Research Article

Enterolactone restricts the proliferation of the LNCaP human prostate cancer cell line *in vitro*

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Ecological data suggest a long-term diet high in plant material rich in biologically active compounds, such as the lignans, can significantly influence the development of prostate cancer over the lifetime of an individual. The capacity of a pure mammalian lignan, enterolactone (ENL), to influence the proliferation of the LNCaP human prostate cancer cell line was investigated as a function of cell density, metabolic activity, expression and secretion of prostate specific antigen (PSA), cell cycle profile, and the expression of genes involved in development and progression of prostate cancer. Treatment with a subcytotoxic concentration of ENL (60 μM for 72 h) was found to reduce: cell density (57.5%, SD 7.23, $p < 0.001$), metabolic activity (55%, SD 0.03, $p < 0.001$), secretion of PSA (48.50% SD 4.74, $p = 0.05$) and induce apoptosis (8.33-fold SD 0.04, $p = 0.001$) compared to untreated cells. Cotreatment with 10 μM etoposide was found to increase apoptosis by 50.17% (SD 0.02, $p < 0.001$). Additionally, several key genes (*e.g.* MCMs, survivin and CDKs) were beneficially regulated by ENL treatment ($p < 0.05$). The data suggest that the antiproliferative activity of ENL is a consequence of altered expression of cell cycle associated genes and provides novel molecular evidence for the antiproliferative properties of a pure lignan in prostate cancer.

Keywords: Apoptosis / Enterolactone / Gene expression / LNCaP / Proliferation

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1 Introduction

In many Western cultures, prostate cancer is the most common cancer in men and the incidence rates are significantly higher than those in Eastern cultures [1]. Ecological data suggest that dietary differences between Eastern and Western cultures may account, at least in part, for the disparity in rates [2–4]. The World Health Organisation proposes that a high fibre, low fat diet is beneficial in the prevention of many cancers, including prostate cancer [5]. The Western diet, typified by the United States and Europe, is high in fat and low in cereals, vegetables and fruit. The Eastern diet is much lower in fat intake and very high in cereals, vegetables and fruits that are known to contain high levels of bioactive compounds such as lignans, which are thought to be protective in endocrine-dependent cancers such as those of the prostate.

Plant lignans are consumed in fruits and vegetables, whole grains and seeds, with linseed (flaxseed) and sesame seed being the richest known natural sources [6]. Enterolactone (ENL), a gut-derived mammalian metabolite of plant lignans (*e.g.*, matairesinol and secoisolariciresinol), is the main circulating phyto-oestrogen in Western populations.

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Abbreviations: CoT, cotreatment; **ENL,** enterolactone; **ETO,** etoposide; **G0,** growth zero (senescence); **G1,** growth one; **G2/M,** growth two/mitosis; **MCM,** minichromosome maintenance; **MTT,** 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; **PSA,** prostate specific antigen; **tPSA,** total PSA

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ENL is a phyto-oestrogen (in addition to the isoflavones) based on its structural similarity to natural oestrogens, despite having little or no oestrogen receptor affinity [7]. ENL, or foods rich in lignans, exert a range of beneficial biological effects with relevance to cancer risk, both *in vitro* and *in vivo* (reviewed in refs. [6, 8]). Given the weak oestrogenicity of ENL, it is unlikely that its reported biological effects are solely a consequence of endocrine activity. There have been several studies examining the relationship between plasma levels and or urinary excretion levels of ENL and the risk of prostate cancer [9–14]. ENL is reported to bioaccumulate in prostatic fluid and tissue, for unknown reasons, suggesting a biological role [15, 16]. Although it is theorised that long-term dietary consumption of lignans over the lifetime of an individual may reduce or delay the onset and progression of prostate cancer, no clear link between ENL and reduced risk of prostate cancer has yet been established.

The focus of this paper was to examine the molecular mechanisms by which ENL may inhibit the proliferation of LNCaP human prostate cancer cells. This cell line is well characterised and widely used as an *in vitro* model of the early androgen-sensitive stage of prostate cancer, as it expresses a promiscuous mutant androgen receptor that responds to both androgens and oestrogens, in addition to oestrogen sensitivity *via* both oestrogen receptors [17–19]. The antiproliferative capacity of ENL in LNCaP cells was investigated as a function of: growth (cell numbers, mitochondrial activity and the expression and secretion of a clinical biomarker of proliferation, prostate specific antigen (PSA)), cell cycle profile and the expression of selected genes implicated as molecular markers in the prognosis and diagnosis of prostate cancer [20–32].

2 Materials and methods

2.1 Cell culture and lignan treatment

The LNCaP cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and cultured in RPMI 1640 medium (Gibco Life Technologies, Paisley, UK) supplemented with 2.0 mM l-glutamine (Gibco), 10.0 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES; Sigma), 1% penicillin–streptomycin (Gibco), 1% of a 45% glucose solution (Sigma) and 10% foetal bovine serum (Gibco), with the media refreshed every 96 h. The negative control for all experiments was LNCaP-specific media.

ENL (~95%, CAS 78473-71-9, Fluka, Schelldorf, Germany) was prepared as a stock solution in dimethylsuphoxide (DMSO, Sigma). A series of working solutions over a concentration range of 0–100 μM prepared in LNCaP-specific media. Etoposide (ETO, ≥98%, CAS 33419-42-0, Sigma–Aldrich, UK) was used as a positive control for DNA content and apoptosis analysis as it blocks the synthesis (S) and G2 phases of the cell cycle resulting in apoptosis [33]. A working solution of 10 μM ETO (from a stock solution of 100 μM) was prepared in LNCaP-specific media. All solutions for all experiments were adjusted to contain the same level of DMSO solvent (0.72% v/v).

2.2 Mitochondrial activity assay

The effect of ENL, over the range of 20–100 μM, on the growth of LNCaP cells over 6 days was determined using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay (Promega, Southampton, UK). This assay measures the capacity of mitochondria to metabolise the yellow soluble MTT dye to the purple insoluble formazan salt, whose absorbance can be measured at 560 nm. A cytotoxic concentration for ENL was defined as a concentration that is significantly lower in absorbance (at 560 nm) to the negative control value. From the data, the maximum subcytotoxic exposure time and the concentration was calculated and used in all subsequent experiments.

An in-house modified version of the Promega standard protocol was used. For each experiment, six 96-well plates were prepared as follows; 1.5×10^4 cells were seeded into eight wells of six columns (negative control and 5 lignan concentrations) and incubated for 48 h at 37°C, 5% CO_2. The plates were then treated with the lignan working solutions and incubated at 37°C, 5% CO_2. An MTT analysis was performed every 24 h as follows; the medium in each well was removed and the cells washed with PBS. Dye solution (15 μL; MTT) was added to each well and the plates incubated at 37°C, 5% CO_2 for 4 h. Finally, 100 μL of solubilising solution was added to each well to stop the reaction and the plates sealed and incubated overnight at 37°C, 5% CO_2. The absorbance, at 560 nm, was measured using a Rainbow thermo spectrophotometer (Tecan, UK). The assay was performed as three independent experiments.

2.3 Estimation of cell viability

The number of LNCaP cells as a consequence of 60 μM ENL treatment over 144 h was examined as a function of trypan blue exclusion staining, which stains nonviable dead cells a distinct blue colour. Cell viability was determined from daily viable cell counts.

For each experiment, 5×10^5 cells (*per* flask) were seeded in 25 cm^2 tissue culture flasks and incubated at 37°C, 5% CO_2 for 24 h to facilitate attachment. The cells were then treated with either the negative control or 60 μM ENL and incubated at 37°C, 5% CO_2. Post-treatment, a viable cell count was performed, using an improved Neubauer haemocytometer, in a set of flasks using trypan blue exclusion staining every 24 h. The numbers of viable (nonblue staining cells) was used to generate a growth curve for each treatment. The assay was performed as three independent

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experiments. After 72 h of exposure, an aliquot of 1×10^6 cells from each treatment was retained for subsequent RNA extraction.

2.4 Prostate specific antigen expression and secretion

The expression of KLK3 gene (which encodes PSA) by LNCaP cells treated with either the negative control or 60 μM ENL for 72 h was determined, relative to 18SrRNA, using Taqman™ real-time PCR.

The total RNA from 1×10^6 LNCaP cells isolated previously was extracted using the RNeasy Mini kit and treated with DNase I (Qiagen, Crawley, UK) following the manufacturer's guidelines. 500 ng of total RNA (purity 1.8 – 2.0) was reverse transcribed into cDNA using the TaqMan™ Multiscribe Reverse Transcription reagent system (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol. The cDNA was stored at −20°C prior to use.

All reagents and instruments were obtained from Applied Biosystems. The expression of KLK3 gene relative to 18SrRNA was assessed using the standard curve method. Primer Express™ was used to design the primers and probe for KLK3 (sense, CAGCATTGAACCAGAGGAGTTCTTGACCC, antisense, GACCACCTGCTACGCCTCA, probe, GGAGGTCCACACTGAAGTTTC). A serial dilution of cDNA from each treatment was used to generate a standard curve for KLK3 and 18SrRNA. Undiluted cDNA from each treatment was used to determine the expression of KLK3 and 18SrRNA. Appropriate no-template and reagent controls were used. All PCRs were prepared as triplicate reactions composed of: 2.5 μL of cDNA, 12.5 μL of TaqMan Universal PCR master mix, 2.5 μL of forward primer, 2.5 μL of reverse primer, 2.5 μL of probe and 2.5 μL of water in a 96 well optical plate and covered with an optical adhesive cover. The plate was then exposed to 45 thermal cycles of: 50°C for 2 min, 95°C for 10 min and 95°C for 15 s followed by 60°C for 1 min. The data were analysed using Relative Expression Software Tool (REST) version 1.9.9 [34].

The concentration of total PSA in the culture media of LNCaP cells from the estimation of cell numbers experiment was measured using the AxSYM Total PSA assay (Abbott, USA), every 24 h according to the manufacturer's protocol by the Clinical Chemistry department in Belfast City Hospital as three independent experiments.

2.5 Cell cycle and cell death analysis

The DNA content of LNCaP cells treated with the negative control, 10 μM ETO, 60 μM ENL or cotreatment (CoT) of 10 μM ETO and 60 μM ENL for 72 h was determined using propidium iodide staining and measured using flow cytometry as described previously by Ormerod [35]. DNA content analysis is based on the incorporation of propidium iodide into DNA and the resultant fluorescence is a measure of the relative proportion of cells in the various stages of the cell cycle. For each experiment, 5×10^5 cells (per flask) were seeded in 25 cm^2 tissue culture flasks and incubated at 37°C, 5% CO_2 for 24 h to facilitate attachment. The cells were then treated with either the negative control, 10 μM ETO, 60 μM or CoT and incubated at 37°C, 5% CO_2 for 72 h. The fluorescence intensity was collected at 585 nm, using CellQuest Software (Becton Dickinson) and analysed for DNA content using WinMDI software (J. Trotter, Scripps Inst.). The experiment was performed as three independent experiments.

The level of cell death (either apoptosis or necrosis) in LNCaP cells was determined using the BD ApoAlert™ Annexin V kit (BD Biosciences, Cowley, UK) and measured using flow cytometry. The assay is based on the externalisation of phosphatidylserine to the outer membrane of the cell during cell death. Annexin V preferentially binds this membrane protein to allow for detection of cell death. Propidium Iodide is also used as a vital dye to distinguish necrotic and apoptotic cell death. Briefly, LNCaP cells were seeded at a density of 1×10^5 cells *per* mL (*per* flask) for 24 h prior to treatment with either negative control, 10 μM ETO, 60 μM ENL or CoT and incubated at 37°C, 5% CO_2 for 72 h. After exposure, the cells were harvested and a suspension of 1×10^6 cells was prepared in 500 μL of 1 × binding buffer with 5 μL of Annexin V-FITC and 5 μL of propidium iodide added and incubated in the dark for 10 min. Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson) and the fluorescence intensity collected using CellQuest Software (Becton Dickinson). The experiment was performed as three independent experiments.

2.6 Gene expression analysis of prostate cancer biomarkers

The total RNA from treated and untreated cells extracted previously was sent in dry ice by overnight courier to SuperArray Bioscience Corporation (Frederick, USA) for microarray analysis using the Oligo GEArray® Human Prostate Cancer Biomarkers Microarray (OHS-403). The experiment was performed as three independent experiments. The microarray data were analysed for significance using the Student's *t*-test, with a value of $p \leq 0.05$ taken as significant.

2.7 Relative quantification of the expression of cell cycle genes

The expression of 84 genes, as a function of ENL treatment, was assessed using the Human RT^2 *Profiler* real-time PCR array (SuperArray Bioscience Corporation, APH-020). The experiment was performed as three independent experiments and the data analysed using the comparative Ct

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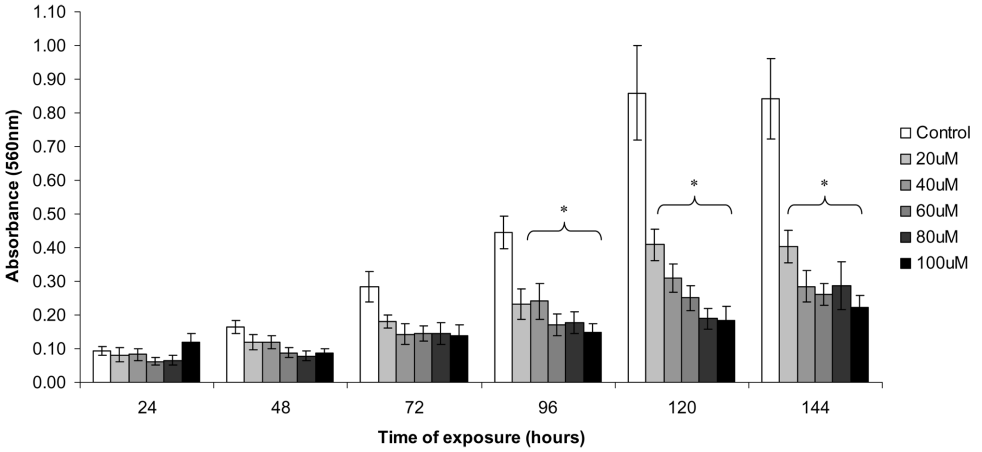

Figure 1. The mitochondrial activity (as measured by the MTT assay) of LNCaP cells treated with negative control (media only) or ENL for 144 h. Data are shown as mean (±SD) of three independent experiments. Later than 72 h, the mitochondrial activity of ENL-treated cells was significantly different to that of untreated cells (*, $p < 0.05$).

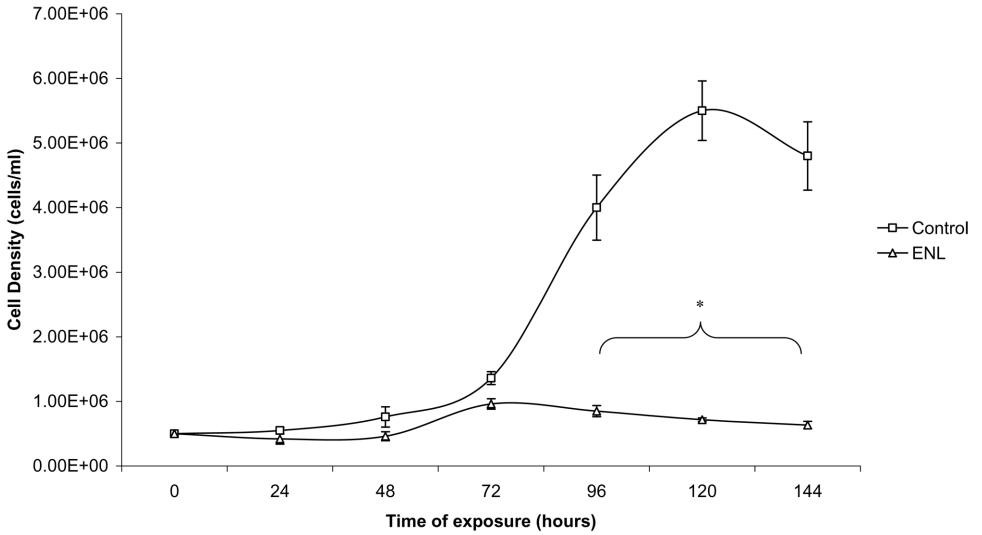

Figure 2. The cell density of LNCaP cells treated with negative control (media only) or 60 μM ENL for 144 h. Data are shown as mean (±SD) of three independent experiments. Later than 72 h, the cell density of ENL-treated cells was significantly different to that of untreated cells (*, $p < 0.05$).

(ΔΔCt) method. The expression of each target gene was calculated relative to the expression of the 18SrRNA, HPRT1, RPL13A, GAPDH and ACTB housekeeping genes. The data were analysed for significance using the Student's *t*-test, with a value of $p \leq 0.05$ taken as significant.

2.8 Statistical analyses

All data were analysed for statistical significance using SPSS (version 12.0) software. The data were tested for normality using the Kolmogorov–Smirnov test. The normal data were analysed with one-way ANOVA (LSD or Games–Howell *post-hoc* tests), the non-normal data were analysed with the Kruskal–Wallis and Mann–Whitney *U* test, unless otherwise stated. The significance level was $p \leq 0.05$.

3 Results

3.1 Mitochondrial activity assay

The cytotoxicity data obtained were used to generate an optimal subcytotoxic dose of ENL in LNCaP cells, defined by both exposure time and corresponding concentration. The exposure time was defined as the longest duration before a significant difference in absorbance (at 560 nm) was measured between the negative control and any of the ENL concentrations used. Significant differences between

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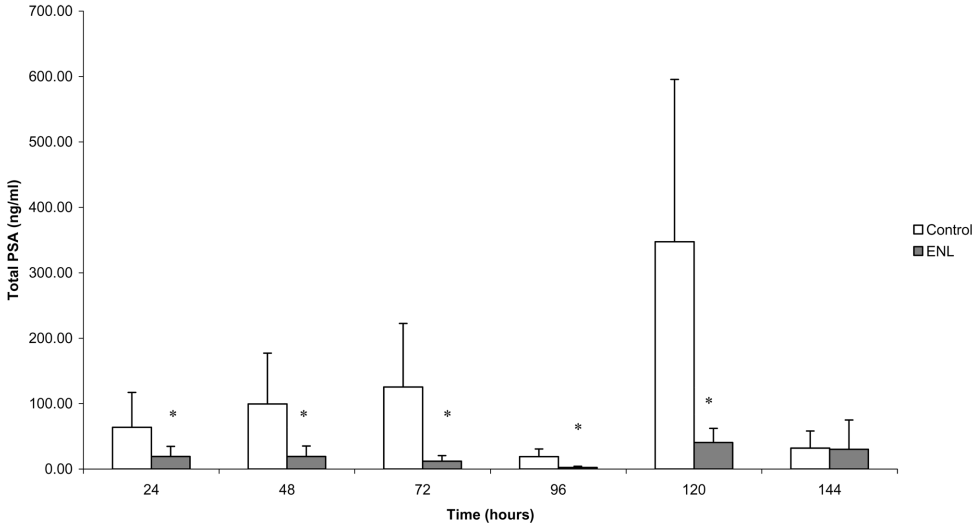

Figure 3. The secretion of tPSA by LNCaP cells treated with negative control (media only) or 60 μM ENL for 144 h. Data are shown as mean (±SD) of three independent experiments. Significant differences in the secretion of tPSA of ENL-treated cells, relative to untreated cells is shown by * ($p < 0.05$).

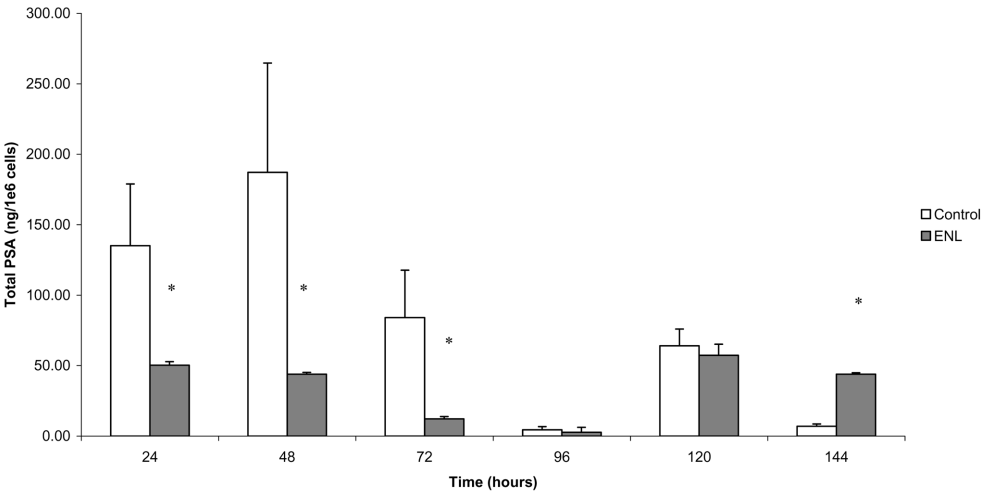

Figure 4. The secretion of tPSA (expressed as ng/1 × 10^6 cells) by LNCaP cells treated with negative control (media only) or 60 μM ENL for 144 h. Data are shown as mean (±SD) of three independent experiments. Significant differences between the treatment and the control are indicated by * ($p < 0.05$).

the negative control and ENL were measured at 96, 120 and 144 h ($p < 0.05$). Therefore, the maximum subcytotoxic exposure time was taken to be 72 h as shown in Fig. 1.

At 72 h, none of the ENL concentrations tested were significantly different from the negative control. The 60 μM concentration was selected as it represented the mid-point of the concentration range and correlated with previous work with ENL (and other lignans) in other prostate cell lines. Therefore, the optimal subcytotoxic dose of ENL in LNCaP cells was 60 μM for 72 h and all further experiments were performed using this dose.

3.2 Estimation of cell viability

At all time points, the number of viable cells was found to comprise ~98% of the total cells counted and these viable cells were used to determine the growth profiles shown in Fig. 2. The cell viability data for the negative control correlated with the expected exponential growth profile. ENL (60 μM) significantly decreased the viability of LNCaP cells over 144 h, as shown in Fig. 2, resulting in a pronounced nonexponential growth profile. Therefore, it was not possible to determine the cellular doubling times of LNCaP cells as a consequence of ENL treatment. The cell

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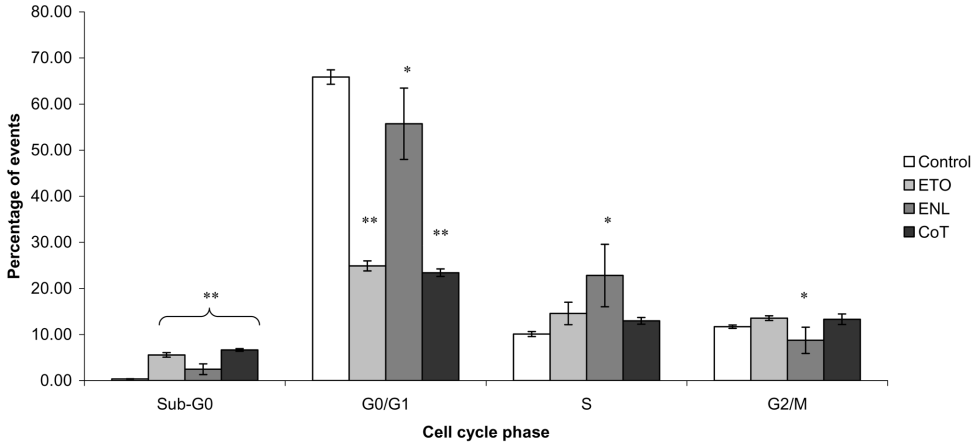

Figure 5. The cell cycle profile of LNCaP cells exposed to negative control (media only), 10 μM ETO, 60 μM ENL, or a CoT 10 μM ETO and 60 μM ENL (CoT) for 72 h. Data are expressed as the mean percentage of events (±SD) of three independent experiments. Significant differences between the treatments and the control are indicated by * ($p < 0.05$) or ** ($p < 0.001$).

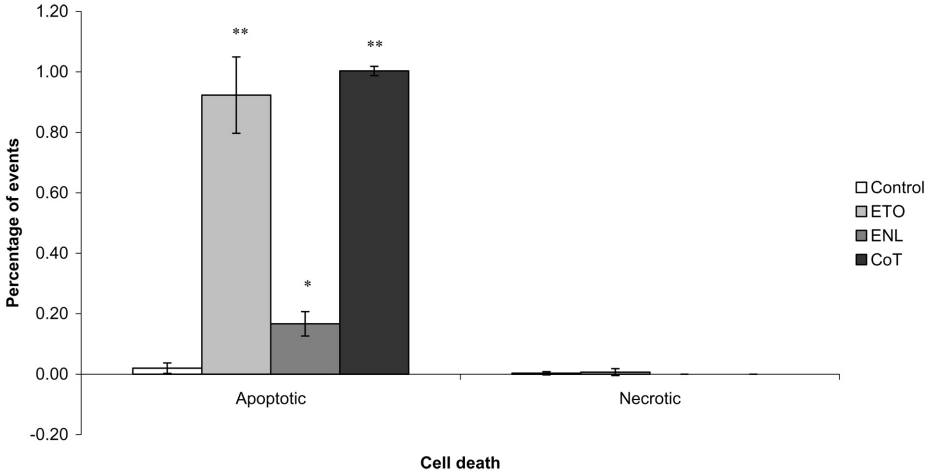

Figure 6. The cell death profile of LNCaP cells exposed to negative control (media only), 10 μM ETO, 60 μM ENL, or a CoT 10 μM ETO and 60 μM ENL (CoT) for 72 h. Data are expressed as the mean percentage of events (±SD) of three independent experiments. Significant differences between the treatments and the control are indicated by * ($p = 0.005$) or ** ($p < 0.001$).

densities of both the control and 60 μM ENL correlated with the reduced proliferation data from the MTT assay ($r^2 = 0.98$ and 0.96, respectively).

3.3 Prostate specific antigen expression and secretion

The secretion of total PSA expressed as ng/mL and standardised to ng/1 × 10^6 cells was reduced by ENL treatment ($p < 0.05$), as shown in Figs. 3 and 4. At 72 h, the expression of KLK3 treated with ENL was significantly reduced to 0.08 (SD 0.01) that of untreated cells (a down-regulation of 12.5-fold, $p = 0.001$) and the secretion of total PSA (tPSA) by 10.5-fold (SD 1.67, $p = 0.001$) (a reduction of 6.87-fold when normalised, $p = 0.001$). The secretion of tPSA at 24 – 72 h, as a function of ENL, correlated with the alterations in cell viability and mitochondrial activity measured previously ($r^2 = 0.97$ and 0.98, respectively).

3.4 Cell cycle and cell death analysis

Significant alterations to the proportion of cells in each phase of the cell cycle was determined for all treatments ($p < 0.05$), as shown in Fig. 5. A significant increase in cellular debris was observed as a result of ETO and CoT ($p < 0.001$, approximately 41% of detected events, data not shown). The cell cycle profile of ETO was in agreement with its reported response in cancer cells [34]. ENL-treated cells accumulated in the sub-G0 and S, and decreased in the G0/G1 and G2/M phases of the cell cycle in LNCaP cells

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Table 1. A listing of the 92 genes in LNCaP cells affected by 60 μM ENL exposure for 72 h, as measured by array analyses

Gene	Fold change	*p*-Value	Gene	Fold change	*p*-Value
ACTB	−1.32 (0.20)	0.15	IGF2	1.06 (0.17)	0.76
AGR2	1.21 (0.73)	0.70	IGFBP3	4.20 (0.89)	0.00
AIG1	1.97 (0.45)	0.02	IL12A	1.01 (0.07)	0.93
AKAP1	−1.56 (0.21)	0.04	IL1B	−1.20 (0.07)	0.17
AKT1	−1.01 (0.27)	0.83	ILK	1.06 (0.30)	0.79
APC	1.14 (0.22)	0.31	JUN	1.29 (0.29)	0.08
AR	1.01 (0.22)	0.97	KAI1	−1.08 (0.07)	0.36
AS1	1.98 (1.47)	0.30	KLK1	1.00 (0.28)	0.98
B2M	−1.33 (0.10)	0.19	KLK3	−4.41 (0.05)	0.00
BAK1	−1.40 (0.06)	0.02	KLK4	−1.72 (0.11)	0.00
BAX	−1.09 (0.11)	0.14	MAP2K4	−1.25 (0.12)	0.05
BCL2L1	−1.13 (0.23)	0.31	MAPK1	1.01 (0.14)	0.91
BRCA1	−1.90 (0.12)	0.00	MAPK12	−1.16 (0.13)	0.12
CANT1	−1.36 (0.13)	0.02	MAPK3	−1.13 (0.15)	0.42
CASP7	1.11 (0.28)	0.47	MAPK6	−1.77 (0.28)	0.06
CD164	−1.59 (0.21)	0.03	MAPK9	1.04 (0.14)	0.65
CDK2	−2.14 (0.17)	0.01	MMP2	−1.24 (0.10)	0.06
CDK4	−1.17 (0.17)	0.34	MYC	−1.02 (0.37)	0.92
CDK5	−1.05 (0.09)	0.48	NCOA4	−1.09 (0.38)	0.63
CDK7	1.18 (0.46)	0.53	NFKB1	1.33 (0.39)	0.21
CDK8	1.31 (0.33)	0.14	NFKBIA	−1.15 (0.18)	0.41
CDK9	−1.32 (0.29)	0.16	NKX3-1	−1.41 (0.16)	0.09
CDKN1A	1.09 (0.19)	0.40	NR1H2	1.01 (0.22)	0.93
CDKN1B	−1.04 (0.30)	0.74	NR1H3	1.06 (0.12)	0.60
CDKN2C	−1.49 (0.16)	0.06	NR2C1	1.06 (0.28)	0.81
CDKN3	−1.84 (0.09)	0.01	NR2F6	−1.33 (0.16)	0.06
CLDN3	1.12 (0.23)	0.36	PCNA	−2.04 (0.07)	0.00
CLN3	1.21 (0.34)	0.20	PIAS1	−1.93 (0.13)	0.01
CLU	−1.61 (0.09)	0.00	PIAS2	−1.10 (0.14)	0.23
COL6A1	2.08 (0.58)	0.01	PIK3CG	−1.21 (0.06)	0.00
CYB5	−1.30 (0.16)	0.18	PPID	−1.17 (0.24)	0.18
CYC1	−1.04 (0.18)	0.85	PRKCD	−1.95 (0.06)	0.00
DAB2IP	−1.32 (0.20)	0.22	PRKCG	−1.29 (0.11)	0.07
DAPK1	1.72 (0.65)	0.08	PRKCH	−1.83 (0.14)	0.01
DNCL1	1.05 (0.25)	0.88	PRKCZ	−1.08 (0.17)	0.29
E2F1	−1.84 (0.11)	0.00	PTEN	−1.06 (0.32)	0.76
EGF	−1.07 (0.20)	0.69	RASSF1	−1.56 (0.19)	0.04
ELAC2	1.30 (0.33)	0.09	RB1	−1.20 (0.23)	0.34
ENO1	−1.13 (0.16)	0.47	RNF14	−1.08 (0.20)	0.42
ENO3	1.02 (0.25)	0.92	RPS27A	−0.35 (1.19)	0.96
ERBB2	1.97 (0.48)	0.02	SLC33A1	−1.25 (3.26)	0.79
EZH2	−1.37 (0.14)	0.02	STEAP	−1.35 (0.10)	0.01
GAPDH	1.09 (0.04)	0.67	STEAP2	1.22 (0.12)	0.15
HIF1A	−1.43 (0.38)	0.18	TMEM37	−1.36 (0.08)	0.01
HRAS	−1.29 (0.13)	0.05	TP53	−1.17 (0.30)	0.29
HSPCB	−1.12 (0.06)	0.30	TPM1	−1.65 (0.05)	0.00

Data are presented as the mean fold change (±SD) of three independent experiments. Positive fold changes indicate up-regulation, whilst negative fold changes indicate down-regulation.

($p < 0.05$), as shown in Fig. 5. CoT with 10 μM ETO and 60 μM ENL appeared to enhance the cell cycle alterations seen with ETO alone ($p < 0.05$).

The increased sub-G0 fraction observed in all treatments was distinguished to be apoptosis (as opposed to necrotic) as shown in Fig. 6. The level of apoptotic cell death as a function of ETO, ENL or CoT was significantly increased (46.17, 8.33 and 50.17-fold, respectively, $p < 0.001$). No significant differences in viability or necrosis were measured ($p < 0.005$). CoT of ETO and ENL was found to be more effective at increasing apoptotic cell death than ETO or ENL alone (1.09 and 6.02-fold, respectively, $p < 0.005$).

3.5 Gene expression analysis of prostate cancer biomarkers

Of the 263 genes spotted on the cDNA microarray, only 92 were affected by ENL treatment in all three experiments, as shown in Table 1. The remaining 171 were excluded from

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Table 2. A functional grouping of 16 genes differentially expressed by LNCaP as a consequence of 60 μM ENL treatment for 72 h as measured by array analyses

Gene name	Accession number	Fold change	Functional grouping (s)
AIG1	NM 016108	+1.96 (0.45)*	Putative prostate cancer marker
BRCA1	NM 007294	−1.92 (0.12)**	Apoptosis, cell cycle, growth/proliferation and differentiation, angiogenesis, extracellular matrix and cell adhesion, transcription factors and regulators and stress response
CDK2	NM 001798	−2.22 (0.17)*	Cell cycle, growth/proliferation and differentiation, proteases, protein kinases and inhibitors
CDKN3	NM 005192	−1.89 (0.09)*	Cell cycle, growth/proliferation and differentiation
COL6A1	NM 001848	+2.04 (0.58)*	Angiogenesis, extracellular matrix and cell adhesion
E2F1	NM 005225	−1.85 (0.11)**	Cell cycle, growth/proliferation and differentiation
IGFBP3	NM 000598	+4.08 (0.05)**	Cell cycle, growth/proliferation and differentiation
KLK3	NM 001648	−4.55 (0.11)**	Angiogenesis, extracellular matrix and cell adhesion, proteases, protein kinases and inhibitors
KLK4	NM 004917	−1.72 (0.07)**	Angiogenesis, extracellular matrix and cell adhesion, proteases, protein kinases and inhibitors
PCNA	NM 182649	−2.04 (0.13)**	Cell cycle, growth/proliferation and differentiation
PIAS1	NM 016166	−1.92 (0.06)*	Transcription factors and regulators
PRKCD	NM 002742	−1.96 (0.14)**	Proteases, protein kinases and inhibitors
PRKCH	NM 006255	−1.85 (0.13)*	Proteases, protein kinases and inhibitors
RASSF1	NM 007182	−1.61 (0.10)*	Cell cycle, growth/proliferation and differentiation
SLC43A1	NM 003627	−2.04 (0.08)**	Putative prostate cancer marker
TPM1	NM 000366	−1.67 (0.03)**	Putative prostate cancer marker

Only genes whose fold change was ±1.5 fold (relative to control) and statistically significant are given. Data is presented as the mean fold change (±SD) of three independent experiments. Positive fold changes indicate up-regulation, whilst negative fold changes indicate down-regulation. Significant differences are given by * $p < 0.05$, ** $p < 0.005$.

further consideration. Biological relevance of the 92 affected genes was determined by considering only those with fold changes of 1.5-fold or more and statistically different to the negative control ($p \leq 0.05$) resulting in 16 differentially regulated genes, as shown in Table 2. These data have been deposited in NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO number GPL4515 [36]. Thirteen genes were found to be differentially down-regulated and the largest proportion (6) are involved in cell cycle, growth/proliferation and differentiation. The three genes up-regulated by ENL exposure are involved in cell growth and angiogenesis.

3.6 Relative quantification of the expression of cell cycle genes

The differential regulation of cell cycle genes by ENL detected by microarray was confirmed and clarified by quantitative real-time PCR using a cell cycle specific array. Of the 84 genes investigated using the RT^2 *Profiler* real-time PCR array, 83 were detected in all three experiments as shown in Table 3. Biological relevance of the 83 affected genes was determined by considering only those with fold changes of 1.5-fold or more and statistically different to the negative control ($p \leq 0.05$) resulting in 34 differentially regulated genes, as shown in Table 4.

The majority of the differentially regulated genes (33 of 34) were down-regulated and cyclin D2 (CCND2) expression was up-regulated by ENL treatment ($p \leq 0.05$). Twenty-four genes are intimately involved in progression through the cell cycle, with the remaining nine involved in cell cycle checkpoints and arrest.

4 Discussion

The selection of the ENL concentration used in this study was based on previous work to investigate the potential bioactivity of matairesinol, secoisolariciresinol, enterolactone and enterodiol in the PC-3, DU 145 and LNCaP human prostate cancer cell lines. The MTT assay was used as a screening tool to determine a common (and therefore comparable) subcytotoxic concentration for each lignan and cell line combination. The only comparable lignan concentration was found to be 60 μM and although this concentration inhibited the growth of the three cell lines, only ENL-treated LNCaP cells had any alterations to their PSA expression and secretion and cell cycle profile. Therefore, this paper focused on examining the molecular mechanisms by which ENL may influence LNCaP proliferation.

The present study provides evidence that a pure lignan inhibits the proliferation of prostate cancer and, to the best of our knowledge, the first study to examine the correlation between the expression of genes associated with prostate cancer growth and biological end-points of proliferation *in vitro*. The data presented here, that ENL can inhibit the proliferation of cancer *in vitro*, agree with the published litera-

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Table 3. A listing of the 83 genes in LNCaP cells affected by 60 μM ENL exposure for 72 h, as measured by real-time PCR analyses

Gene	Fold change	*p*-Value	Gene	Fold change	*p*-Value
ABL1	−1.20 (0.11)	0.05	CKS1B	−2.75 (0.04)	0.00
ANAPC2	−1.29 (0.19)	0.11	CKS2	−2.22 (0.11)	0.00
ANAPC4	−1.14 (0.02)	0.00	CUL1	0.90 (0.17)	0.29
ARHI	1.17 (0.48)	0.57	CUL2	2.20 (2.27)	0.41
ATM	1.03 (0.19)	0.78	CUL3	−1.05 (0.21)	0.70
ATR	−1.28 (0.12)	0.04	DDX11	−4.25 (0.08)	0.00
BAX	−1.45 (0.14)	0.02	DNM2	1.04 (0.14)	0.64
BCCIP	−1.08 (0.14)	0.44	E2F4	−1.28 (0.09)	0.01
BCL2	1.25 (0.09)	0.01	GADD45A	−1.66 (0.01)	0.00
BIRC5	−2.46 (0.04)	0.00	GTF2H1	1.10 (0.19)	0.41
BRCA1	−2.50 (0.03)	0.00	GTSE1	13.30 (22.54)	0.40
BRCA2	−2.64 (0.13)	0.00	HERC5	4.01 (2.78)	0.13
CCNB1	−2.41 (0.03)	0.00	HUS1	−1.19 (0.11)	0.07
CCNB2	−2.81 (0.09)	0.00	KNTC1	−2.00 (0.01)	0.00
CCNC	1.03 (0.12)	0.66	KPNA2	−1.90 (0.06)	0.00
CCND1	−1.07 (0.19)	0.60	MAD2L1	−2.33 (0.01)	0.00
CCND2	4.22 (0.95)	0.00	MAD2L2	−1.25 (0.06)	0.00
CCNE1	1.19 (0.23)	0.21	MCM2	−2.01 (0.02)	0.00
CCNF	−2.03 (0.20)	0.01	MCM3	−1.76 (0.11)	0.00
CCNG1	−2.43 (0.06)	0.00	MCM4	−2.37 (0.11)	0.00
CCNG2	1.19 (0.11)	0.04	MCM5	−2.87 (0.05)	0.00
CCNH	−2.03 (0.06)	0.00	MKI67	−3.07 (0.08)	0.00
CCNT1	−1.01 (0.16)	0.89	MNAT1	1.07 (0.12)	0.37
CCNT2	−1.09 (0.17)	0.45	MRE11A	−1.65 (0.07)	0.00
CDC16	1.13 (0.22)	0.39	NBN	1.17 (0.02)	0.00
CDC2	−2.80 (0.00)	0.00	PCNA	−2.49 (0.07)	0.00
CDC20	−3.16 (0.03)	0.00	RAD1	−1.23 (0.11)	0.05
CDC34	−1.04 (0.08)	0.42	RAD17	1.06 (0.20)	0.65
CDK2	−1.99 (0.04)	0.00	RAD51	−1.36 (0.84)	0.61
CDK4	−1.66 (0.11)	0.00	RAD9A	1.10 (0.48)	0.73
CDK5R1	−1.76 (0.03)	0.00	RB1	1.62 (0.59)	0.14
CDK5RAP1	−1.44 (0.02)	0.00	RBBP8	−1.18 (0.09)	0.03
CDK6	−1.48 (0.05)	0.00	RBL1	−1.61 (0.06)	0.00
CDK7	−1.05 (0.03)	0.09	RBL2	−1.11 (0.05)	0.03
CDK8	1.43 (0.16)	0.01	RPA3	−1.70 (0.14)	0.01
CDKN1A	−1.48 (0.02)	0.00	SERTAD1	1.47 (0.71)	0.31
CDKN1B	−2.41 (0.03)	0.00	SKP2	−2.07 (0.07)	0.00
CDKN2A	1.63 (0.15)	0.00	SUMO1	1.22 (0.14)	0.05
CDKN2B	1.27 (0.46)	0.37	TFDP1	−1.22 (0.32)	0.39
CDKN3	−2.59 (0.04)	0.00	TFDP2	−1.44 (0.06)	0.00
CHEK1	−1.94 (0.08)	0.00	TP53	−1.39 (0.01)	0.00
CHEK2	−1.52 (0.38)	0.19	UBE1	−1.31 (0.17)	0.07

Data are presented as the mean fold change (±SD) of three independent experiments. Positive fold changes indicate up-regulation, whilst negative fold changes indicate down-regulation.

ture currently available [6, 14, 37]. The antiproliferative activity of ENL has been demonstrated as a function of reduced cell density, mitochondrial activity, PSA expression and secretion, increased apoptosis and an altered cell cycle profile, as given in Figs. 1–6. Although the cell cycle profiling of ENL-treated cells determined a number of interesting alterations, it cannot be excluded that these changes occurred prior to 72 h and the increase in the S-phase fraction may be a result of partial synchronisation of cells from an earlier event. Of interest is the finding that CoT with ETO and ENL appears to induce a higher level of apoptotic cell death than either treatment alone ($p < 0.05$), as seen in Fig. 6. Although it has been reported that CoT with ETO and ENL statistically increases the level of apoptosis in contrast to ETO alone, the similarity of the profiles shown in Fig. 6, raises the question of the biological relevance of this finding. It is conceivable that it is a function of statistical analysis and not biological response. Additionally, a notable increase in cellular debris was measured as a function of ETO and CoT exposure ($p < 0.001$). This is attributed to an accumulation of debris as a function of cell death prior to the 72 h sampling point. A previous *in vitro* study into the effect of ENL and enterodiol on prostate cancer cells by Lin *et al*. [37], also found that a 72 h exposure to ENL was nontoxic and that cell density (measured as propidium iodide incorporation) was significantly reduced.

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Table 4. Changes in expression by LNCaP cells of genes associated with cell cycle phases as a consequence of 60 μM ENL treatment for 72 h as measured by real-time PCR

Gene	Name	Fold change	Function (cell cycle phase)
BIRC5	Survivin	−2.46 (0.04)**	Apoptosis (G2 and G2/M)
BRCA1	Breast cancer 1	−2.50 (0.03)**	Genomic integrity/DNA repair
BRCA2	Breast cancer 2	−2.64 (0.13)**	Genomic integrity/DNA repair
CCNB1	Cyclin B1	−2.41 (0.03)**	Cell cycle (G2 and G2/M)
CCNB2	Cyclin B2	−2.81 (0.09)**	Cell cycle (M)
CCND2	Cyclin D2	+4.22 (0.95)**	Cell cycle (G1 and G1/S)
CCNF	Cyclin F	−2.03 (0.20)*	Cell cycle (M)
CCNG1	Cyclin G1	−2.43 (0.06)**	Cell cycle (G2 and G2/M)
CCNH	Cyclin H	−2.03 (0.06)**	Cell cycle (G2 and G2/M)
CDC2	Cell division cycle 2	−2.80 (0.00)**	Cell cycle (G1/S and G2/M)
CDC20	Cell division cycle 20	−3.16 (0.03)**	Cell cycle (M)
CDK2	Cyclin-dependent kinase 2	−1.99 (0.04)**	Cell cycle (G1 and G1/S)
CDK4	Cyclin-dependent kinase 4	−1.66 (0.11)**	Cell cycle (G1 and G1/S)
CDK5R1	CDK5, regulatory subunit 1	−1.76 (0.03)**	Cell cycle (G2 and G2/M)
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	−2.41 (0.03)**	Cell cycle (G1 and G1/S)
CDKN3	Cyclin-dependent kinase inhibitor 3 (Cip2)	−2.59 (0.04)**	Cell cycle (G1, G1/S, G2 and G2/M)
CHEK1	CHK1 checkpoint homolog	−1.94 (0.08)**	Cell cycle checkpoint/arrest
CKS1B	CDC28 protein kinase regulatory subunit 1B	−2.75 (0.04)**	CDK activity (G2 and G2/M)
CKS2	CDC28 protein kinase regulatory subunit 2	−2.22 (0.11)**	CDK activity (G2 and G2/M)
DDX11	DEAD/H box polypeptide 11	−4.25 (0.08)**	RNA helicase (G2 and G2/M)
GADD45A	Growth arrest and DNA-damage-inducible, alpha	−1.66 (0.01)**	Genomic integrity/DNA repair
KNTC1	Kinetochore associated 1	−2.00 (0.01)**	Chromosome segregation (M)
KPNA2	Karyopherin alpha 2	−1.90 (0.06)**	Protein transport (G2 and G2/M)
MAD2L1	MAD2 mitotic arrest deficient-like 1	−2.33 (0.01)**	Spindle assembly checkpoint (M)
MCM2	Mini-chromosome maintenance 2, (mitotin)	−2.01 (0.02)**	Genomic replication (S)
MCM3	Mini-chromosome maintenance 3	−1.76 (0.11)**	Genomic replication (S)
MCM4	Mini-chromosome maintenance 4	−2.37 (0.11)**	Genomic replication (S)
MCM5	Mini-chromosome maintenance 5	−2.87 (0.05)**	Genomic replication (G0/G1 and S)
MKI67	Antigen identified by mAb Ki-67	−3.07 (0.08)**	Cell cycle
MRE11A	Meiotic recombination 11 homolog A	−1.65 (0.07)**	Cell cycle (M)
PCNA	Proliferating cell nuclear antigen	−2.49 (0.07)**	DNA polymerase cofactor (S)
RBL1	Retinoblastoma-like 1 (p107)	−1.61 (0.06)**	Cell cycle (G1/S and G2/M)
RPA3	Replication protein A3	−1.70 (0.14)*	DNA replication/repair (S and M)
SKP2	S-phase kinase-associated protein	−2.07 (0.07)**	Cyclin A/CDK2 activity (G1, G1/S, S)

Data are presented as the mean fold change (±SD) of three independent experiments. Positive fold changes indicate up-regulation, whilst negative fold changes indicate down-regulation. Significant differences are given by * $p < 0.05$, ** $p < 0.005$.

The IC_{50} (inhibitory concentration 50%) of 57 μM for ENL in LNCaP cells calculated by Lin *et al*. is in good agreement with IC_{50} of 60 μM found in the present study. The present study confirms the data of Lin *et al*., but also expands upon and provides a gene expression analysis for the reported antiproliferative effects.

A number of gene expression alterations induced by ENL may explain the reduced proliferation observed as seen in Tables 1–4. The microarray experiments were performed to investigate a range of prostate cancer biomarkers and based on the results, which indicated cell cycle genes to be affected, a focused real-time array was used to investigate the expression of cell cycle-associated genes. A list of those genes differently regulated according to the filtering criteria applied in both microarray and real-time array analyses are given in Table 5.

A decrease in IGFBP3 expression (and resultant increase in IGF-1) is linked with the progression to androgen insensitivity in LNCaP cells [38]. IGFBP3 is normally expressed at low levels by the LNCaP cell line [39] and ENL increased the expression of this gene by 4.08-fold ($p = 0.002$). Physiologically, the IGFBP3 protein binds to and inhibits the activity of IGF1 and II, which are potent mitogens. Additionally IGFBP3 is in involved inducing apoptosis [40, 41]. The expression change reported here may provide a mechanism whereby abnormal IGF signalling could be restrained and also may explain the increased apoptosis reported here.

The secretion of tPSA was significantly reduced by ENL over 120 h ($p < 0.05$), as shown in Fig. 3. When the PSA secretion is normalised to 1×10^6 cells, as shown in Fig. 4, ENL significantly reduces PSA secretion up and including 72 h ($p < 0.05$). At 72 h (which is prior to ENL toxicity) the expression of KLK3 and secretion of PSA are significantly reduced relative to the untreated cells ($p < 0.05$). The PSA secretion of untreated cells correlates well ($r^2 = 0.77$) with the changes in cell density up to 96 h where the relationship between PSA secretion and cell density breaks down. It is not clear why the PSA measured between 96 and 144 in

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Table 5. The eight genes differentially regulated in LNCaP cells exposed to 60 μM ENL for 72 h in both micro- and real-time arrays

Gene	Fold change	
	Microarray	Real-time array
BRCA1	−1.90 (0.12)[a)]	−2.50 (0.03)[a)]
CDK2	−2.14 (0.17)[a)]	−1.99 (0.04)[a)]
CDK4	−1.17 (0.17)	−1.66 (0.11)[a)]
CDK7	1.18 (0.46)	−1.05 (0.03)
CDKN3	−1.84 (0.09)[a)]	−2.59 (0.04)[a)]
KLK3	−4.41 (0.05)[a)]	−12.5 (0.01)[a),b)*]
PCNA	−2.04 (0.07)[a)]	−2.49 (0.07)[a)]
TP53	−1.17 (0.30)	−1.39 (0.01)

Only genes whose fold change was ±1.5-fold (relative to control) and statistically significant are given. Data are presented as the mean fold change (±SD) of three independent experiments. Positive fold changes indicate up-regulation, whilst negative fold changes indicate down-regulation.

a) Only genes whose fold change was ±1.5-fold (relative to control) and statistically significant ($p \leq 0.05$) were taken to be biologically relevant.

b) KLK3 expression was measured using TaqMan real-time PCR.

both the untreated and ENL samples does not correlate with the cell density measured in the previous experiments and at present cannot be explained. It is probable that confluencey and toxicity issues may be involved. The data in Figs. 3 and 4, however, do show that PSA secretion and cell density do not directly correlate and other factors influence the secretion of PSA. The PSA data presented here only show that up to and including 72 h ENL reduces the secretion of a widely used marker of cell proliferation.

Several studies using dietary interventions with foods rich in lignans have linked reduced growth with reduced levels of PSA (reviewed in ref. [6]). The reduction in KLK3 expression and secretion of PSA has also been seen with genistein [42, 43]. It is unclear whether the reduced KLK3 and PSA levels reported here directly effect the proliferation of the cell line used or are merely a marker of proliferation, although PSA can restrict tumour proliferation, progression to an invasive phenotype and increase apoptosis [44, 45].

Several of the expression changes detected were in genes associated with cell cycle regulation. Defects in the regulation of the cell cycle are a common feature of tumourigenic cells. Progression of the cell cycle process is regulated by appropriate sequential activation and inactivation of cyclin-dependent kinases (CDKs) during the cell cycle. The activity of CDKs is positively regulated by cyclins and negatively by cyclin-dependent kinase inhibitors (CDKIs). The present study found altered expression of several CDKs, cyclins and CDKIs in a manner consistent with impairing the cell cycle (as seen in Tables 1 and 2) as a result of ENL treatment. It is probable that this deregulation of the expression of these genes in LNCaP cells may result in reduced proliferation. Additionally, ENL was found to regulate other key genes involved in the cell cycle. The expression of E2F was down-regulated by 1.85-fold ($p < 0.005$). E2F is required for progression of the cell cycle into S phase and activates several genes responsible for cellular proliferation in the S and subsequent phases. The activity of this gene is negatively regulated by retinoblastoma and several cyclin–CDK complexes can hyperphosphorylates RB, leading to its release from E2F [46, 47]. Any deregulation of the normal CDKI/CDK/cyclin interactions, whether direct or indirect, that interferes with the Rb/E2F complex would alter the progression of cells from G1 to S phase of the cell cycle. Array and real-time PCR analysis of ENL-treated LNCaP cells found several alterations in CDKI/CDK/cyclin expression, which may reduce the hyperphosphorylation of RB, keeping it bound to E2F and thereby blocking cell proliferation and inducing cell growth arrest. This may also explain the altered G1 fraction seen with DNA content analysis. The disruption of the CDKI/CDK/cyclin expression may also impact on the other phases of the cell cycle, as shown in Tables 1 and 2.

The minichromosome maintenance genes (MCMs) encode for a number of proteins that are essential in maintaining genome integrity as they are critical for the initiation of replication of DNA [48, 49]. MCMs are vital components of the replication complex and also ensure that only one replication cycle occurs *per* cell cycle [48, 49]. MCMs are normally not expressed in quiescent cells, but are up-regulated in cycling cells, resulting in their use as proliferation markers [48–54]. The present study found a down-regulation ($p < 0.001$) of MCMs 2–5 as a result of ENL exposure (2.0, 1.75, 2.38 and 2.86, respectively). Reduced expression of MCMs have been linked with a favourable prognosis in prostate cancer [53, 54] and other cancers [50, 52]. The altered expression of MCMs reported may provide an explanation of the overall reduced proliferation and S-phase accumulation detected in this study. Additionally, other traditional markers of proliferation, such as PCNA and MKI67 (Ki-67), were also down-regulated by ENL (2.04 and 3.03-fold, respectively, $p < 0.05$). However, PCNA is also expressed during DNA repair, not just in cycling cells and MKI67 expression is affected by nutritional deprivation [50]. Our study shows that ENL reduces proliferation using sophisticated markers of proliferation, the MCMs.

The increase in apoptosis seen with ENL may be due the reduced expression of BIRC5 (survivin). Survivin is a member of the inhibitor of apoptosis family and functions by binding to and preventing the activity of initiator and effector caspases, such as caspase 9 [55, 56]. Undetectable in quiescent tissues, survivin is abundantly expressed in proliferating cells and has shown to be linked with poor prognosis in many cancers including those of the prostate [55–59]. In addition to its antiapoptotic properties it linked with microtubule formation in mitosis and in angiogenesis

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[60, 61]. LNCaP cells have been shown to express low levels of survivin mRNA, but high levels of survivin protein [59]. Normally the activity of survivin is negatively regulated by phosphorylation of the protein by the CDC2/CCNB1 complex. All three genes, BIRC5, CDC2 and CCNB1, were down-regulated by ENL treatment (2.46, 2.80 and 2.41-fold respectively). This down-regulation may serve to explain the increased level of apoptosis seen in ENL and CoT (ETO and ENL) reported here (8.33- and 50.17-fold, respectively). Interestingly, LNCaP cells have previously been shown to be resistant to ETO-mediated apoptosis [62, 63]. The finding that CoT with ENL and ETO results in an additive effect may be of further interest.

However, the present study is limited by for a number of reasons. Firstly, the concentration at which the ENL exerted its effects is significantly higher than that found in the plasma and urine of humans. It is reasonable to question whether the concentration used is achievable *in vivo*. Based on the MTT data presented here a lower concentration may have been more appropriate. However, ENL has been shown to accumulate in the prostate [15, 16]. Secondly, only one cell line was used to investigate the antiproliferative activity of ENL. We have previously determined that ENL restricts the growth of both DU 145 and PC-3 cells (unpublished), no cell cycle alterations were seen in these cell lines and so were not further investigated. Interestingly, preliminary data from our laboratory have found that the cell cycle profile (including the increased sub-G0 fraction) is also seen in LNCaP cells at an ENL concentration range of 6×10^{-5} to 10^{-9} M. The lower concentrations (6×10^{-7} to 10^{-9}) have been reported in the plasma and prostate fluid. Therefore, ENL may potentially possess antiproliferative properties at physiologically relevant concentrations. Thirdly, although ENL is weakly oestrogenic, such a high dose of ENL may have undesirable oestrogenic effects. Finally, although it has been shown that ENL regulates the expression of several genes, it has not been shown that ENL itself directly effects the gene expression. It is possible that ENL indirectly influences gene expression via other cellular molecules not investigated here.

As LNCaP cells are a model of the early androgen-sensitive stages of prostate cancer and our working hypothesis is that long-term consumption of lignans may be protective against prostate cancer risk, it would be necessary to investigate the effect of ENL on primary and benign prostate cell lines. It is proposed that this would provide sufficient evidence for *in vivo* investigations. Despite these limitations, this study provides, for the first time, a molecular mechanism by which the reported anticancer properties of lignans may occur.

Prostate cancer initiation and progression is a complex multifactoral process that involves altered gene expression resulting in abnormal cell proliferation, inhibition of apoptosis, invasion, angiogenesis and metastasis. Whilst the accumulation of genetic lesions, both somatic and hereditary, is critical in tumourigenesis, the influence of epigenetic factors such as diet and lifestyle are also important. Chemoprevention involving the use of dietary compounds to inhibit or delay the process of carcinogenesis could be an effective approach to reduce the incidence of prostate cancer [63]. Prostate cancer represents an excellent candidate disease for dietary chemoprevention, due to its typical diagnoses in elderly men. The lifetime consumption of foods high in anticancer compounds, such as the lignans, may reduce the risk of developing cancer, resulting in substantial reduction in the incidence of the disease. We have shown that ENL can reduce the growth of a widely used model of prostate cancer and can also influence the expression of genes that are associated with detrimental cellular behaviour. If the concentration used in our study could be achieved and maintained *in vivo*, it may provide a mechanism by which lignans reduce the risk of developing prostate cancer over the lifetime of an individual.

The authors have declared no conflict of interest.

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