# Lycopene acts through inhibition of IκB kinase to suppress NF-κB signaling in human prostate and breast cancer cells

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# Abstract

We studied the effect of the potent dietary antioxidant lycopene on multiple points along the nuclear factor kappa B (NF-κB) signaling pathway in prostate and breast cancer cells. Lycopene significantly inhibited prostate and breast cancer cell growth at physiologically relevant concentrations of ≥1.25 μM. Similar concentrations also caused a 30-40% reduction in IκB phosphorylation in the cells, as determined by western blotting. Furthermore, the same degree of inhibition by lycopene was observed for NF-κB transcriptional activity, as determined by reporter gene assay. Concomitant with this, immunofluorescence staining of lycopene-treated cells showed a significant suppression (≥25%) of TNF-induced NF-κB p65 subunit nuclear translocation. Further probing of lycopene's effects on upstream elements of the NF-κB pathway showed a 25% inhibition of both activity of recombinant IKKβ kinase in a cell-free *in vitro* assay, as well as activity of IKKβ immunoprecipitated from MDA-MB-231 cells treated with lycopene. In conclusion, the anti-cancer properties of lycopene may occur through inhibition of the NF-κB signaling pathway, beginning at the early stage of cytoplasmic IKK kinase activity, which then leads to reduced NF-κB–responsive gene regulation. Furthermore, these effects in cancer cells were observed at concentrations of lycopene that are relevant and achievable *in vivo*.


**Keywords**: Prostate cancer; Breast cancer; IκB kinase; Lycopene; NF-κB; Nutrition.

**Abbreviations:** NF-κB (nuclear factor kappa B); IκB (inhibitor of kappa B); IKK (IκB kinase).

# Introduction

Prostate and breast cancer are two of the most common cancers worldwide. Prostate cancer is the most common cancer in men in the UK whilst breast cancer is the second most common cancer worldwide [1]. Although age and genetic factors play key roles, many studies indicate that lifestyle and diet are also significant aspects involved in cancer risk [2]. While preventative measures for both cancers are not well known, increased tomato intake, as highlighted first by the Health Professionals Follow-Up Study (HPFS) and a few other studies, has been linked to a reduced risk of prostate cancer [3, 4]. This potential chemo-preventative effect of increased tomato intake has been attributed to their richness in the red pigment carotenoid lycopene [5-8]. An association between plasma levels of lycopene and reduced risk of aggressive prostate cancer was reported in the Physicians Health study, which showed a 25% reduction in overall prostate cancer with plasma lycopene concentrations of 388 ng/ml (0.72 μM) and 44% lower incidence of aggressive prostate cancer at plasma lycopene concentrations of 356 ng/ml (0.66 μM) [5]. Due to its lipophilic nature, lycopene accumulates in the prostate, adrenal gland, testes and liver [9], which could explain its particular connection to prostate cancer risk. However, those findings led to investigations in a number of other cancer types and diseases. Although no significant link between dietary lycopene intake and breast cancer has yet been made [6, 7], a number of studies have demonstrated its chemo-preventative effects in breast cancer cell cultures [8, 10-12].

The putative chemo-preventative activity of lycopene may be attributed to it potent antioxidant properties, derived from an electron-rich conjugated polyene chain, making it the most effective single oxygen-quenching carotenoid. Thus, it is highly effective in deactivating electrophiles such as oxygen and free radicals, formed as cellular oxidative

by-products, and which can cause oxidative stress and damage to cellular components [13]. Several cell culture-based investigations as well as some *in vivo* studies have shown lycopene to reduce DNA, protein and lipid damage from oxidative injury [14, 15]. However, in the case of prostatic carcinoma cell lines, different studies have reported variable inhibitory efficacies of lycopene as regards cell proliferation [16-19]. One study found lycopene at 1 μM to be sufficient for reducing proliferation in LNCaP cells [16], whereas in another study using PC3, DU145 and LNCaP cells, lycopene reduced cell viability and growth only at high concentrations (20μM) which is too high to be realistically achievable *in vivo* [17]. Within the physiologically relevant concentration range, no effect of lycopene was observed on LNCaP cell proliferation [18], whilst in contrast, a decrease in DU145 cell growth was reported at particularly low concentrations (10 nM) [19].

Therefore, there remains an uncertainty as to the actual efficacy of lycopene as an anticancer molecule at physiologically relevant concentrations. We recently showed that lycopene inhibited angiogenesis of endothelial cells at physiologically relevant concentrations [20]. In prostate cancer cells, we showed that lycopene at 1.15 μM inhibited motility of PC3 and DU145 cells by 40% and 58% respectively, as well as inhibiting cell adhesion at ≥1.25 μM [21].

The redox-sensitive transcription factor nuclear factor-κB (NF-κB) controls the transcriptional activation of many fundamental genes that regulates cell proliferation, apoptosis, metastasis, angiogenesis, inflammation, cellular adhesion and cell invasion [22]. Consequently, aberrant signaling along the NF-κB pathway is believed to mediate several major cellular processes that occur in carcinogenesis. Constitutive activation of the NF-κB pathway has been reported in several cancer cell lines [22], including from prostate tumors [23]. Over-activity of the NF-κB pathway has been reported during the

early developmental stages of prostate cancer [24, 25], whilst inhibition of the NF-κB pathway was shown to reduce the malignant properties of PC3 cells [26]. Furthermore, constitutive activation of NF-κB has been linked to tumor progression in hormone-independent breast cancer cell lines such as MDA-MB-231 [27], and breast gland development and breast carcinogenesis have been linked with NF-κB-induced cyclin D1 expression [28, 29] and selective activation of Bcl-3 and NF-κB subunits, p50 and p52 [30]. Furthermore, mutations in NFKB1, IKK2, IκBα and IκBε have also been identified in breast cancer [31] as well as defective IκBα in solid breast tumours [32]. Therefore, therapeutic targeting the NF-κB pathway may represent a potential and multifaceted approach to combating cancers.

Several studies have shown the ability of a variety of antioxidant compounds to suppress NF-κB signaling in reproductive cancers including ovarian [33], breast [34] and prostate [35] cancers. Likewise, lycopene has been shown to inhibit Ras-dependent activation of the NF-κB pathway in prostate cancer cells, resulting in cell cycle inhibition and apoptosis [36]. In SK-Hep-1 cells, lycopene reduced the binding affinity of the NF-κB transcription factor complex to *MMP-9* gene promoter sequences [37]. Therefore, these clues warrant further mechanistic investigation into the effects of lycopene on the NF-κB signaling pathway in cancer cells. Importantly, whether lycopene can exert these potential effects at physiologically attainable concentrations also requires clarification. In the present study, we have conducted such an investigation, examining the NF-κB signaling pathway at multiple levels in both human prostate and breast cancer cells. Here, we report that lycopene at physiologically relevant concentrations inhibits NF-κB signaling through direct inhibition of the upstream activating kinase IKK, thus linking with suppression of the pathway observed at multiple points further downstream.

# Materials and methods

*Lycopene preparation*

Pre-filtered tetrahydrofuran (THF; Sigma-Aldrich, Poole, UK), stabilized with butylated hydroxytoluene (BHT) at 0.0025% (w/v) to avoid peroxide formation, was used for the preparation of lycopene stock solutions and aliquots of solution were stored in dark in amber vials at -80°C. Lycopene was a gift of DSM Nutritional Products, Basel, Switzerland. For each experiment, the concentration of stock solution of lycopene was checked using an extinction coefficient of 196145 $M^{-1}$ $cm^{-1}$ for a solution of lycopene in THF. Prior to treatment of cells, the stock solution was first diluted 1:10 in FBS (fetal bovine serum; Lonza, Slough, UK), which acts an effective delivery vehicle for the cellular uptake and stability of lycopene [38]. This was then further diluted in DMEM medium (Dulbecco's Modified Eagle Medium with 4.5 g/L L-glucose and L-glutamine; (Life Technologies Gibco, Paisley, UK) to achieve the desired experimental concentrations (0.5-5 μM). THF/BHT was used as a solvent vehicle control, and was also prepared through further dilution in the same manner as lycopene. We found that replenishing lycopene on a daily basis, which was performed in one of our experiments (Figure 1b), did not alter the experimental outcomes significantly compared to when the replenishment step was omitted.

*Cell culture*

Two human cancer cell lines were used in this investigation; PC3 cell line and MDA-MB-231 cell line. The PC3 cell line is androgen-independent, highly malignant, pro-metastatic, and is an established human prostate cancer cell line that was initiated from a bone metastasis of a 62-year old Caucasian. The MDA-MB-231 cell line is also pro-

metastatic, estrogen-receptor negative and initiated from a breast metastasis of a 51-year old female Caucasian. Both cell lines were purchased from European Collection of Cell Cultures (ECACC) in 2013, and were authenticated in-house by short tandem repeat (STR) analysis as previously described [39]. Cells were maintained in culture in complete medium composed of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Lonza). Cells were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Cells were seeded at $0.5x10^3$ for MTS assays in 96-well plates. Cells were seeded at concentrations of $1x10^4$ cells/ml and $3x10^3$ cells/ml in 24-well plates, for western blot and luciferase assay, respectively. Cells used for IP were plated in 100mm dishes and treated once 80-90% confluence was reached. A cocktail of phosphatase inhibitors (Sigma-Aldrich), calyculin A and Sodium orthovanadate was added to cell lysates for all downstream applications.

*MTS cell growth assay*

Cell survival/growth in the presence of lycopene (0.5-5 μM, 48 h) was measured using the colorimetric MTS assay method. MTS tetrazolium compound (CellTiter 96® Aqueous MTS reagent; Promega, Southampton, UK) was mixed with PMS (phenazine methosulfate; Sigma-Aldrich) to form MTS assay reagent. 20 μL MTS-PMS complex was added per well to the 96-well plates at the end of the incubation periods with test agents. The catalytic activity of viable cells results in formazan dye production, which is then quantified. Cells were incubated with the dye for 1h, followed by absorbance reading at 492nm on a spectrophotometer (BioTek, Potton, UK).

*Western blot*

Cells were seeded into tissue culture multiwell plates, and on the following day, the growth medium was replaced with serum-free medium containing various concentrations of either lycopene (1.25-5 μM) or THF as vehicle control. The cells were incubated further for 20 h. Recombinant human TNFα (R&D Systems, Abingdon, UK) was added to cells at 40ng/ml 30 mins prior to lysis for use as a positive control inducer of the NF-κB pathway. Cells were washed with ice-cold PBS and lysed in 75 μl/well lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 nM Tris pH 8.0) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich), Calyculin A and Sodium orthovanadate. Cell lysates were removed from the well and clarified by centrifugation at maximum speed in a microfuge for 10 min at 4°C. The soluble cell extracts were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), alongside a molecular weight marker sample spanning the 10-250kDa range (PageRuler Plus pre-stained protein ladder; Thermo Scientific, Loughborough, UK). Separated proteins on the gels were transferred to an activated PVDF membrane (Immun-Blot®; Bio Rad, Hercules, CA) using a fast semi-dry protein transfer method (Pierce G2 Fast Blotter and 1-Step Transfer Buffer; Thermo Scientific). Membranes were first incubated in blocking buffer (TBS-Tween 20 wash buffer containing 3% BSA) for 1 h at room temperature, with the exception of blots probed for phosphorylated IκBα (p-IκBα), which were not blocked so as to avoid over-quenching of the signal. Membranes were incubated with primary antibody overnight at 4°C, following which they were washed 3 x 5 min in wash buffer, and then incubated with HRP-conjugated secondary antibody for 2h at room temperature. Primary antibodies that had been characterized and reported in previous studies were used at recommended dilutions as follows: rabbit anti-p-IκB-α (Ser 32/36) (Cat. No. sc-101713, Santa Cruz Biotechnology, Heidelberg, Germany) [40], and anti-actin rabbit polyclonal

(Cat. No. A2066-.2ML) (Sigma-Aldrich) [41]. Secondary antibody was horseradish peroxidase (HRP)-conjugated swine anti-rabbit Ig (Product No. p021702-2, Dako, Glostrup, Denmark) [42]. After washing, the membrane was developed by incubation with an enhanced chemiluminescence reagent followed by imaging of the blot under a CCD camera imaging system (G:BOX Chemi XL1.4, Syngene, Cambridge, UK).

*Cell fixing and Immunofluorescence-staining*

MDA-MB-231 and PC3 cells were seeded onto coverslips placed in 6 well culture plates. Cells at ~60% confluence were serum-starved overnight, after which they were pre-treated with lycopene (5 μM) or THF vehicle for 2 h. Cells were then stimulated with TNFα (40ng/ml) over a time-course of 1-5 h. Cells were fixed with 4% paraformaldehyde diluted in PBS and then permeabilized with 0.2% Triton X-100 diluted in PBS; 2 x 5 min PBS washes were performed in between fixing and permeabilization. Cells were blocked for 1 h at room temperature with 10% horse serum diluted in PBS, followed by 1 x 5 min PBS wash. Cells on coverslips were incubated with rabbit polyclonal NF-κB p65 primary antibody (C-20; Santa Cruz) [43] for 1 h at room temperature. The secondary antibody was Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A-21206; Life Technologies) [44], incubated for 1 h at room temperature in the dark. Three x 5 min washes were performed between antibody incubation stages. Cells were mounted onto microscope slides using Vectashield (Vector Laboratories, Burlingame, CA). Images were obtained using a fluorescence microscope (FSX100; Olympus, Southend-on-Sea, UK).

*NF-κB-responsive gene activation reporter assay*

The NF-κB activity level in cell lysates at the level of NF-κB–responsive gene expression was measured by luciferase-based reporter assay (Promega dual-luciferase® reporter assay system). The firefly luciferase reporter plasmid contains 5 copies of an NF-κB response element (PGL4.32[*luc2P*/NF-κB-RE/Hygro] vector; Promega). This plasmid drives transcription of the luciferase reporter gene *luc2P* (*Photinus pyralis*). Cell treatment incubation time for luciferase assay lysates was approximately 72 h and did not include a serum starvation period. Each concentration of lycopene (0.5-5 μM) had an equivalent dilution of THF as matched control. Cells were transfected 24 h after plating with firefly and transfection control *Renilla* luciferase reporter plasmids using jetPRIME® transfection reagents according to manufacturer's protocol (Polyplus, Nottingham, UK). Cells were incubated with transfection reagent mixed with firefly and transfection control *Renilla* luciferase reporter plasmids, and 72 h later were washed with PBS and lysed with luciferase assay lysis buffer (Promega). Luciferase levels were measured for 10 sec using a luminometer (1450 microbeta, PerkinElmer, Seer Green, UK). Background readings of untransfected control lysates were subtracted from luminescence values. Values were normalized against positive transfection control (*Renilla* luciferase).

*Immunoprecipitation*

Once cells reached 80-90% confluence in 100mm dishes, cells were treated accordingly, by incubation overnight with THF, lycopene and staurosporine (10nM) as an IKK inhibitor positive control. Cells were washed with ice-cold PBS and lysed with 1 ml Pierce IP lysis buffer (Thermo Scientific) per dish. Cell lysates were clarified by centrifugation and pre-cleared with 10ul protein A/G-agarose beads (Santa Cruz) 1 h at 4°C with constant rotation. Lysates were then incubated with 2ug anti-IKKβ rabbit

polyclonal (Cat. No. SAB1300467-100UG) [45] 1 h on ice. Anti-Trx rabbit polyclonal antibody (Santa Cruz) was used as a negative control pulldown antibody. After this, 10ul protein A/G-agarose beads was added to each sample and incubated further for 1 h at 4°C with constant rotation. Beads were pelleted by centrifugation at 8000 x *g* for 1 min and the supernatant removed. Beads were washed by suspension in ice-cold IP lysis buffer followed by centrifugation at 8000 x g for 1 min, repeated three times, discarding the supernatant each time.

*In vitro IκB kinase (IKK) activity assay*

The IκB kinase β (IKKβ) Kinase Enzyme System and ADP-Glo™ Kinase assay were performed according to the manufacturer's instructions for each assay (Promega) in 96-well plates. The beads pellet obtained from immunoprecipitation of IKKβ was washed a final time with IKK reaction buffer (8000 x g for 1 min), discarding the supernatant. The ADP-Glo™ Kinase assay reaction buffer, containing ATP (25 μM) and the IKKβ substrate peptide IKKtide (0.2 ug/ul) (Promega) were added directly to the pellet, and the kinase reaction incubated for 1 h at room temperature, to allow IKK to convert ATP to ADP. After this, the beads were centrifuged (8000 x g for 1 min) to separate them from the supernatant, to be used for western blot analysis (see above). Subsequent probing of the pulldowns with the anti-IKKβ antibody in western blot revealed that the IKKβ IPs from each sample were successful and even. The supernatant was added to a 96-well plate and ADP-Glo™ reagent (Promega) was added to the wells and incubated at room temperature for 40 min, to halt the kinase reaction and deplete any remaining ATP. Kinase detection reagent was then added to wells and left to incubate for a final 30 min at room temperature. This final step converts ADP to newly synthesized ATP via a luciferase reaction. The same assay was also performed in a cell-free system. 100ng

recombinant IKKβ (Sigma-Aldrich) was pre-incubated with lycopene (5 μM) and assay reagents for 1 h. The reaction was then induced and incubated with IKKtide for a further hour. Luminescence was read using a microplate reader (BMG Labtech Fluorstar Optima, Offenburg, Germany).

*Statistical analysis*

*ImageJ* software (http://imagej.nih.gov/ij/) was used to perform densitometric analysis of relative nuclear:cytoplasmic ratio of immunostained cells and western blot protein bands, and density values of bands of interest were normalized against those of bands for actin. Paired T-test was used for pairwise analysis of THF- and lycopene-treated cells at varying concentrations; a $p$ value ≤0.05 was considered statistically significant. One way ANOVA with Tukey *post hoc* test was used for dose-response values. Prism software (GraphPad, La Jolla, CA) was used for all statistical analyses.

# Results

*Lycopene inhibits proliferation in PC3 and MBA-MD-231 cells*

We began our investigation by studying the effect of lycopene on the proliferation of prostate cancer cells. We found that incubation of PC3 and MBA-MD-231 cells for 48 h with lycopene (0.5-5 μM) caused a concentration-dependent decrease in growth of both cells lines, relative to THF vehicle (ANOVA, $p < 0.05$, DU145 ($p < 0.0001$) and PC3 ($p < 0.001$) *vs* control from 1.25 μM concentration upwards (Figure 1a). We found that the growth inhibitory effect of lycopene was significant only after incubation with cells for 48h. MBA-MD-231 cells that were treated with lycopene (5 μM) or THF for 24 h showed

no significant differences in growth, whereas 48 h incubation with lycopene resulted in an approximately 16% reduction versus THF (Paired T-test, $p < 0.05$) (Figure 1b).

*Lycopene inhibits IκB phosphorylation in PC3 and MBA-MD-231 cells*

To determine whether lycopene has an effect on NF-κB signaling pathway we initially investigated its effect on phosphorylation of inhibitor of kappa Bα (IκBα). PC3 and MBA-MD-231 cells were serum-starved and treated for 20 h with a range of concentrations of lycopene (1.25-5 μM), or THF solvent vehicle. We employed western blotting technique and found that in both cell lines a 30-40% reduction in p-IκBα protein levels was observed in lycopene-treated cells relative to THF. This effect was significant at all concentrations tested (1.25-5 μM) (Figure 2a). Furthermore, lycopene also significantly inhibited p-IκBα protein levels in both cancer cell lines pretreated for 30 min with TNFα in a concentration-dependent manner (Figure 2b; Suppl Fig. S1). Therefore, lycopene inhibited NF-κB signaling in prostate cancer cells at the level of IκBα phosphorylation by IKK, under both basal and TNF-stimulated conditions (Paired T-test, $*p < 0.05$ vs THF). We initially included a third cell line (human epithelial prostate cell line PNT2), however p-IκBα protein levels in this non-tumour cell line were too low to be detected (data not shown).

*Lycopene inhibits TNFα induced NF-κB p65 nuclear translocation in MDA-MB-231 and PC3 cells*

We then tested if the inhibition of IκBα phosphorylation by lycopene was concomitant with a decrease in NF-κB nuclear translocation. To determine this, we performed immunofluorescence-staining for the NF-κB p65 subunit (Figure 3). MDA-MB-231 and PC3 cells were serum starved overnight and then treated with lycopene (5 μM) or THF

vehicle the next day for 2 h. Following this, p65 nuclear translocation was induced in cells with TNFα (40ng/ml) over a time-course of 1-5 h. A peak effect by TNFα was found to occur at 2 h in both cell lines (MDA-MB-231, Figure 3a; PC3 Figure 3b) where the p65 nuclear:cytoplasmic ratio was ~2.5x greater than basal levels. Lycopene significantly suppressed p65 nuclear translocation from 2-5 h by at least ~25%, which almost mirrored basal levels in the presence of THF vehicle and absence of TNFα stimulation. The most significant effect of lycopene in both cell lines also occurred at 2 h, where p65 nuclear translocation was inhibited by approximately ~60% compared to cells in vehicle only (paired T-test, *$p < 0.05$ vs THF).

*Lycopene inhibits NF-κB transcriptional activity in MBA-MD-231 and PC3 cells*

We investigated whether the inhibition of p65 nuclear translocation caused by lycopene occurred at a transcriptional level downstream of the NF-κB pathway. For this we utilized a NF-κB-responsive gene activation reporter assay. PC3 and MBA-MD-231 cells were treated with a range of concentrations of lycopene (0.5-5 μM) or THF solvent vehicle alone for 72 h. Incubation of prostate cancer cells with lycopene caused a concentration-dependent decrease in NF-κB transcriptional activity, with reductions of 20-40% and 20-50% in MDA-MB-231 ($p < 0.05$) and PC3 cells ($p < 0.001$), respectively, in comparison to cells incubated with vehicle at the same dilutions (Figure 4). The inhibitory effects were significant starting from 1.25 μM (ANOVA, $p < 0.05$), with 0.5 μM lycopene showing no significant effect.

*Lycopene reduces IKKβ kinase activity in a cell-free system and in intact MBA-MD-231 cells*

Finally we examined whether lycopene's inhibitory effect on the NF-κB pathway also occurred at an upstream level by studying its effect on IKKβ kinase activity. We tested

this in a cell-free system as well as using MBA-MD-231 cells. Pure recombinant IKKβ was pre-incubated with 5 μM lycopene or THF solvent for 1 h, then a further incubation for 1 h in the presence of IKKtide substrate. Lycopene reduced IKKβ kinase activity by ~25% when compared to THF control ($p < 0.01$) (Figure 5a). Similarly, IKKβ from MBA-MD-231 cells treated overnight (~15 h) with lycopene (5 μM) showed a reduction in activity by ~25% (Figure 5b) compared to THF-treated cells. A vehicle effect was observed in both cell-free system and in MBA-MD-231 cells; THF reduced IKKβ kinase activity by ~20%. Staurosporine (10 nM) reduced IKKβ activity in cells by ~25% compared to baseline levels, and by ~40% in the presence of THF. This indicates that although there was a vehicle effect, this did not mask the effect of either lycopene or staurosporine. Therefore, these data indicate that lycopene directly acts to inhibit IKKβ kinase activity.

## Discussion

In this study, we have investigated the effects of lycopene specifically on the NF-κB signaling pathway. Here, we demonstrate that lycopene acts via inhibiting NF-κB signaling, the influence of which have observed at multiple levels along the pathway. MTS proliferation assays showed that lycopene reduced proliferation in both PC3 and MBA-MD-231 prostate cancer cells, which was apparent only after 48 h incubation. The inhibition was concentration-dependent, including concentrations that are physiologically relevant (1.25-5 μM). A conceivable mechanism for this growth inhibition is through the induction of cell cycle arrest and apoptosis [14, 30, 46-48]. Several studies have previously reported lycopene to inhibit growth of prostate cells, including DU145, LNCaP and human prostate epithelial cells [14, 48, 49]. Additionally,

studies using MCF-7 breast cancer cell line have also noted anti-proliferative activity from lycopene [11, 50].

We found that 20 h incubation with lycopene reduced phosphorylation of IκBα by approximately one third in both PC3 and MBA-MD-231 cells. This effect occurred both in the absence and presence of TNFα, a strong inducer of the NF-κB pathway [22]. The phosphorylation of IκBα occurs early in the NF-κB pathway; by which it instigates activation of this signal pathway, releasing NF-κB from sequestration by IκBα, and permitting translocation of NF-κB to the nucleus, hence driving target gene expression [22]. This therefore demonstrates that lycopene interferes with an early event in the NF-κB pathway, specifically the stage where IKK phosphorylates IκBα and thereby affects the cellular localization of NF-κB. The inhibition of IκBα phosphorylation occurred at all concentrations tested, including the lowest concentration (1.25 μM), which is an achievable physiological concentration of lycopene that has been measured in the blood [51, 52]. Furthermore, the maximum effect was observed at 2.5 μM; a higher concentration of 5 μM did not increase the effect. This effect was concomitant with lycopenes influence on nuclear translocation of NF-κB p65 subunit. We found that lycopene (5 μM) was able to dramatically inhibit TNFα-induced p65 nuclear translocation by as much as ~60% in MDA-MB-231 and PC3 cells. A similar effect has also been reported in LPS-stimulated murine dendritic cells, where lycopene (10 μM) was shown to suppress nuclear translocation of p65 [53].

We also probed the influence of lycopene on the downstream effects of the NF-κB pathway, where we employed a luciferase reporter assay to determine the transcriptional activity of cells bearing an NF-κB responsive promoter. In this experiment we uncovered that lycopene inhibited the NF-κB pathway (~20-40%) in a concentration-dependent manner in both cell lines tested, which was consistent with

our findings for upstream IκBα phosphorylation. This effect was most evident from concentrations 1.25 μM and upwards.

Finally we investigated the influence of lycopene on IKK, an upstream component of the activation of the NF-κB pathway. The IKK enzyme complex phosphorylates IκBα in response to inflammatory stimuli such as TNFα and lipopolysaccharide (LPS) [54]. There is evidence for a correlation between inflammation and prostate cancer [55, 56] and that cytokine-induced activation of IKK (IκB kinase) is majorly involved in NF-κB pathway activation [57-63]. Furthermore, there are also high levels of of IKK-i/IKKε and kinase activity in multiple breast cancer cell lines compared to MCF-10F breast epithelial cells [64], and increased IKKα and IKKβ activity in breast cancer cells and tumor specimens [65]. We employed an enzyme activity assay to observe the effects of lycopene on IKKβ activity. We found that lycopene (5 μM) significantly reduced IKKβ kinase activity by ~25% compared to vehicle in a cell-free system. This indicates a direct interaction between the IKKβ enzyme complex and lycopene. We then tested if a similar effect was achievable in whole prostate cancer cells. IKKβ obtained from MBA-MD-231 cells treated with lycopene (5 μM) ~15 h showed reduced kinase activity by ~25% compared to cells treated with THF. Therefore, these data confirm that lycopene directly inhibits the activity of IKKβ and that this can also occur within prostate and breast cancer cells *in vivo*.

While we have observed inhibition by lycopene of the NF-κB pathway in prostate and breast cancer cells at different levels, our data indicate that lycopene acts at an early stage in the pathway. Lycopene inhibits phosphorylation of IκBα by reducing IKK activity in the cytoplasm. This then has the expected consequence of a reduced translocation of NF-κB into the nucleus, and hence reduced NF-κB–responsive gene regulation. Recently, one study has found that 1 h incubation with synthetic lycopene

derivatives reduces TNFα induced NF-κB activity in T47D mammary carcinogenic cells and osteoblasts (MC3T3 and HOS) [60]. A preparation of intact lycopene (2.7 μM) with part-oxidised lycopene and derivatives resulted in 20-30% TNFα induced NF-κB activity inhibition. Furthermore 1 h incubation with lycopene derivatives (~6-10 μM) resulted in attenuated p-IκB protein levels and IKKβ activity. However this study found that intact lycopene alone did not have any inhibitory effect.

Our results showing lycopene suppression of the NF-κB pathway are in keeping with the role of lycopene as a potent antioxidant alongside several other antioxidants that have also been shown to act as NF-κB inhibitors, such as melatonin [33], grape seed extract [34] and nobiletin [35]. However, rather than through free radical scavenging, most of these antioxidants could possibly be acting *in vivo* through the ubiquitin proteasome system. Specifically, a target of lycopene could be the Keap1-Cul3-Rbx ligase complex, which ubiquitinates IKK and prepares it for degradation by the proteasome [66]. Under oxidative stress, or possibly through Keap1 mutations in certain cancers, Keap1 is modified and releases IKK, thus allowing it to phosphorylate IκB and activate the NF-κB pathway. Therefore, in addition to targeting IKK activity, lycopene may be causing dysregulation of the Keap1-mediated IKK ubiquitination process, thereby preventing NF-κB activation and subsequent tumor progression.

It is also possible that antioxidants such as lycopene could be acting directly on the Keap1 complex. Keap1 is also a sensor of oxidative stress through its interaction with the major antioxidant gene regulator Nrf2 [66-68]. Under stress conditions, Nrf2, which is a transcription factor, is released from Keap1 and translocates to the nucleus, where it binds to the antioxidant response element (ARE) and stimulates expression of antioxidative gene products that protect the cell [66, 67]. Lycopene has previously been shown to activate Nrf2 [69] and therefore could be doing this via the Keap1 complex. In

addition, as the Keap1 complex appears to perform a pro-tumorigenic role through its concomitant activation of NF-κB signaling and repression of Nrf2-ARE signaling, Keap1 may represent an attractive therapeutic target in cancer [70]. Therefore, the targeting of Keap1 by lycopene confers an added advantage to lycopene beyond its recognised free radical scavenging functions.

The results from our study indicate lycopene has an inhibitory effect on NF-κB signaling at concentrations ≥1.25 μM. A lower concentration of 0.5 μM had no effect, which may explain why in previous studies lycopene failed to show significant protective effects in cancer patients at plasma lycopene concentrations of ~0.60 μM [61, 62]. Plasma levels of lycopene can be raised easily with the consumption of processed tomatoes to 1.2 μM [63, 71, 72], and with one paper reporting an increase of up to 2.1 μM [73]. One study looking at the effect of tomato paste on cultured PC3 cells and nude mice with PC3 xenografts fed on a Western-like diet with 10% tomato paste for 6.5 weeks reported an inhibition of NF-κB activity, lycopene accumulation in the xenografts, and modulation of cancer and immune related genes [74]. Therefore, lycopene intervention studies of cancer patients should aim for raising the plasma lycopene levels above 1 μM and nutritionists should be looking into ways of increasing the bioavailability of lycopene from food/supplements so that plasma levels can be easily raised above 1.25μM.

This study helps to expand our understanding of the chemo-preventative attributes of lycopene and provides further explanation of reduced risk of prostate cancer and decreased prostate and breast tumour aggression in those with higher dietary intake of tomato products. The putative properties of this compound provide grounds for future research into its clinical usefulness, as there are currently too few clinical investigations that have examined the effectiveness of lycopene in prostate cancer patients. Therefore this, and the fact that our study has shown effectiveness of lycopene at physiological

concentrations, warrants the need for further investigation in regards to its clinical efficacy. Furthermore, this study is the first to use prostate and breast cancer cells, as well as using physiological concentrations.

In conclusion, we have shown in this study that lycopene inhibits the NF-κB signaling pathway at multiple levels in human prostate and breast cancer cells *in vitro*. Therefore, our data indicate the possibility that physiologically relevant levels of plasma lycopene, achievable through diet supplementation, could act through suppression of the NF-κB pathway in prostate and breast tumours in patients. Further, translational studies are required to determine this phenomenon in the clinical scenario.

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## Author contributions

EA and MCV performed the experiments, analyzed the results and wrote the paper. MC and SH conceived of the study, designed the experiments, analyzed the results and wrote the paper.

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**Conflict of Interest**: *None*.

## Figure Legends

**Fig. 1.** Effect of lycopene on survival/growth of PC3 and MDA-MB-231 cells. (**A**) Concentration-response experiment of effect of lycopene (0.5-5 μM) on PC3 and MDA-MB-231 cells treated for 48 h, compared to THF vehicle. Viable cell number was measured via MTS assay as described. Values are mean±SEM normalized percentage viable cells for MDA-MB-231 cells ($p < 0.0001$ vs THF; n=4 experiments) and PC3 ($p < 0.001$ vs THF; n=3 experiments). (**B**) Effect of lycopene (L) (5 μM) on MDA-MB-231 cells after 24 vs 48 h, compared to THF (T) vehicle. Viable cell number was measured via MTS assay as described. Values are mean±SEM of optical density (quadruplicate wells per treatment; 4 independent experiments). * $p < 0.05$; lycopene vs THF at 48 h.

**Fig. 2.** Effect of lycopene on phosphorylation of IκBα (p-IκBα) in PC3 and MDA-MB-231 cells. (**A**) Expression levels of p-IκBα protein in serum-starved PC3 and MDA-MB-231 cells treated with lycopene (1.25-5 μM) for 20 h compared THF vehicle. Band densitometric values for p-IκBα were normalized against band densities for actin. Values are expressed as percentage change from the control for that particular concentration. Values are mean±SEM of normalized densitometric value (n=4 separate experiments); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs THF). (**B**) Effect of lycopene on TNFα–stimulated IκBα phosphorylation in PC3 and MDA-MB-231 cells. Serum-starved cells were pre-treated with lycopene (1.25-5 μM) (or THF vehicle) for 20 h, after which TNFα (40ng/ml) was added for 30 min prior to lysis. Band densitometric values for p-IκBα were normalized against band densities for actin. Values are expressed as percentage change from the control for that particular concentration. Values are mean±SEM of normalized densitometric value (n=4 separate experiments); *$p < 0.05$,

**p < 0.01, ***p < 0.001 vs THF). (**C**) Western blot of p-IκBα protein level in lysates of serum-starved MDA-MB-231 cells treated with lycopene (L) at a range of concentrations (1.25-5 μM) or with THF vehicle (T) for 20 h, in the absence and presence of TNFα (40 ng/ml; added 30 min prior to lysis). Bands show expression of p-IκBα (37 kDa; upper panel) and actin (42 kDa; lower panel). The blot has been cropped and is representative of 4 separate experiments performed, the analyzed data from which is presented in (**A**) and (**B**).

**Fig. 3.** Effect of lycopene on TNFα induced NF-κB p65 nuclear translocation in MDA-MB-231 and PC3 cells. Serum-starved MDA-MB-231 (**A**) and PC3 (**B**) cells were pre-treated with lycopene (5 μM) or THF vehicle for 2 h, following which cells were stimulated with TNFα (40ng/ml) over a time-course of 1-5 h. (**A**; left panel) Representative p65 immunofluorescence micrographs of MDA-MB-231 cells either untreated (Basal) or following 3h treatment with TNFα, with either THF or lycopene pre-treatment. No staining was visible when anti-p65 primary antibody was omitted. Scale bar represents 15 μm. (**A**; right panel) Densitometric analysis from MDA-MB-231 fluorescence micrographs showing p65 nuclear:cytoplasmic ratio values as % change from basal. (**B**) Densitometric analysis from PC3 fluorescence micrographs. Results are the mean (±SD) of densitometric values from multiple cells (*p < 0.05, **p < 0.01), from a representative experiment.

**Fig. 4.** Effect of lycopene on NF-κB transcription factor activity in PC3 and MDA-MB-231 cells. Cells were treated with lycopene (0.5-5 μM) or THF vehicle for 72 h, with each concentration of lycopene having an equivalent dilution of THF as matched control. Following this, cell lysates were subjected to luciferase assay, as described. Luciferase

activity is expressed as the ratio of NF-κB-dependent firefly luciferase activity divided by transfection control (*Renilla*) luciferase activity. Values are given as percentage as normalized against THF control for each particular concentration. Results are the mean±SEM of luminescence from 3 separate experiments.

**Fig. 5**. Effect of lycopene on kinase activity of IKKβ, *in vitro* as well as IKKβ derived from breast cancer cells. (**A**) Cell-free, *in vitro* kinase activity of recombinant IKKβ (100 ng) either alone or in the presence of lycopene (L) (5 μM) or THF vehicle (T). Values are normalized against IKKβ activity and expressed as a percentage of that activity. Results are the mean±SEM of luminescence values from 3 separate experiments (**$p < 0.01$ lycopene vs THF treated cells). (**B**) The effect of lycopene (Lyc) (5 μM) on the activity of IKKβ obtained by immunoprecipitation from lysates of treated MDA-MB-231 cells, as compared to treatment with THF vehicle. Staurosporine (10 nM) (Stauro) acts to inhibit IKK as a positive control. Cells treated with both THF vehicle and 10 nM staurosporine (THF+Stauro) act as a control to determine if an effect still occurs in the presence of THF. IP control represents cell lysate immunoprecipitated with anti-Trx antibody as a negative control for the pulldown. Values are normalized against MDA-MB-231 baseline levels and expressed as a percentage. Results are the mean± SD of luminescence values from a representative experiment with triplicate readings of samples.