, though reduced cell number due to inhibition of proliferation was observed in HBL100 cells. Our data are in agreement with other studies. Recent investigation with downregulation of RelA, IKK α , IKK β and IKK γ by siRNAs found no correlation between NF- κ B and apoptosis in MDA-MB-231 cells [73]. In particular, a decrease in p65 protein had no effects on apoptosis or cell proliferation, thus questioning the importance of NF- κ B alone in the maintenance of these cells. It is plausible, that fairly small decrease (35 $\pm$ 4 %) in NF- κ B-regulated reporter gene expression in MDA-MB-231 cells with extremely high constitutive activity may be not sufficient to reduce viability or decrease gene expression of NF- κ B-regulated genes.

Altogether, our data indicate that there are some similarities in the effect of LY294002 on the NF- κ B-regulated reporter gene expression and caspase 3/7 activity in MDA-MB-231 and HBL100 cells in contrast to MDA-MB-468 cells. Likewise, the pattern of expression of the Rel proteins and the mRNA levels of IL-6 and uPA were much higher in the former cell lines. Both of them belong to the basal B subtype, in contrast to MDA-MB-468 cells with the basal A subtype [50], therefore, investigation of contribution of the NF- κ B pathway to the subtype characteristics will be of great interest and potential clinical importance.

Modulation of NF- κ B signalling by I3C was not related to I3C-induced apoptosis

An extensive study conducted by Aggarwal and co-authors [33] demonstrated that I3C suppressed constitutive NF- κ B activation, TNF α -induced NF- κ B DNA binding and NF- κ B-regulated reporter gene expression, without affecting cell viability, in myeloid and leukaemia cells. TNF α -induced NF- κ B activation was prevented by I3C via inhibition of IKK, which resulted in reduced I κ B α phosphorylation in that study. The response of HBL100 cells with regard to I3C-mediated downregulation of constitutive NF- κ B activation, as well as sensitivity to TNF α -induced cell death was similar to those of myeloid and leukaemia cells. However, TNF α -induced NF- κ B activation was reduced by I3C in myeloid and leukaemia cells, but not in HBL100. Possibly I3C treatments longer than used in this study (6 h) are required to produce this effect in HBL100 cells.

Recently Sarkar and co-authors examined the role of NF- κ B in I3C-induced inhibition of MDA-MB-231 cells in cell culture and an animal model [12]. They found that I3C decreased levels of Bcl2 and increased Bax levels, decreased NF- κ B DNA binding and expression of NF- κ B regulated targets (MMP9 and CXR4) after 72 h treatment in culture. The reduced Bcl2/Bax ratio is likely to reflect ongoing apoptosis. These cells do undergo apoptosis in response to I3C (Fig. 5 and [30]). The authors also show decreased NF- κ B DNA binding in two tumours and similar protein changes in one tumour from I3C-fed mice. Thus, I3C decreased NF- κ B and expression of metastatic NF- κ B-regulated biomarkers in MDA-MB-231 cells both *in vitro* and *in vivo* [12].

Other data on modulation NF- κ B by I3C in cancer cells are fragmented and lacking quantification. Decreased NF- κ B DNA binding, coinciding with significant I3C-induced apoptosis, was found in prostate PC3 cells by Sarkar and co-authors [32]. Since NF- κ B p50 and p65 are substrates for caspases [74], reduction in NF- κ B DNA binding during apoptosis is likely to be a result of increased caspase activity, but not an initiating event in I3C-induced cell death. Similarly, I3C decreased NF- κ B DNA binding at 5 hour treatment with 250-1000 μ M I3C in MDA-MB-468, but not HBL100 cells [28]. Extremely high I3C concentrations up to 500-1000 μ M, used in that study, induce apoptosis at 5 h [29] and are likely to produce artificial results. Another study by Rahman et al [75], reporting that NF- κ B DNA binding and reporter gene expression in malignant breast MCF10CA1a cells were regulated by overexpression of Akt and influenced by I3C via Akt downregulation, did not provide functional evidence of reduced NF- κ B in apoptosis [75].

Our data suggest that the effect of I3C or specific inhibitors on NF- κ B signalling varies in breast cancer cell lines. NF- κ B-regulated reporter gene expression was downregulated in HBL100 cells, but was transiently increased in MDA-MB-468 cells. NF- κ B DNA binding (LB) was also increased at 6.5 h, the time equivalent to the reporter gene rise in these cells, and was reduced by Src inhibition. I3C-induced increase in DNA binding was also preceded by physical association of IKK β with the activated Src-EGFR complex and increased phosphorylation of I κ B α . Interestingly, an increased IKK activity at 5 h treatment with 250-750 μ M I3C was shown previously [28]. On the

contrary, no changes in NF- κ B DNA binding were found in other cells, some increase was observed in STAT5 DNA binding in this study. This may be related to differential Src signalling in those cells; e.g. I3C-induced Src activation involved CAS signalling in HBL100 cells [31]. Transcriptional activation of STAT5b is shown to be related to Src activity in MDA-MB-231 cells [76]. Hence, these data support the important roles of Src and EGFR in I3C-induced signalling. Although the mechanism of I3C-induced downregulation of NF- κ B in HBL100 cells is not clear; it is not related to Akt, since no changes in total or pS473 Akt were detected in HBL100 cells treated with I3C for 18-30 h [30]. Neither has the cause of I3C-induced NF- κ B downregulation in MDA-MB-231 cells, myeloid or leukaemia cells been elucidated.

I3C-induced transient increase in the NF- κ B signalling in MDA-MB-468 cells is unlikely to cause apoptosis, since prominent LY294002-induced upregulation of this pathway caused only a slight increase in caspase3/7 activity, but did not change either the cell number or phosphatidylserine externalisation (compare Fig. 3 and Fig. 2). Likewise, I3C-induced decrease in the NF- κ B signalling in HBL100 cells was not related to apoptosis, as a similar decrease, induced by LY294002, reduced the cell number without cell death in HBL100 cells (compare Fig. 3 and Fig. 2). Hence, there was no correlation between I3C-induced modulation of NF- κ B and I3C-induced apoptosis in breast cancer cells in our study. There are no data in this study to support a causal role of NF- κ B in I3C-induced apoptosis. However, I3C potentiated TNF α -induced cell death in TNF α -sensitive myeloid and leukaemia cells [33] and HBL100 cells. Moreover, I3C-induced apoptosis was enhanced by TNF α in TNF α -resistant MDA-MB-468 and MDA-MB-231 cells. Therefore, combinations of I3C with other cytotoxic agents should be investigated with regard to cancer cell death.

Collectively, our data show that the regulation and consequences of constitutive nuclear NF- κ B are cell-specific and the pathway is regulated in a complex way, which results in cell-specific modulation of expression of NF- κ B-regulated reporter gene and endogenous target genes in breast cancer cells. I3C caused cell-specific modulation of this pathway, which was not related to I3C-

induced cell death. Our data warrant further investigation into chemopreventive efficacy of I3C in combined treatments.

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References

1. Parkin D M, Bray F, Ferlay J et al (2005) Global cancer statistics, 2002. *CA Cancer J Clin* 55:74-108
2. Gandini S, Merzenich H, Robertson C et al (2000) Meta-analysis of studies on breast cancer risk and diet: the role of fruit and vegetable consumption and the intake of associated micronutrients. *Eur J Cancer* 36:636-646
3. Dos Santos Silva I, Mangtani P, McCormack V et al (2002) Lifelong vegetarianism and risk of breast cancer: a population-based case-control study among South Asian migrant women living in England. *Int J Cancer* 99:238-244
4. Hermann S, Linseisen J, Chang-Claude J (2002) Nutrition and breast cancer risk by age 50: a population-based case-control study in Germany. *Nutr Cancer* 44:23-34
5. Hirose K, Takezaki T, Hamajima N et al (2003) Dietary factors protective against breast cancer in Japanese premenopausal and postmenopausal women. *Int J Cancer* 107:276-282
6. Sieri S, Krogh V, Pala V et al (2004) Dietary patterns and risk of breast cancer in the ORDET cohort. *Cancer Epidemiol Biomarkers Prev* 13:567-572
7. Fung T T, Hu F B, Holmes M D et al (2005) Dietary patterns and the risk of postmenopausal breast cancer. *Int J Cancer* 116:116-121
8. Ambrosone C B, McCann S E, Freudenheim J L et al (2004) Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. *J Nutr* 134:1134-1138

9. Pathak D R, He J P, Charzewska J (2005) Joint association of high cabbage/sauerkraut intake at 12-13 years of age and adulthood with reduced breast cancer risk in Polish migrant women: Results from the US component of the Polish Womens Health Study (PWHs). *Cancer Epidemiology Biomarkers & Prevention* 14:2755S-2755S
10. Malloy V L, Bradlow H L, Orentreich N (1997) Interaction between a semisynthetic diet and indole-3-carbinol on mammary tumor incidence in Balb/cfC3H mice. *Anticancer Res* 17:4333-4337.
11. Bradlow H L, Michnovicz J, Telang N T et al (1991) Effects of dietary indole-3-carbinol on estradiol metabolism and spontaneous mammary tumors in mice. *Carcinogenesis* 12:1571-1574.
12. Rahman K M, Sarkar F H, Banerjee S et al (2006) Therapeutic intervention of experimental breast cancer bone metastasis by indole-3-carbinol in SCID-human mouse model. *Mol Cancer Ther* 5:2747-2756
13. Wattenberg L W, Loub W D (1978) Inhibition of polycyclic aromatic hydrocarbon-induced neoplasia by naturally occurring indoles. *Cancer Res* 38:1410-1413.
14. Kojima T, Tanaka T, Mori H (1994) Chemoprevention of spontaneous endometrial cancer in female Donryu rats by dietary indole-3-carbinol. *Cancer Res* 54:1446-1449.
15. Grubbs C J, Steele V E, Casebolt T et al (1995) Chemoprevention of chemically-induced mammary carcinogenesis by indole-3-carbinol. *Anticancer Res* 15:709-716.
16. Bell M C, Crowley-Nowick P, Bradlow H L et al (2000) Placebo-controlled trial of indole-3-carbinol in the treatment of CIN. *Gynecol Oncol* 78:123-129
17. Naik R, Nixon S, Lopes A et al (2006) A randomized phase II trial of indole-3-carbinol in the treatment of vulvar intraepithelial neoplasia. *Int J Gynecol Cancer* 16:786-790
18. Rosen C A, Bryson P C (2004) Indole-3-carbinol for recurrent respiratory papillomatosis: long-term results. *J Voice* 18:248-253

19. Malejka-Giganti D, Parkin D R, Bennett K K et al (2007) Suppression of mammary gland carcinogenesis by post-initiation treatment of rats with tamoxifen or indole-3-carbinol or their combination. *Eur J Cancer Prev* 16:130-141
20. Donald S, Verschoyle R D, Greaves P et al (2004) Dietary agent indole-3-carbinol protects female rats against the hepatotoxicity of the antitumor drug ET-743 (trabectedin) without compromising efficacy in a rat mammary carcinoma. *Int J Cancer* 111:961-967
21. Anderton M J, Manson M M, Verschoyle R D et al (2004) Pharmacokinetics and tissue disposition of indole-3-carbinol and its acid condensation products after oral administration to mice. *Clin Cancer Res* 10:5233-5241
22. Reed G A, Arneson D W, Putnam W C et al (2006) Single-dose and multiple-dose administration of indole-3-carbinol to women: pharmacokinetics based on 3,3'-diindolylmethane. *Cancer Epidemiol Biomarkers Prev* 15:2477-2481
23. Crowell J A, Page J G, Levine B S et al (2006) Indole-3-carbinol, but not its major digestive product 3,3'-diindolylmethane, induces reversible hepatocyte hypertrophy and cytochromes P450. *Toxicol Appl Pharmacol* 211:115-123
24. Zhang X, Malejka-Giganti D (2003) Effects of treatment of rats with indole-3-carbinol on apoptosis in the mammary gland and mammary adenocarcinomas. *Anticancer Res* 23:2473-2479.
25. Parkin D R, Malejka-Giganti D (2004) Differences in the hepatic P450-dependent metabolism of estrogen and tamoxifen in response to treatment of rats with 3,3'-diindolylmethane and its parent compound indole-3-carbinol. *Cancer Detect Prev* 28:72-79
26. Leibelt D A, Hedstrom O R, Fischer K A et al (2003) Evaluation of chronic dietary exposure to indole-3-carbinol and absorption-enhanced 3,3'-diindolylmethane in sprague-dawley rats. *Toxicol Sci* 74:10-21
27. Chinni S R, Sarkar F H (2002) Akt inactivation is a key event in indole-3-carbinol-induced apoptosis in PC-3 cells. *Clin Cancer Res* 8:1228-1236.

28. Howells L M, Gallacher-Horley B, Houghton C E et al (2002) Indole-3-carbinol inhibits protein kinase B/Akt and induces apoptosis in the human breast tumor cell line MDA MB468 but not in the nontumorigenic HBL100 line. *Mol Cancer Ther* 1:1161-1172.
29. Howells L M, Hudson E A, Manson M M (2005) Inhibition of phosphatidylinositol 3-kinase/protein kinase B signaling is not sufficient to account for indole-3-carbinol-induced apoptosis in some breast and prostate tumor cells. *Clin Cancer Res* 11:8521-8527
30. Moiseeva E P, Fox L H, Howells L M et al (2006) Indole-3-carbinol-induced death in cancer cells involves EGFR downregulation and is exacerbated in a 3D environment. *Apoptosis* 11:799-812
31. Moiseeva E P, Heukers R, Manson M M (2007) EGFR and Src are involved in indole-3-carbinol-induced death and cell cycle arrest of human breast cancer cells. *Carcinogenesis* 28:435-445
32. Chinni S R, Li Y, Upadhyay S et al (2001) Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. *Oncogene* 20:2927-2936.
33. Takada Y, Andreeff M, Aggarwal B B (2005) Indole-3-carbinol suppresses NF- κ B and I κ B α kinase activation causing inhibition of expression of NF- κ B-regulated antiapoptotic and metastatic gene products and enhancement of apoptosis in myeloid and leukemia cells. *Blood*
34. Kucharczak J, Simmons M J, Fan Y et al (2003) To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene* 22:8961-8982
35. Hoffmann A, Natoli G, Ghosh G (2006) Transcriptional regulation via the NF-kappaB signaling module. *Oncogene* 25:6706-6716
36. Karin M, Cao Y, Greten F R et al (2002) NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2:301-310
37. Rayet B, Gelinas C (1999) Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* 18:6938-6947

38. Romieu-Mourez R, Kim D W, Shin S M et al (2003) Mouse mammary tumor virus c-rel transgenic mice develop mammary tumors. *Mol Cell Biol* 23:5738-5754
39. Varela L M, Stangle-Castor N C, Shoemaker S F et al (2001) TNFalpha induces NFkappaB/p50 in association with the growth and morphogenesis of normal and transformed rat mammary epithelial cells. *J Cell Physiol* 188:120-131
40. Landesman-Bollag E, Song D H, Romieu-Mourez R et al (2001) Protein kinase CK2: signaling and tumorigenesis in the mammary gland. *Mol Cell Biochem* 227:153-165.
41. Bhat-Nakshatri P, Newton T R, Goulet R, Jr. et al (1998) NF-kappaB activation and interleukin 6 production in fibroblasts by estrogen receptor-negative breast cancer cell-derived interleukin 1alpha. *Proc Natl Acad Sci U S A* 95:6971-6976
42. Pederson L, Winding B, Foged N T et al (1999) Identification of breast cancer cell line-derived paracrine factors that stimulate osteoclast activity. *Cancer Res* 59:5849-5855
43. Bhat-Nakshatri P, Sweeney C J, Nakshatri H (2002) Identification of signal transduction pathways involved in constitutive NF-kappaB activation in breast cancer cells. *Oncogene* 21:2066-2078
44. Biswas D K, Cruz A P, Gansberger E et al (2000) Epidermal growth factor-induced nuclear factor kappa B activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. *Proc Natl Acad Sci U S A* 97:8542-8547
45. Madrid L V, Wang C Y, Guttridge D C et al (2000) Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. *Mol Cell Biol* 20:1626-1638
46. Ali S, Kaur J, Patel K D (2000) Intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and regulated on activation normal T cell expressed and secreted are expressed by human breast carcinoma cells and support eosinophil adhesion and activation. *Am J Pathol* 157:313-321.

47. Newton T R, Patel N M, Bhat-Nakshatri P et al (1999) Negative regulation of transactivation function but not DNA binding of NF-kappaB and AP-1 by IkappaBbeta1 in breast cancer cells. *J Biol Chem* 274:18827-18835.
48. Mendez O, Martin B, Sanz R et al (2006) Underexpression of transcriptional regulators is common in metastatic breast cancer cells overexpressing Bcl-xL. *Carcinogenesis* 27:1169-1179
49. Espana L, Fernandez Y, Rubio N et al (2004) Overexpression of Bcl-xL in human breast cancer cells enhances organ-selective lymph node metastasis. *Breast Cancer Res Treat* 87:33-44
50. Neve R M, Chin K, Fridlyand J et al (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 10:515-527
51. Rice N R, MacKichan M L, Israel A (1992) The precursor of NF-kappa B p50 has I kappa B-like functions. *Cell* 71:243-253
52. Patel N M, Nozaki S, Shortle N H et al (2000) Paclitaxel sensitivity of breast cancer cells with constitutively active NF-kappaB is enhanced by IkappaBalpha super-repressor and parthenolide. *Oncogene* 19:4159-4169.
53. Chen L F, Greene W C (2004) Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol* 5:392-401
54. Glasgow J N, Qiu J, Rassin D et al (2001) Transcriptional regulation of the BCL-X gene by NF-kappaB is an element of hypoxic responses in the rat brain. *Neurochem Res* 26:647-659
55. Gordon L A, Mulligan K T, Maxwell-Jones H et al (2003) Breast cell invasive potential relates to the myoepithelial phenotype. *Int J Cancer* 106:8-16.
56. Yin M J, Yamamoto Y, Gaynor R B (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* 396:77-80
57. Das R, Mahabeleshwar G H, Kundu G C (2003) Osteopontin stimulates cell motility and nuclear factor kappaB-mediated secretion of urokinase type plasminogen activator through

- phosphatidylinositol 3-kinase/Akt signaling pathways in breast cancer cells. *J Biol Chem* 278:28593-28606.
58. Sliva D, Rizzo M T, English D (2002) Phosphatidylinositol 3-kinase and NF-kappaB regulate motility of invasive MDA-MB-231 human breast cancer cells by the secretion of urokinase-type plasminogen activator. *J Biol Chem* 277:3150-3157.
 59. Huang W C, Chen J J, Chen C C (2003) c-Src-dependent tyrosine phosphorylation of IKKbeta is involved in tumor necrosis factor-alpha-induced intercellular adhesion molecule-1 expression. *J Biol Chem* 278:9944-9952
 60. Huang W C, Chen J J, Inoue H et al (2003) Tyrosine phosphorylation of I-kappa B kinase alpha/beta by protein kinase C-dependent c-Src activation is involved in TNF-alpha-induced cyclooxygenase-2 expression. *J Immunol* 170:4767-4775
 61. Jalal D I, Kone B C (2006) Src activation of NF-kappaB augments IL-1beta-induced nitric oxide production in mesangial cells. *J Am Soc Nephrol* 17:99-106
 62. Qing G, Qu Z, Xiao G (2005) Regulation of NF-kappa B2 p100 processing by its cis-acting domain. *J Biol Chem* 280:18-27
 63. Saccani S, Pantano S, Natoli G (2003) Modulation of NF-kappaB activity by exchange of dimers. *Mol Cell* 11:1563-1574
 64. Moorthy A K, Ghosh G (2003) p105.Ikappa Bgamma and prototypical Ikappa Bs use a similar mechanism to bind but a different mechanism to regulate the subcellular localization of NF-kappa B. *J Biol Chem* 278:556-566
 65. Lombaerts M, van Wezel T, Philippo K et al (2006) E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. *Br J Cancer* 94:661-671
 66. Funakoshi-Tago M, Tago K, Andoh K et al (2005) Functional role of c-Src in IL-1-induced NF-kappa B activation: c-Src is a component of the IKK complex. *J Biochem (Tokyo)* 137:189-197

67. Abu-Amer Y, Ross F P, McHugh K P et al (1998) Tumor necrosis factor- α activation of nuclear transcription factor- κ B in marrow macrophages is mediated by c-Src tyrosine phosphorylation of I κ B. *J Biol Chem* 273:29417-29423
68. Fan C, Li Q, Zhang Y et al (2004) I κ B α and I κ B β possess injury context-specific functions that uniquely influence hepatic NF- κ B induction and inflammation. *J Clin Invest* 113:746-755
69. Habib A A, Chatterjee S, Park S K et al (2001) The epidermal growth factor receptor engages receptor interacting protein and nuclear factor- κ B (NF- κ B)-inducing kinase to activate NF- κ B. Identification of a novel receptor-tyrosine kinase signalosome. *J Biol Chem* 276:8865-8874
70. Kapoor G S, Zhan Y, Johnson G R et al (2004) Distinct domains in the SHP-2 phosphatase differentially regulate epidermal growth factor receptor/NF- κ B activation through Gab1 in glioblastoma cells. *Mol Cell Biol* 24:823-836
71. Beraud C, Henzel W J, Baeuerle P A (1999) Involvement of regulatory and catalytic subunits of phosphoinositide 3-kinase in NF- κ B activation. *Proc Natl Acad Sci U S A* 96:429-434
72. Davies S P, Reddy H, Caivano M et al (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95-105
73. Monks N R, Pardee A B (2006) Targeting the NF- κ B pathway in estrogen receptor negative MDA-MB-231 breast cancer cells using small inhibitory RNAs. *J Cell Biochem*
74. Chang H Y, Yang X (2000) Proteases for cell suicide: functions and regulation of caspases. *Microbiol Mol Biol Rev* 64:821-846
75. Rahman K M, Li Y, Sarkar F H (2004) Inactivation of akt and NF- κ B play important roles during indole-3-carbinol-induced apoptosis in breast cancer cells. *Nutr Cancer* 48:84-94
76. Kloth M T, Laughlin K K, Biscardi J S et al (2003) STAT5b, a Mediator of Synergism between c-Src and the Epidermal Growth Factor Receptor. *J Biol Chem* 278:1671-1679

Figure legends

Fig. 1. Constitutive activation of NF- κ B in breast cancer cells

A. Western blots of cytoplasmic (c) and nuclear (n) extracts of breast cancer cells (40 μ g/lane) were developed with the antibodies against NF- κ B p50, p52, p65, RelB, cRel (from Dr Rice) and lamin A/C, indicated on the right. Antibodies against p50 and p52 recognised p50/p105 and p52/p100, respectively. A nuclear envelop component lamin A/C was used as a control of the fractionation procedure. **B.** EMSA of nuclear extracts (10 μ g/lane) incubated with the [32 P]-labelled NF- κ B oligonucleotide, separated by PAGE and autoradiographed. UB, upper band; MB, middle band; LB, lower band; SS, supershift band. **C.** EMSA of nuclear extracts (5 μ g/lane), pre-incubated with/without antibodies, followed by incubation with the [32 P]-labelled NF- κ B oligonucleotide with/without non-labelled oligonucleotide (x15 excess). Antibodies from Santa Cruz are marked as SC. **D.** The levels of reporter gene expression in cells transfected with the plasmid, containing pNF- κ B-regulated reporter gene, are shown. The ratio of activities of firefly luciferase to renilla luciferase was estimated for each cell line and normalised to the ratio in HBL100 cells. n=16. **E.** mRNA levels of IL-6, ICAM1, uPA, Bcl-xL and I κ B α , measured by real-time PCR, were normalised to the levels in HBL100 cells. n=3 in triplicates. *, P<0.05 vs. HBL100; #, P<0.05 vs. MDA-MB-468; “, P=0.0602 vs. HBL100.

Fig. 2. Effect of inhibitors on constitutive activation of NF- κ B and viability

A. Expression of NF- κ B-regulated-reporter gene in cells, transfected for 36 hours followed by treatments with specific inhibitors of PI3K (10 μ M LY294002), Src (5 μ M PP2), EGFR (1 μ M PD 153035) or IKK β (100 μ M aspirin) for 24 hours, is shown. The data are expressed as a percentage of control DMSO-treated cells. n=23. **B.** mRNA levels of IL-6, ICAM1, uPA, Bcl-xL and I κ B α in cells, treated with DMSO or 10 μ M LY294002 for 30 h, were normalised to the levels in control DMSO-treated cells. n=3 in triplicates. **C.** Cell viability of cells, treated with the inhibitors of PI3K (10 μ M LY294002), Src (5 μ M PP2), EGFR (1 μ M PD 153035) or IKK β (100 μ M aspirin) for 48

hours, is shown. n=8. **D.** Caspase3/7 activity in cells treated with the inhibitors, as in **C**, is shown. n=8. **E.** Number of live, apoptotic and necrotic cells was measured by phosphatidylserine externalisation in cells treated with DMSO or 10 μ M LY294002 for 48 hours. n=8. *, P<0.05 vs. untreated control.

Fig. 3. Effect of I3C on NF- κ B-regulated reporter gene expression

NF- κ B-regulated reporter gene expression in MDA-MB-468 (**A**) and HBL 100 (**B**) cells, transfected with pNF- κ B luc and pRL-SV40 plasmids and incubated in the presence or absence of indicated concentrations (μ M) of I3C for indicated number of hours, is shown. n=16. *, P<0.05. **C.** TNF- α -induced NF- κ B-regulated reporter gene expression is shown. MDA-MB-468 and HBL100 cells, transfected with pNF- κ B luc and pRL-SV40 for 48 hours, were treated with 250 μ M I3C for 1 or 6 hours and 10 ng/ml TNF α for another 30 min, followed by incubation in DMEM for 5 h. n=16. #, P<0.05 “I3C” vs. “no I3C”; *, P<0.05 “TNF α ” vs. “no TNF α ”.

Fig. 4. I3C-induced activation of NF- κ B pathway in MDA-MB-468 cells

A. MDA-MB-468 cells were treated with 125 μ M I3C for 3.5-7 hours. Western blots were developed with the antibodies indicated on the left. **B.** MDA-MB-468 cells were treated with 125 μ M I3C for 5 h. Proteins, immunoprecipitated (500 μ g/lane) with anti-c-Src antibodies, were detected with antibodies against Src, EGFR or IKK β . Results for Src, EGFR and actin (**A** and **B**) are reproduced from Fig. 2, published in [31]. **C.** Quantified results for NF- κ B DNA binding in the lower band of two EMSA experiments. **D.** EMSA. The cells were treated with indicated concentration of I3C for 5.5 or 6.5 hours with/without 10 μ M PP2. Nuclear extracts (10 μ g/lane) were incubated with the [32 P]-labelled NF- κ B (top panel) or STAT5 (bottom panel) oligonucleotide duplex with/without non-labelled oligonucleotides (x5 excess), separated by PAGE and autoradiographed. Specific upper (UB) and lower (LB) bands of NF- κ B are indicated on the right. Amounts of radioactivity in the LB are shown as percentage of control under the top panel.

Fig. 5. Effect of I3C in combination with TNF α on caspase3/7 activation in breast cancer cells

Caspase 3/7 activity in breast cancer cells, treated with 125 or 250 μ M I3C for 6 hours with 20 ng/ml TNF α , added in the same medium for another 24 hours. n=6. *, P<0.05 "I3C" vs. "no I3C"; #, P<0.05 "TNF α " vs. "no TNF α ".

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5