A key regulator of cholesterol homoeostasis, SREBP-2, can be targeted in prostate cancer cells with natural products

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There is growing evidence showing that prostate cancer cells have perturbed cholesterol homoeostasis, accumulating cholesterol to promote cell growth. Consequently, cholesterol-lowering drugs such as statins are being evaluated in prostate cancer treatment. Furthermore, natural products such as betulin (from birch tree bark) and tocotrienols (a minor form of vitamin E) have been shown to lower cholesterol levels. Using these drugs and oxysterols, we have determined which aspects of cholesterol homoeostasis should be targeted in prostate cancer, e.g. cellular cholesterol levels are increased by the transcription factor SREBP-2 (sterol-regulatory-element-binding protein isoform 2), whereas LXR (liver X receptor) promotes cholesterol efflux. Whereas betulin exerted non-specific effects on cell viability, tocotrienols produced a strong direct correlation between SREBP-2 activity and cell viability. Mechanistically, tocotrienols lowered SREBP-2 activity by degrading mature SREBP-2 independently of the proteasome. In contrast, no correlation was seen between LXR activity and cell viability, implying that SREBP-2 is a better target than LXR for prostate cancer treatment. Lastly, androgen-dependent and -independent LNCaP cells were both sensitive to tocotrienols. Overall, this suggests that tocotrienols and other drugs targeting the SREBP-2 pathway are a potential therapeutic option for prostate cancer.

Key words: betulin, cholesterol, prostate cancer, sterol-regulatory-element-binding protein isoform 2 (SREBP-2), tocotrienol, vitamin E.

INTRODUCTION

In developed countries, prostate cancer is the most common cancer in males and contributes significantly to cancer-related mortality [1]. Epidemiological evidence has associated prostate cancer risk with high cholesterol levels [2,3], and laboratory studies have observed that the aging prostate and prostate cancer cells have elevated intracellular cholesterol levels [4]. Such cholesterol accumulation could promote prostate cancer development by providing raw material for membrane synthesis, androgen production and other signalling pathways [5]. Consequently, there has been considerable interest in cholesterol-lowering drugs in prostate cancer treatment [2,3]. One example is the statins, a class of drugs that target HMGCR (3-hydroxy-3-methylglutaryl-CoA reductase), which catalyses a rate-limiting step in cholesterol synthesis. Statins have proven effective in animal models (see, e.g., [6]), and there is some evidence that statins reduce the risk of advanced prostate cancer (reviewed in [3]). However, statin treatment has limitations, including their side effects [7] and the potential inability to reach sufficient concentrations at the tumour site [3]. Thus there is a need to find better methods of manipulating cholesterol levels in prostate cancer.

Cellular cholesterol levels are largely regulated by two master transcription factors: SREBP-2 (sterol-regulatory-element-binding protein isoform 2) and LXR (liver X receptor). The regulation of SREBP-2 has been thoroughly elucidated by the laboratory of Brown and Goldstein [8]. Briefly, SREBP-2 initially exists in its precursor form, tethered to the endoplasmic reticulum. When cholesterol levels are low, SREBP-2 is escorted by SCAP (SREBP-2 cleavage activation protein) to the Golgi, where SREBP-2 is cleaved. This releases the active N-terminal portion of SREBP-2, which migrates to the nucleus to up-regulate genes involved in cholesterol synthesis (e.g. HMGCR) and uptake (e.g. LDLR (low-density lipoprotein receptor)). This increases cholesterol levels, which promotes the binding of SCAP to Insig (insulin-induced gene), retaining SREBP-2 in the endoplasmic reticulum. Thus SREBP-2 increases cholesterol levels, which then feed back on its activity.

In contrast, LXR is activated by oxygenated cholesterol derivatives (oxysterols), and serves to lower cellular cholesterol levels by up-regulating genes involved in cholesterol efflux, such as ABC (ATP-binding cassette transporter) A1 and G1. Consequently, LXR agonists have been put forward as a therapeutic option for prostate cancer [9,10]. However, given that LXR also up-regulates fatty acid synthesis (e.g. via fatty acid synthase), this could cause side effects such as enhancing lipid levels in the liver (hepatic steatosis), or even in prostate cancer cells.

Recent studies have characterized novel lipid-lowering drugs isolated from natural products. For example, the active ingredient in birch tree bark, betulin, inhibits SREBP-2 activation by promoting SCAP–Insig binding [11]. In vivo, betulin improved the outcome of diet-induced metabolic diseases, such as atherosclerosis and obesity [11]. Another natural product of interest is vitamin E, which consists of two families of lipophilic compounds: tocotrienols and tocopherols. Tocotrienols are sourced from cereal
grains and palm oil, whereas tocopherols are found in vegetable oil [12]. Both families share a chromanol ring and an isoprenoid-derived side chain, but the side chain is saturated in tocopherols and unsaturated in tocotrienols [12]. Well-known as antioxidants, they have also been investigated in cholesterol homoeostasis (see, e.g., [13–15]). The tocotrienols, particularly the γ and δ isomers, blocked SREBP-2 activation and degraded HMGCR by an Insig-dependent mechanism [15]. There have been intense efforts evaluating tocotrienols for cancer therapy (reviewed in [16]), but not in the context of manipulating prostate cancer cholesterol levels.

In the present study, we examined the potential of toggling cholesterol homoeostasis in prostate cancer, using betulin and tocotrienol. We correlate this with their mechanism of action, finding that SREBP-2 activity determines resistance to the tocotrienols. Furthermore, we propose that SREBP-2 is a better target than LXR for treating prostate cancer, supporting the potential for tocotrienols in prostate cancer therapy.

EXPERIMENTAL

Materials

FBS (fetal bovine serum) was obtained from Bovogen, NCS (newborn calf serum) and penicillin/streptomycin were from Life Technologies, and all other media components were from Sigma–Aldrich. NCLPDS (lipoprotein-deficient NCS) and FBLPDS (lipoprotein-deficient FBS) were prepared from NCS and FBS respectively, as described previously [17,18]. α-, γ- and δ-tocotrienol were obtained from Cayman Chemical. Betulin, compactin (mevastatin), casodex (bicalutamide) and 25-HC (25-hydroxycholesterol) were obtained from Sigma–Aldrich. Zeocin was obtained from Life Technologies. 24,25-(S)-epoxycholesterol was obtained from Enzo Life Sciences. Testosterone was a gift from Dr David Handelsman (ANZAC Research Institute, Concord, NSW, Australia).

Cell culture

The prostate cancer cell lines LNCaP and PC-3 were a gift from Dr Pamela Russell (Australian Prostate Cancer Research Center, Buranda, QLD, Australia), and were maintained in medium A [RPMI 1640, supplemented with 10 % (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin]. Before plating LNCaP and PC-3 cells, plates and dishes were treated with polyethyleneimine (Sigma–Aldrich) to enhance cellular adhesion as described previously [19], with slight modifications: polyethyleneimine was prepared as a 25 μg/ml solution in 0.15 mM NaCl, with the pH neutralized using HCl. As specified in experiments, prostate cancer cells were treated in medium B [RPMI 1640, supplemented with 10 % (v/v) FBLPDS, 100 units/ml penicillin and 100 μg/ml streptomycin], to remove the influence of exogenous cholesterol.

The CHO (Chinese-hamster ovary) cell line CHO-7 and SRD-1 were a gift from Dr Michael Brown and Dr Joseph Goldstein (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). SRD-15 cells were a gift from Dr Russell DeBose-Boyd (University of Texas Southwestern Medical Center). CHO-7 cells were maintained in medium C [DF-12, supplemented with 5 % (v/v) NCLPDS, 100 units/ml penicillin and 100 μg/ml streptomycin], and SRD-1 and SRD-15 cells were maintained in medium C supplemented with 1 μg/ml 25-HC. As specified in experiments, these cells were treated in medium D (medium C, supplemented with 5 μM compactin and 50 μM mevalonate), to maximize SREBP-2 activity [20].

Plasmid constructs and transfection

The sources of plasmids are listed in Table 1. For simplicity, the plasmid names have been condensed. To generate the SCAP-Myc expression construct, PIPE (polymerase incomplete primer extension) cloning [21] was used to clone the SCAP gene into pcDNA4-MycHisC, retaining the Myc and His tags within the open reading frame. SCAP-Y234A-Myc was generated from SCAP-Myc using PCR-based SDM (site-directed mutagenesis) [22]. To generate the HA-SREBP-2(M) expression construct, the HA-SREBP-2(M) gene was cloned from pCMV5-HA-SREBP-2(M) into pcDNA4-MycHisC using PIPE cloning; in this construct, the stop codon is located upstream of the His and Myc tags, thus excluding these tags from the open reading frame. Point mutations were made in the DNA-binding domain (Y342R [23]) and CPD (Cdc4 phosphodegron) motif (S432A/S436A [24]) by PCR-based SDM [22]. The primers used in these cloning and mutagenesis protocols are available from A.J.B. upon request. Successful constructs were confirmed by sequencing and expression by Western blotting.

For plasmid transfection, cells were plated and transfected in their maintenance medium, using TransIT-2020 reagent (MirusBio) according to the manufacturer’s instructions. In addition, the medium was refreshed before adding the TransIT-2020–DNA complexes.

Generation of stable cell lines

To generate stable clones LNCaP-EV, LNCaP-SCAP-WT and LNCaP-SCAP-mut, LNCaP cells were each plated in a 100-mm-diameter dish and transfected with pcDNA4-MycHisC, SCAP-Myc or SCAP-Y234A-Myc constructs respectively. Transfected cells were selected in medium A using 250 μg/ml zeocin (Life Technologies), according to the manufacturer’s instructions. Single clones were selected and expanded, and stable expression was assessed by Western blotting. Stable cells were maintained in medium A, supplemented with 250 μg/ml zeocin.

To generate androgen-independent LNCaP cells, LNCaP cells were initially grown for eight passages in medium A, supplemented with 10 nM testosterone, generating the LNCaP-305 cell line. These cells can tolerate physiological androgen concentrations (~10 nM) (see, e.g., [25]). To simulate androgen ablation, LNCaP-305 cells were grown for eight passages in medium A, supplemented with 10 μM casodex, generating the LNCaP-364 cell line. These cells grow independently of the medium androgen levels. These stable populations were maintained in their respective selection medium.

Luciferase assay

Cells were plated in 60-mm-diameter dishes for transfection. Cells were transfected with 6 μg of firefly luciferase plasmid and 0.12 μg of Renilla luciferase plasmid. Following transfection, cells were trypsinized and seeded into 24-well plates in medium B, and allowed to adhere overnight. As described previously [17], the treatment was delivered in a small quantity of plating medium, added to the existing medium in the wells. After treatment, the luciferase assay was conducted using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer’s instructions. The firefly luciferase values were divided by their corresponding Renilla luciferase values and made relative to the vehicle condition to obtain ‘Renilla/vehicle-normalized luciferase activity’.

Furthermore, since LXRE-luc and SRE-luc are driven by both their respective LXR or SREBP-2 response elements and the
viral TK (thymidine kinase) promoter [26], additional cells were transfected with the TK-luc construct in the same experiment and treated in parallel. Renilla/vehicle-normalized luciferase activity values of LXRE-luc or SRE-luc were divided by those of TK-luc to determine LXRE-specific and SRE-specific promoter activity. For simplicity, this has been depicted as ‘relative luciferase activity’ in the Figures.

**WST-1 (water-soluble tetrazolium salt 1) assay**

Cells were seeded in 96-well plates in Phenol Red-free RPMI 1640 medium, supplemented with 0.1% fatty-acid-free BSA, 100 units/ml penicillin and 100 μg/ml streptomycin. Phenol Red was avoided since it was found to interfere with the WST-1 assay, and cells were seeded in serum-free conditions to provide an equal baseline when treatment began in different serum conditions. Owing to different growth rates between cell lines, PC-3 cells have been described previously [26,28,29].

**qRT-PCR (quantitative real-time reverse transcription–PCR)**

Cells were plated and treated as described in the Figure legends. RNA was harvested and reverse-transcribed, and mRNA levels were quantified by qRT-PCR as described previously [19]. All primers for human genes have been described previously [19], except that different ABCG1 primers were used, with the ABCG1 primer sequences provided by Dr Etienne Lefai (Faculté de Médecine Lyon Sud, Lyon, France). All primers for hamster genes have been described previously [26,28,29].

**siRNA (small interfering RNA) knockdown**

Cells were plated in medium A at 350 000 cells/well in six-well plates. The next day, the medium was refreshed and cells were transfected with 25 nM siRNA, using Lipofectamine™ RNAi-MAX (Life Technologies) according to the manufacturer’s instructions. As a negative control (siNC), scrambled siRNA (SIC001, Sigma–Aldrich) was used. siRNA oligonucleotides against SCAP (siSCAP) were designed by Sigma–Aldrich and their sequences (sense strand provided only) are as follows: siSCAP1, 5′-CACUCAAAGACUUGUUAUUTT-3′; siSCAP2, 5′-GCAUCAUCUAGCGGAGUUT-3′; siSCAP3, 5′-GACUAGCAGCUUGCGUUT-3′; siSCAP4, 5′-CCGCUUGCUUGUUT-3′; siSCAP5, 5′-GGAAAUGUCUCCGCGCGC-3′; siSCAP6, 5′-GUGCUGUUCGCGCGC-3′. Their efficiency was assessed by Western blotting (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/446/bj4460191add.htm).

**RESULTS**

**Cell viability correlates with changes in SREBP-2 activity**

Previously, we have shown that PC-3 cells have higher basal SREBP-2 activity than LNCaP cells [17]. Thus we hypothesized that SREBP-2 activity would be less affected by betulin and tocotrienols in PC-3 cells. We tested this using a luciferase reporter assay: in this experiment, cells were co-treated with the HMGCR inhibitor compactin to eliminate HMGCR-specific effects (Figure 1A) and increase basal SREBP-2 activity. As
expected, SREBP-2 activity in PC-3 cells was less affected than in LNCaP cells (Figure 1B), with γ- and δ-tocotrienol being more potent than the other drugs. In contrast, the drugs had little effect on LXR activity in either cell line, in contrast with the LXR agonist GW68395 (Figure 1C).

Next, we tested whether tocotrienols reduce SREBP-2(M) by interfering with cholesterol homeostasis, lowering cholesterol levels by reducing SREBP-2 activity, activating LXR and degrading HMGCR [30]. Thus these tocotrienols were used to determine the dependence of cell viability on SREBP-2 compared with LXR activity. In terms of SREBP-2 activity, 25-HC was more potent, and LNCaP cells were more affected (Figure 3A). The reverse trend could be seen for LXR activity, with 24,25-EC being more agonistic and PC-3 cells more responsive (Figure 3B). Furthermore, cell viability was more affected in LNCaP cells, with 25-HC being more potent (Figure 3C), correlating better with SREBP-2 rather than LXR activity (Figure 3D). In addition, 1 μM GW68395, which activated LXR more than 25-HC (Figures 1C and 3B), had little effect on LNCaP or PC-3 cell viability (results not shown). Overall, these data suggest that cell viability is more influenced by SREBP-2 than by LXR activity.

**Androgen-independence does not completely determine tocotrienol sensitivity**

We explored why PC-3 cells were more resistant to tocotrienols. Given that LNCaP cells are androgen-sensitive, whereas PC-3 cells are androgen-independent, could it be related to their androgen-independence? To test this, we generated LNCaP-364 cells, an LNCaP subline that grows independently of medium androgen status.

In response to betulin, LNCaP-364 cells resembled PC-3 cells (Figures 4A and 4B), particularly with SREBP-2 activity being more resistant than in LNCaP cells (Figure 4A, leftmost panel). For the tocotrienols, LNCaP-364 cells exhibited a similar SREBP-2 response to LNCaP cells (Figure 4A), with their SREBP-2 activities having a comparable correlation with cell viability (Figure 4C). Furthermore, at high tocotrienol concentrations, LNCaP-364 cells displayed an intermediate sensitivity between LNCaP and PC-3 cells (Figure 4B). Together, these data suggest that androgen-dependence only partially determines tocotrienol sensitivity.

**Tocotrienols act by degrading mature SREBP-2**

To provide insight into the determinants of tocotrienol resistance, we explored the mechanism by which tocotrienols inhibit SREBP-2 activity. We employed the CHO-7 cell line and its mutant derivatives, which are commonly used to study cholesterol homeostasis (see, e.g., [20,31]).

Since tocotrienols degrade HMGCR by promoting HMGCR–Insig binding [15], we hypothesized that tocotrienols would reduce active SREBP-2 by inhibiting its processing in an Insig-dependent mechanism. To test this, we compared CHO-7 cells with Insig-deficient SRD-15 cells [32]. For instance, 25-HC ablates SREBP-2 processing in CHO-7 cells (Figure 5A, left-hand panel), demonstrated by a loss of the active mature form (M) and accumulation of the precursor form (P). This effect was severely blunted in SRD-15 cells (Figure 5A, right-hand panel), demonstrating by a loss of the active mature form (M) and accumulation of the precursor form (P). This effect was severely blunted in SRD-15 cells (Figure 5A, right-hand panel), confirming that 25-HC acts through Insig to inhibit SREBP-2 processing [33]. In contrast, the tocotrienols had a similar effect in both cell lines (Figure 5A), suggesting that tocotrienols reduce SREBP-2(M) independently of Insig.

Next, we tested whether tocotrienols reduce SREBP-2(M) by inhibiting SREBP-2 processing. We focused on δ-tocotrienol, since it had the most pronounced effect in CHO-7 cells (Figure 5A). We used the SRD-1 cell line, a mutant CHO-7 cell line that constitutively expresses SREBP-2(M) [34]. Its
Figure 2  
Betulin and tocotrienols influence cell viability in a cholesterol-dependent manner

(A) Cells were treated with compactin (CPN), betulin (Bet), α-tocotrienol (αT3), γ-tocotrienol (γT3) or δ-tocotrienol (δT3) at the concentrations indicated, in the presence of FBLPDS. After 3 days, cell viability was measured using the WST-1 assay. (B) The effects of α-tocotrienol (αT3), γ-tocotrienol (γT3) and δ-tocotrienol (δT3) on cell viability (from A) are plotted against their respective effects on SREBP-2 activity (from Figure 1B) for both cell lines (PC-3 and LNCaP). For the combined dataset, the correlation is $y = 0.85x$ ($R^2 ≈ 0.81$, $P < 0.01$, regression analysis). (C) Cells were treated and assayed as in (A), except using 10 μM drug (indicated) with or without mevalonate (5 mM). The change in viability with mevalonate treatment is presented as a percentage, relative to the vehicle (Veh) condition. (D) Cells were treated and assayed as in (A), except using 10 μM drug (indicated) with either cholesterol-deficient (FBLPDS) or full (FBS) serum. Viability is relative to the vehicle (Veh)/FBLPDS condition for each cell line. (A–D) Results are means ± S.E.M. from at least three separate experiments for each cell line, each performed with triplicate wells per condition.

To test this, we used ectopic SREBP-2(M), with mutations in the DNA-binding domain (Y342R [23], denoted ‘DNABmut’) and CPD motif (S432A/S436A [24], denoted ‘CPDmut’). The phosphorylation doublet seen with wild-type SREBP-2(M) (WT) was lost with CPDmut (Figure 5C). Treatment with δ-tocotrienol was accompanied by co-treatment with a translation inhibitor, cycloheximide, to ensure only effects on degradation were being observed. δ-Tocotrienol promoted the degradation of both wild-type and mutant SREBP-2(M), suggesting that this occurs independently of the Akt/GSK3/Fbw7 axis. Furthermore, although the proteasomal inhibitor MG132 increased SREBP-2(M), it could not rescue the δ-tocotrienol effect (Figure 5D). Together, these data suggest that tocotrienols degrade SREBP-2(M) independently of the proteasome.

To confirm that this SREBP-2 degradation has downstream effects, we examined the tocotrienol effect on SREBP-2 target gene expression. Similarly to SREBP-2 processing (Figure 5A), δ-tocotrienol reduced the expression of SREBP-2 target genes higher basal SREBP-2(M) levels required a longer treatment time before a clear fall in SREBP-2(M) levels was observed (8 h, compared with 4 h in CHO-7 cells) (Figure 5B). Interestingly, this δ-tocotrienol effect was enhanced by 25-HC. This oxysterol could promote SREBP-2(M) degradation by the Akt/GSK-3 (glycogen synthase kinase 3)/Fbw7 (F-box and WD repeat domain-containing 7) axis. This axis has been elucidated for SREBP-1 and generalized to SREBP-2 [5,35]: when mature SREBP binds DNA, it is phosphorylated by GSK-3 at the CPD motif. This primes SREBP for ubiquitination by the E3 ligase Fbw7 and subsequent proteasomal degradation [24]. This is prevented by Akt, which phosphorylates GSK-3. In turn, 25-HC degrades Akt [36], which would derepress GSK-3 and promote SREBP-2(M) degradation. Given that δ-tocotrienol has been shown to reduce Akt activity [37,38], and 25-HC and δ-tocotrienol act synergistically (Figure 5B), could δ-tocotrienol influence the Akt/GSK-3/Fbw7 axis of SREBP-2(M) degradation?
Figure 3  The influence of oxysterols on cell viability correlates better with SREBP-2 activity than with LXR activity

(A and B) Cells were transfected with SRE-luc (A) or LXRE-luc (B), and treated for 24 h with 24,25-EC or 25-HC at the concentrations indicated. In (A), cells were also co-treated with compactin (10 μM). Following treatment, cells were assayed for luciferase activity. (C) Cells were treated with 24,25-EC or 25-HC (3.3 μM) in the presence of FBS. After 3 days, cell viability was measured using the WST-1 assay and made relative to the vehicle condition. (D) Summary of trends in (A)–(C). (A–C) Results are means ± S.E.M. from at least three separate experiments, each performed with triplicate wells per condition.

(LDLR and HMGCR) in CHO-7 cells (Figure 6). Similar effects were observed at 4 h (n = 3, results not shown). In SRD-1 cells, δ-tocotrienol reduced LDLR expression by 37 ± 3% at 8 h under the same experimental conditions (n = 3). These effects could be translated to a prostate setting, with SREBP-2 target gene expression being suppressed in LNCaP cells (Figure 6). In contrast, LXR target gene (ABCA1 and ABCG1) expression was unaffected by δ-tocotrienol (Figure 6), but increased with an LXR ligand (25-HC), together showing a similar trend to LXR-driven luciferase activity (Figure 1C). Overall, tocotrienols degrade SREBP-2(M), having downstream effects on target gene expression in both the CHO system and prostate cancer cells.

Modulating SCAP levels alters sensitivity to tocotrienols

Since tocotrienols degrade SREBP-2(M), could higher SREBP-2 processing provide resistance to tocotrienols? In support of this, we found that SCAP mRNA levels were higher in PC-3 than LNCaP cells (Figure 7A). Higher SCAP levels would promote SREBP-2 processing, generating more SREBP-2(M) to protect against tocotrienols.

To confirm that increased SCAP mRNA levels translate into more SCAP protein, we used the R139 antibody [27]. This antibody was raised against hamster SCAP; we have characterized it for use against human SCAP in the present study (Supplementary Figure S1 at http://www.BiochemJ.org/bj/446/bj4460191add.htm). We found that PC-3 cells have SCAP protein levels comparable with those in LNCaP cells (Figure 7B), despite differences at the mRNA level. In addition, we observed a 110 kDa band that was expressed prominently in PC-3 cells, but found it to be not specific to SCAP (Supplementary Figures S2 and S3 at http://www.BiochemJ.org/bj/446/bj4460191add.htm). Overall, it is unlikely that differences in SCAP expression explain the tocotrienol resistance in PC-3 cells.

Nevertheless, to confirm the role of SREBP-2 activity in tocotrienol resistance, we manipulated the levels of its regulator, SCAP. First, SCAP knockdown with siRNA lowered basal viability in PC-3 cells, which could not be reduced further with δ-tocotrienol (Figure 7C). Thus eliminating SCAP expression ablates the effect of δ-tocotrienol. Secondly, we generated LNCaP clones that stably express either ectopic human wild-type SCAP or mutant SCAP (Y234A), which cannot mediate SREBP-2 processing [39] (Figure 7D). Overexpressing wild-type SCAP increased resistance to δ-tocotrienol with regard to SREBP-2 activity and cell viability, whereas overexpressing mutant SCAP did not (Figure 7E). Again, a strong correlation exists between SREBP-2 activity and cell viability (Figure 7E, right-hand panel). Together, these results demonstrate that altering SCAP expression influences sensitivity to tocotrienols.

DISCUSSION

In the present study, we have used natural products to toggle cholesterol homeostasis in prostate cancer cells, determining potential targets for prostate cancer therapy. We found that (i) tocotrienols degrade mature SREBP-2, (ii) altering SREBP-2 activity, such as by adjusting SCAP expression, influences sensitivity to tocotrienols, and (iii) targeting SREBP-2 is more lethal to prostate cancer cells than is LXR.

Initially, we focused our attention on both betulin and the tocotrienols. Betulin influenced SREBP-2 activity more in LNCaP cells than PC-3 cells (Figure 1B), but had a similar effect on cell viability (Figure 2A), suggesting that it exerts other effects that influence cell viability. Betulin has been shown to induce apoptosis in PC-3 cells [40], but we did not consider it further because, in contrast, tocotrienols demonstrated a strong correlation between SREBP-2 activity and cell viability (Figures 2B, 4C and 7E).
We found that \(\gamma\)- and \(\delta\)-tocotrienols were more potent inhibitors of SREBP-2 than was \(\alpha\)-tocotrienol. This trend was also observed with HMGCGR degradation [15], and given that tocotrienols degrade HMGCGR via Insig-1, we hypothesized that SREBP-2 activity was also reduced in an Insig-dependent manner. However, this was not the case, instead degrading SREBP-2(M) (Figure 5). It has been demonstrated that SREBP-2(M) is ubiquitinated [41] and degraded by the proteasome [42]. Furthermore, a well-characterized GSK-3-dependent degradation pathway has been elucidated for SREBP-1, with evidence suggesting that this also occurs for SREBP-2 [5,35]. However, \(\delta\)-tocotrienol promoted SREBP-2(M) degradation independently of GSK-3 and the proteasome (Figure 5), with further work required to elucidate this mechanism. Nevertheless, this has downstream effects on SREBP-2 target gene expression (Figure 6), adding a new dimension to previous work that showed tocotrienols regulate cholesterol metabolism.

Furthermore, this implies that PC-3 cells could be more resistant to tocotrienols than LNCaP cells (Figure 1 and 2) because they have higher mature SREBP-2 levels than LNCaP cells [17]. We hypothesized the latter was due to high mRNA levels of the SREBP-2 activator, \(SCAP\), in PC-3 cells (Figure 7A), but found this did not translate to the protein level (Figure 7B). Despite this, manipulating \(SCAP\) levels via siRNA knockdown or overexpression constructs had noticeable effects on tocotrienol sensitivity (Figure 7). Although we have not ruled out effects of tocotrienols on other transcription factors such as PPARs (peroxisome-proliferator-activated receptors) and RXR (retinoid X receptor), these \(SCAP\) modulation experiments (Figure 7) demonstrate that tocotrienol sensitivity is dependent on SREBP-2 activity. Moreover, mevalonate treatment rescued cell viability against HMGCGR inhibition (compactin), but not against tocotrienols (Figure 2C), suggesting that the tocotrienols acted largely through SREBP-2, which regulates cholesterol levels more broadly than HMGCGR. In addition to cholesterol synthesis, degrading SREBP-2 also reduces cholesterol uptake.

\[ y = 0.99x \ (R^2 \approx 0.89) \]

\[ y = 1.21x - 0.15 \ (R^2 \approx 0.92) \]
by down-regulating LDLR expression (Figure 6). Combined with the oxysterol data (Figure 3), this supports the notion that targeting SREBP-2 would be preferable over targeting LXR.

Several studies have explored LXR as a prostate cancer target, finding that LXR ligands arrested prostate cancer cell growth, via ABCA1, p27 and Akt inhibition [9,10]. However, these studies used the LXR ligand TO-901317, which has been shown to antagonize the androgen receptor [43] and activate nuclear receptors FXR (farnesoid X receptor) [44] and PXR (pregnane X receptor) [45]. Oxysterols, which additionally inhibit HMGCR and SREBP-2 activity [30], were also used. In contrast, we found that a more specific LXR ligand, GW683965, had little effect on cell viability and that SREBP-2 activity has a greater influence over cell viability than LXR activity. However, we have only shown this in a prostate cancer setting. Unlike prostate cells is the androgen receptor, a key regulator of prostate cell growth and differentiation, which also reduces LXR activity [19]. This is evident with GW683965 (Figure 1C), and oxysterols (Figure 3) being less potent LXR ligands in LNCaP cells compared with androgen-receptor-null PC-3 cells. The androgen receptor/LXR cross-talk would dampen the effect of LXR ligands on prostate cell viability, but targeting LXR may still be useful for other cancers.

In the context of tocotrienols, other studies have also found that γ- and δ-tocotrienol are more lethal to prostate cancer cells than α-tocotrienol [46–48], with tocotrienols being more effective than tocopherols [38,46,48]. In contrast with the present study, one study found the viability of PC-3 cells to be more sensitive than LNCaP to tocotrienols [47], but this could be attributed to shorter treatment times (24–48 h, compared with 72 h in the present study) and serum conditions (FBS compared with FBLPDS). In fact, all other studies used full serum, achieving IC50 values above the low-micromolar range assessed in the present study. Despite this, similarly high concentrations had little effect on prostate epithelial cells [47,48], as seen with the tocotrienol-rich fraction of palm oil [49], suggesting a cancer-specific phenomenon. Nevertheless, attainable serum tocotrienol levels are in the high-nanomolar range (see, e.g., [50]), with postprandial levels peaking at ∼2–3 μM after 4 h and returning to a submicromolar baseline after 8 h [50], which also suggests a low bioavailability. Thus physiologically relevant concentrations of tocotrienols only seem to influence cell viability under cholesterol-deficient conditions (compare Figures 2A and 2D).

Consequently, serum cholesterol levels may need to be lowered to enhance the effective tocotrienol dose. It should be noted that, although tocotrienols lower LDLR expression (Figure 6), this does not necessarily eliminate uptake of serum cholesterol by other means, such as via passive diffusion. Thus lowering serum cholesterol in vivo would confirm that the deficiency of cholesterol

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**Figure 5** Tocotrienols lower SREBP-2 activity by degrading nuclear SREBP-2.

(A) CHO-7 and SRD-15 cells were plated in medium C, and starved for 16 h in medium D. The cells were then treated for 4 h with vehicle (Veh) or betulin (Bet), α-tocotrienol (αT3), γ-tocotrienol (γT3), δ-tocotrienol (δT3) or 25-HC, all at 10 μM in medium D. Protein was harvested and subjected to SDS/PAGE and Western blotting as in (A). CHO-7 cells were seeded in 60-mm-diameter dishes and transfected with HA-SREBP-2(M), seeded and starved as in (A). (B) SRD-1 cells were plated in medium C, and starved for 16 h in medium D. The cells were then treated with δ-tocotrienol (δT3) (10 μM) and/or 25-HC (10 μM) as indicated, in medium D. Following treatment, protein was harvested and subjected to SDS/PAGE and Western blotting as in (A). (C) CHO-7 cells were seeded in 60-mm-diameter dishes and transfected with HA-SREBP-2(M). (D) CHO-7 cells were transfected with wild-type HA-SREBP-2(M), seeded and starved as in (C). The cells were then treated for 6 h with or without δ-tocotrienol (δT3) (10 μM) or MG132 (10 μM), in the presence of cycloheximide (CHX) (10 μg/ml). Following treatment, protein was harvested and subjected to SDS/PAGE and Western blotting as in (A). (A–D) Blots are each representative of at least two separate experiments. Molecular masses are indicated in kDa.
**Figure 6 δ-Tocotrienol has downstream effects on SREBP-2 target gene expression**

CHO-7 cells were plated in medium C, and starved for 16 h in medium D. LNCaP cells were plated in medium A, and then starved for 16 h in medium B, supplemented with 5 μM compactin and 50 μM mevalonate to be consistent with the CHO-7 cells. Cells were treated for 8 h with 10 μM δ-tocotrienol (δT3) or 10 μM 25-HC in their respective starvation medium. Following treatment, RNA was harvested and mRNA expression was determined by qRT-PCR, normalized to the vehicle (Veh) condition. Results are means ± S.E.M. from three separate experiments per cell line, each performed with triplicate wells per condition.

**Figure 7 Changing SCAP levels alters sensitivity to tocotrienols**

(A) and (B) LNCaP and PC-3 cells were grown in medium A. (A) RNA was harvested and SCAP mRNA levels were determined by qRT-PCR, normalized to LNCaP cells. (B) Protein was harvested and subjected to SDS/PAGE and Western blotting against SCAP and α-tubulin. The asterisk (*) denotes a non-specific band explored further in Supplementary Figures S2 and S3 at [http://www.BiochemJ.org/bj/446/bj4460191add.htm](http://www.BiochemJ.org/bj/446/bj4460191add.htm). Densitometry was performed on the SCAP band (150 kDa) and α-tubulin, and relative density was calculated as SCAP/α-tubulin, normalized to LNCaP cells. (C) PC-3 cells were transfected with negative-control scrambled siRNA (siNC) or SCAP-specific siRNA (siSCAP2), then seeded into 96-well plates. The next day, cells were treated with different concentrations of δ-tocotrienol (δT3) in FBLPDS for 3 days, after which the WST-1 assay was performed. (D) LNCaP cells stably transfected with empty vector (EV), wild-type SCAP (WT) or Y243A-mutant SCAP (mut) were grown in medium A, supplemented with 250 μg/ml zeocin. Protein was harvested and subjected to SDS/PAGE and Western blotting against Myc, SCAP and α-tubulin. For SCAP, the 'low exposure' was 5 s and 'high exposure' was 30 s. (E) Left-hand panel: LNCaP clones described in (D) were transfected with SRE-luc and treated with different concentrations of δT3, in the presence of compactin (5 μM). Following treatment, cells were assayed for luciferase activity. Middle panel: LNCaP clones were treated with different concentrations of δT3 for 3 days as in (C), after which the WST-1 assay was performed. Right-hand panel: the effects of δT3 on cell viability and SREBP-2 activity were correlated as described in Figure 2(B). The correlation for the combined dataset is \( y = 1.21x - 0.24 \) (\( R^2 \approx 0.90, P < 0.01 \), regression analysis). (B and D) Blots are representative of at least two separate experiments. Molecular masses are indicated in kDa. (A-C and E) Results are means ± S.E.M. from at least three separate experiments, with each experiment performed with triplicate wells per condition.
in FBLPDS is what causes tocotrienol sensitivity (compare Figures 2A and 2D), as opposed to other elements removed during FBLPDS preparation. As proof-of-principle, co-treatment of a statin with γ- or δ-tocotrienol worked synergistically to impair melanoma growth in vivo [51]; this could be tested in prostate cancer. Furthermore, although we found no changes in the expression of cholesterol-efflux genes (Figure 6), in vivo studies should consider other whole-body compensatory mechanisms, such as cholesterol reabsorption. In this respect, tocotrienols have been found to reduce serum-cholesterol levels (see, e.g., [52]), and could thus enhance their own observed effects against prostate cancer cells in vitro.

In addition, mechanistic insights from previous studies should be tested in this setting, such as increased apoptotic signalling [46,47], reduced metastasis by enhanced E-cadherin expression [47], increased PPARγ activity, and reduced TGFβ2 (transforming growth factor β2) signalling [48] by tocotrienols. Interestingly, one study observed tocotrienols that induced caspase-dependent apoptosis in LNCaP, but not PC-3 or DU-145, cells, from which the authors proposed that androgen-dependence influences sensitivity [46]. Using our progression model, we have shown that both androgen-dependent (LNCaP) and -independent (LNCaP-364) cells were sensitive to tocotrienols (Figure 5C) and oxysterols (results not shown), supporting the idea that cholesterol homeostasis could be an alternative therapeutic target for androgen-independent prostate cancer [53].

There is very little in the way of treatment options to substantially extend survival in patients with advanced recurrent prostate cancer, with existing strategies including chemotherapeutic and immunotherapeutic approaches [54]. Could tocotrienols be useful here? For instance, γ-tocotrienol preferentially accumulated in prostate cancer xenografts, reduced xenograft growth, and sensitized xenografts to chemotherapy [55,56]. To our knowledge, no large-scale clinical trials have been conducted to evaluate tocotrienols for prostate cancer treatment. The potential for tocotrienols, and other drugs that manipulate cholesterol homeostasis via SREBP-2, should be explored further in a prostate cancer setting. Given that SREBP-2 has also been considered for metabolic diseases such as diabetes, cardiovascular disease and hyperlipidaemia [11,57], this has implications for other disease settings.

AUTHOR CONTRIBUTION
The study was conceived and designed by James Krycer and Andrew Brown. Experimentation and data analysis was performed by James Krycer and Lisa Phan. The paper was written by James Krycer and Andrew Brown, and proofread by Lisa Phan before submission.

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Targeting cholesterol in prostate cancer with tocotrienols


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A key regulator of cholesterol homoeostasis, SREBP-2, can be targeted in prostate cancer cells with natural products

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Characterization of the R139 antibody for use against human SCAP

To the best of our knowledge, the R139 antibody has not been properly characterized for use against human SCAP. The R139 antibody was raised against hamster SCAP [1], but given the high homology between hamster and human SCAP (Figure S1A), we found that the R139 antibody detected a 150 kDa band in PC-3 and LNCaP cells (Figure S1B, lanes 1 and 6). This band could be knocked down by siRNA against SCAP (siSCAP, lanes 2–5) and was the same size as ectopic human SCAP (SCAP-Myc, lane 7). This putative human SCAP band matched the size of the ectopic hamster SCAP (haSCAP, lane 8), which was shifted by a GFP (green fluorescent protein)-fusion (GFP–haSCAP, lane 9). Thus the R139 antibody can be used to detect human SCAP.

Furthermore, we observed that this antibody detected a 110 kDa band that was uniquely prominent in PC-3 cells. We hypothesized this to be a truncated SCAP isoform that has been documented previously (GenBank® accession number EAW64825.1), and which should be of comparable size (this truncated isoform is 905 amino acids long, compared with 1279 amino acids for wild-type SCAP). Interestingly, it is missing the Insig-binding domain (Figures S2 and S3A), potentially explaining three observations: (i) that siSCAP1–siSCAP3 failed to knock it down (Figure S1B, lanes 3–5), whereas it could be detected by qRT-PCR (Figure 6A of the main text) and Western blotting (Figure 6B of the main text); (ii) that SCAP mRNA levels differed between LNCaP and PC-3 cells (Figure 6A of the main text), yet “full-length” SCAP protein levels remained the same (Figure 6B of the main text); (iii) PC-3 cells have higher SREBP-2 processing than LNCaP cells [2]. However, using siRNA oligonucleotides against the C-terminal half of SCAP (siSCAP4–siSCAP6) had no effect on this 110 kDa band (Figure S3B), suggesting that it was not a putative SCAP isoform, but an unrelated non-specific band. Furthermore, we could not amplify the truncated isoform from PC-3 cDNA, even when sourced from siSCAP-treated cells, suggesting that it is unlikely to be expressed in PC-3 cells.
Figure S1  The R139 antibody can detect human SCAP

(A) ClustalW2 alignment of the human (Uniprot Q12770) and Chinese hamster (‘hamster’, Uniprot P97260) protein sequences. The R139 antibody was raised against CHO-7 SCAP amino acids 54–277 and 540–707 (in bold and underlined).

(B) Lanes 1–5, PC-3 cells were either untransfected (−) or transfected with scrambled siRNA (siNC) or SCAP-specific siRNA (siSCAP1–siSCAP3), as described in the Experimental section of the main text; lane 6, LNCaP cells were grown in medium A; lanes 7–9, HeLaT (highly transfectable HeLa) cells were a gift from Dr Noel Whitaker (University of New South Wales, Sydney, NSW, Australia) and were maintained in medium A. These cells were seeded in a six-well plate and transfected with 1 μg of SCAP-Myc, haSCAP or GFP–haSCAP expression constructs for 24 h. The medium was refreshed and, 24 h later, the cells were harvested. Lysates (50 μg for PC-3 and LNCaP, 15 μg for HeLaT) were subjected to SDS/PAGE and Western blotting against SCAP (R139) and α-tubulin. The asterisk (*) denotes a non-specific band investigated further in Figures S2 and S3. Molecular masses are indicated in kDa.
The region predicted to be deleted in the putative truncated SCAP isoform was deduced from a ClustalW2 alignment of the protein sequence (GenBank® accession number EAW64825.1) with the wild-type protein sequence (GenBank® accession number EAW64826.1) (not shown). Similarly, the R139-binding regions were deduced from the amino acid sequences used to raise this antibody (detailed in Figure S1A). A simplified schematic diagram is presented in Figure S3A.
Figure S3  The 110 kDa band detected by the R139 antibody is not a putative SCAP isoform

(A) Schematic diagram of the deleted region of the putative truncated SCAP isoform, relative to the positions of the R139-, qRT-PCR primer- and siSCAP-binding sites. A more detailed version is presented in Figure S2. (B) PC-3 cells were transfected with negative-control scrambled siRNA (siNC) or SCAP-specific siRNA (siSCAP1–siSCAP6). Following transfection, protein was harvested and handled as described in Figure 6(B) of the main text. The asterisk (*) denotes the non-specific band detected by the R139 antibody. Densitometry was performed on the R139 bands (110 and 150 kDa) and α-tubulin band, and relative density was calculated as SCAP/α-tubulin, normalized to the siNC condition. Results are means ± S.D. representative of two separate experiments for each siRNA.

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