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RESEARCH PAPER

The sphingosine kinase inhibitor 2-(*p*-hyroxyanilino)-4-(*p*-chlorophenyl)thiazole reduces androgen receptor expression via an oxidative stress-dependent mechanism

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BACKGROUND AND PURPOSE
Sphingosine kinase catalyses the formation of sphingosine 1-phosphate and is linked with androgen receptor signalling in prostate cancer cells. Therefore, we investigated the effect of sphingosine kinase inhibitors on androgen receptor expression.

EXPERIMENTAL APPROACH
Androgen-sensitive LNCaP cells were treated with SKi (2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole), which inhibits sphingosine kinases 1 and 2 activity, and the effect on androgen receptor expression was measured.

KEY RESULTS
Treatment of cells with SK1 inhibitors reduced the expression of the androgen receptor and prostate-specific antigen, while (*R*)-FTY720 methyl ether (a sphingosine-kinase-2-selective inhibitor), at a concentration that eliminates sphingosine kinase 2 from cells, had no significant effect on androgen receptor expression. The effect of SKi on androgen receptor expression was independent of the SKi-induced proteasomal degradation of SK1 and was post translational, although androgen receptor mRNA transcript was reduced. Fumonisin B1 (a ceramide synthase inhibitor) also failed to reverse the effect of SKi on androgen receptor expression, thereby excluding a role for ceramide derived from the salvage pathway. The effect of SKi on androgen receptor expression was reversed by N-acetylcysteine, which was used to scavenge reactive oxygen species.

CONCLUSION AND IMPLICATIONS
Inhibition of sphingosine kinase 1 activity abrogates androgen receptor signalling via an oxidative stress-induced, p53-independent mechanism in prostate cancer cells. Therefore, SK1 inhibitors may offer therapeutic potential in promoting the removal of AR receptors from prostate cancer cells, resulting in an increased efficacy, which is likely to be superior to inhibitors that simply reversibly inhibit AR signalling.

Abbreviations

AR, androgen receptor; GSSG, oxidized glutathione; LC3, microtubule associated protein 1 light chain 3; MDM2, murine double minute 2; NAC, *N*-acetyl-L-cysteine; PSA, prostate-specific antigen; ROS, reactive oxygen species; SK1, sphingosine kinase 1; S1P, sphingosine 1-phosphate

Introduction

There is a considerable body of evidence to demonstrate that sphingosine 1-phosphate (S1P) and sphingosine kinase (SK), which catalyses the formation of S1P from sphingosine, are involved in cancer (Pyne and Pyne, 2010). Therefore, the S1P/SK pathway is an attractive target for cancer chemotherapy. There are two isoforms of sphingosine kinase (SK1 and SK2) that are encoded by distinct genes and possess different functions (Pyne and Pyne, 2001; 2010). S1P is degraded by S1P lyase but can be recycled to sphingosine by the action of lipid phosphate- and S1P-specific phosphatases. The expression of SK1 is increased in stomach, lung, brain, colon, kidney and breast cancers and non-Hodgkin's lymphoma (Pyne and Pyne, 2010). Indeed, high expression of SK1 in ER^+ breast cancer tumours and grade 4 astrocytoma is associated with poor prognosis (Li *et al.*, 2008; Long *et al.*, 2010; Watson *et al.*, 2010). Moreover, the deletion of SK1 in p53-deficient mice reduces thymic lymphomas and prolongs the survival of these mice by 30% (Heffernan-Stroud *et al.*, 2011). Indeed, a number of studies showed that the injection or implantation of SK1 overexpressing cancer cells results in the formation of larger vascularized tumours (see Pyne and Pyne, 2010 and references cited therein). In addition, SK1 can functionally interact with and is regulated by oncogenes such as Ras (Xia *et al.*, 2000). Prostate tumour inducer-1 (PTI-1), when over-expressed in cells, also activates SK1 (Leclercq *et al.*, 2011). SK1 also functionally interacts with tumour suppressors. For instance, deactivation of SK1 occurs via its dephosphorylation catalysed by protein phosphatase 2A (PP2A) (Barr *et al.*, 2008), involving the tumour suppressor B'α (B56α/PR61α/PPP2R5A) regulatory subunit of PP2A (Arnold and Sears, 2008; Pitman et al. 2011). The finding that p53 induces a caspase-2-dependent proteolytic degradation of SK1 in response to genotoxic stress (Heffernan-Stroud *et al.*, 2011) suggests that p53 inactivating mutations in tumours might lead to an increase in SK1 expression, which in turn provides a selective growth and survival advantage to cancer cells. Therefore, there is a strong rationale for the development of SK1 inhibitors for the treatment of cancer.

In this regard, several SK1 inhibitors have been synthesized. SK1-I (BML-258, (2*R*,3*S*,4*E*)-*N*-methyl-5-(4′-pentylphenyl)-2-aminopent-4-ene-1,3-diol) is specific for SK1 and enhances survival of mice in an orthotopic intracranial tumour model (Kapitonov *et al.*, 2009). SKi (or SKI-II, 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole) inhibits both SK1 and SK2 activity and induces apoptosis of various cancer cell lines (French *et al.*, 2003). FTY720 (2-amino-[2-(4-*n*-octylphenyl)ethyl]-1,3-propanediol) and *(S)*-FTY720 vinylphosphonate are also inhibitors of SK1 activity (Tonelli *et al.*, 2010; Pyne *et al.*, 2011; Lim *et al.*, 2011a).

SK2 also has an important role in cancer. Thus, siRNA knock down of SK2 enhances doxorubicin-induced apoptosis of breast or colon cancer cells (Sankala *et al.*, 2007) and reduces proliferation and migration/invasion in A498, Caki-1 or MDA-MB-231 cells (Gao and Smith, 2011), and this is more effective than knock down of SK1. Three new SK2-selective inhibitors, namely (*R*)-FTY720 methyl ether ((*R*)-FTY720-OMe), ABC294640 [3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl) amide] and *trans*-*N*-(4-(4-octylphenyl)-cyclohexyl)-*N,N,N*-trimethylammonium iodide, have been synthesized recently (French *et al.*, 2010; Lim *et al.*, 2011b; Raje *et al.*, 2012). (*R*)-FTY720-OMe is a competitive (with sphingosine) inhibitor of SK2. Treatment of MCF-7 cells with (*R*)-FTY720-OMe prevents actin enrichment into lamellipodia in response to S1P (Lim *et al.*, 2011b). ABC294640 is a competitive inhibitor (with sphingosine) of SK2 activity (French *et al.*, 2010). ABC294640 inhibits proliferation and migration of several tumour cell lines and reduces mammary adenocarcinoma growth (French *et al.*, 2010).

The S1P signalling pathway is involved in regulating the proliferation of androgen-independent prostate cancer PC-3 cells (Akao *et al.*, 2006). Indeed, SK1 inhibitors sensitize radiation-resistant LNCaP cells to irradiation-induced apoptosis (Nava *et al.*, 2000). Chemotherapeutic agent-induced reduction in cell viability *in vivo* is also correlated with the efficacy of SK1 inhibition via an unidentified mechanism (Pchejetski *et al.*, 2005). Moreover, overexpression of recombinant SK1 induces chemotherapeutic resistance in prostate cancer cells (Pchejetski *et al.*, 2008). In addition, LNCaP cells overexpressing recombinant SK1 do not undergo growth inhibition when androgen is removed (Dayon *et al.*, 2009). These findings suggest that SK1 promotes the survival and androgen-independent proliferation of prostate cancer cells.

We have also shown that the expression of two N-terminal variants of SK1, termed SK1a (GenBank number: NM_001142601) and SK1b (GenBank number: NM_182965, as SK1a but with an additional N-terminal 86 amino acid extension), are increased in androgen-independent LNCaP-AI cells compared with androgen-sensitive LNCaP cells (Loveridge *et al.*, 2010). We have reported that the SK1 inhibitors SKi, FTY720 and (*S*)-FTY720 vinylphosphonate induce the proteasomal degradation of SK1a and SK1b in prostate cancer cells (Loveridge *et al.*, 2010; Tonelli *et al.*, 2010). The creation of prostate cancer cells with severely reduced SK1 expression results in their apoptosis. We also found that SK1 inhibitors reduce the expression of the androgen receptor in LNCaP-AI cells (Tonelli *et al.*, 2010). This provides a means for inhibiting the growth of prostate cancer cells and could represent an additional facet for enhancing the efficacy of these anti-cancer agents. In the current study, we have further characterized the mechanism by which SK1 inhibitors reduce the expression of androgen receptor in prostate cancer cells.

Methods

Materials

All general biochemicals, PMSF, *N*-acetyl-L-cysteine (NAC), diphenyleneiodonium (DPI), anti-p53 and anti-actin antibodies were from Sigma (Poole, UK). RPMI 1640 medium, European FCS (EFCS), penicillin–streptomycin (10 000 U·mL^{-1} penicillin and 10 000 μg·mL^{-1} streptomycin), L-glutamine and Lipofectamine2000™ were from Invitrogen (Paisley, UK). Charcoal-filtered FBS was from Lonza (Switzerland). LNCaP and LNCaP-AI cells were gifts from Professor Hing Leung (Beatson Institute, Glasgow). LNCaP-AI cells were derived by culturing LNCaP cells in androgen-deprivation conditions (Halkidou *et al.*, 2003). AR receptor is still expressed in LNCaP-AI cells but this cell line is androgen-independent in terms of growth and proliferation, and its cell cycle remains unaffected by androgen manipulation. Prostate-specific antigen (PSA) expression is preserved in LNCaP-AI cells, and the gene remains regulated by androgen (Halkidou *et al.*, 2003). Anti-androgen receptor and anti-myc antibodies were from Santa Cruz (CA). Anti-ERK2 antibody was from BD Transduction Laboratories (Oxford, UK). Anti-PSA antibody was a kind gift from Professor Hing Leung. MG132, Z-VAD-FMK, Ac-DEVD-CHO, fumonisin B1 (FB1), myriocin, PD150606, C2-ceramide, C2-dihydroceramide and dihydrosphingosine were from Enzo Life Sciences (Exeter, UK). (*S*)-FTY720 vinylphosphonate was synthesized according to Lu *et al*. (2009), and (*R*)-FTY720 methyl ether was synthesized according to Lim *et al*. (2011b). SKi, cycloheximide, CA074Me and the caspase-2 inhibitor ICH-1 were from Merck Biosciences (Nottingham, UK). S1P was from Avanti Polar Lipids (Alabaster, AL). Myc-tagged SK2 plasmid construct was generated as described previously (Lim *et al.*, 2011b).

Cell culture and transfection

Human prostate cancer LNCaP and LNCaP-AI cell lines were maintained in RPMI 1640 medium supplemented with 10% EFCS or 10% delipidated serum, respectively, 1% penicillin–streptomycin and 1% L-glutamine. All cells were maintained in a humidified atmosphere at 37°C with 5% CO_2. Cells were treated with inhibitors or vehicle for 24–48 h, as indicated for each experiment. When cells were treated for 48 h, the inhibitor or vehicle was replaced after 24 h. LNCaP cells were transfected with Lipofectamine2000™ reagent and myc-tagged SK2 plasmid constructs and grown for 24 h before being treated with (*R*)-FTY720 methyl ether.

Preparation of whole cell extracts

LNCaP and LNCaP-AI cell extracts for SDS-PAGE and Western blot analysis were prepared by washing treated cells with 5 mL of PBS and then re-suspending the cell pellets in lysis buffer [(137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% v/v NP40, 10% v/v glycerol, 20 mM Tris) (pH 8.0) containing 0.2 mM PMSF, 0.2 mM leupeptin, 0.2 mM aprotinin, 0.5 mM Na_3VO_4, 100 μM NaF and 10 mM β-glycerophosphate]. Samples were repeatedly (×10) passed through a 25-gauge needle using a syringe and rotated for 30 min at 4°C to allow for efficient lysis. Cell debris was pelleted by centrifugation at 22 000× *g* for 10 min at 4°C, and the supernatant (whole cell extract) was collected. The protein content was measured using the Pierce BCA assay kit (Fisher Scientific UK, Loughborough). For each sample, 10–20 μg of protein, combined with sample buffer (125 mM Tris, pH 6.7, 0.5 mM $Na_4P_2O_7$, 1.25 mM EDTA, 0.5% w/v SDS containing 12.5% v/v glycerol, 0.06% w/v bromophenol blue and 50 mM dithiothreitol) was used for SDS-PAGE and Western blotting. When preparing samples for the detection of LC3 conversion (LC3-I to LC3-II) by immunoblotting for autophagy analysis, cells were washed with ice-cold PBS and resuspended in TNTE lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.3% Triton X-100, 50 μg·mL^{-1} PMSF, protease inhibitor cocktail; pH 7.5). Samples were repeatedly (×10) passed through a 25-gauge needle using a syringe and left for 5 min at 4°C to allow for efficient lysis. Cell debris was then pelleted by centrifugation at 22 000× *g* for 10 min at 4°C, and the supernatant collected. The protein content was measured using the BCA Assay. For each sample, 80 μg of protein was combined with sample buffer and subjected to SDS-PAGE and Western blotting.

Western blotting

Analysis of proteins by SDS-PAGE and Western blotting was performed as previously described (Alderton *et al*. 2001) using anti-AR, anti-p53 or anti-myc antibodies, as required. Blots were stripped and re-probed with anti-ERK2 or anti-actin antibodies to confirm comparable protein loading.

Real-time PCR

Total RNA extraction of LNCaP cells grown in T-25 cell culture flasks was carried out using the NucleoSpin® RNA II kit (Abgene/Thermo Fisher Scientific, Epsom, UK), according to the manufacturer's instructions. The procedure included an on-column DNase treatment of the RNA. The extracted total RNA was quantified and measured with a NanoDrop-2000C spectrophotometer. Each RNA sample (5 μg) was used in a first-strand Superscript III cDNA synthesis reaction (Invitrogen) and primed with a NV-clamped Oligo $d(T)_{18}$ primer (Eurofins, Germany). cDNA synthesis reactions lacking Superscript III were also set up in parallel to act as controls for genomic DNA contamination assessment. cDNA (100 ng) was used in each 25 μl of Solaris qPCR reaction (Thermo Fisher Scientific, Epsom, UK) with the exception of the no-template control (H_2O) reactions. The qPCR reactions were run on a Bio-Rad DNA Engine with Chromo4 Real-Time PCR Detection System (Bio-Rad, Hemel Hempstead, UK) under the required Solaris cycling conditions. The data were collected using Opticon Monitor 3.1 (Bio-Rad). The primers were from Dharmacon (Epsom, UK). These are AR, for: AAGACCTGCCTGATCTGTGGA, rev: GCGCACAGGTACTTCTGTT, probe: AGCCGCTGAAGGGAAAC; GAPDH, for: GCCTCAAGATCATCAGCAATG, rev: CTTCCACGATACCAAAGTTGTC, probe: GCCAAGGTCATCCATGA. Experiments were performed in triplicate, and results were expressed as relative AR mRNA expression, normalized to GAPDH (reference gene) mRNA levels, according to the Comparative C_T method ($\Delta\Delta C_T$ method).

Isolation of cell extracts and liquid chromatography mass spectrometry

Cells (1×10^6) were plated in T-25 cell culture flasks and grown until the cell number doubled (48 h) before being treated with SKi (10 μM) or vehicle for 24 h. Cell extracts were prepared by washing the cells twice with 37°C PBS before harvesting the cells into pre-cooled extraction solution (methanol/acetonitrile/H_2O 50:30:20) (1 mL per 2×10^6 cells). Cell lysates were mixed at 4°C at 1440 r.p.m. for 12 min, before being centrifuged at 0°C at 13 000 r.p.m. for 15 min. The supernatants were collected and transferred into HPLC vials for loading into the LC-MS auto-sampler. LC-MS analysis was carried out using a ZICpHILIC column (150 × 4.6 mm × 5 μm; HiChrom, Reading, UK), which was eluted with a linear gradient over 30 min between 20 mM ammonium carbonate (pH 9.2)/acetonitrile (20:80) at 0 min and 20 mM ammonium carbonate (pH 9.2)/acetonitrile (20:80) at 30 min with a flow rate of 0.3 mL·min^{-1}, followed by washing with 20 mM ammonium carbonate (pH 9.2)/acetonitrile (95:5) for 5 min and then re-equilibrated with the starting conditions for 10 min. LC-MS was carried out with an Accela HPLC pump coupled to an Exactive (Orbitrap) mass spectrometer from Thermo Fisher Scientific (Bremen, Germany). Nitrogen as sheath and auxiliary gas was produced by a NM32LA nitrogen generator from Peak Scientific (Scotland, UK). The ESI interface was operated in a positive/negative polarity switching mode. The spray voltage was 4.5 kV for positive mode and 4.0 kV for negative mode. The temperature of the ion transfer capillary was 275°C and sheath and auxiliary gas was 50 and 17 arbitrary units, respectively. The full scan range was 75 to 1200 m/z for both positive and negative modes with settings of AGC target and resolution as Balanced and High (1e^6 and 50 000), respectively. The data were recorded using the Xcalibur 2.1.0 software package (Thermo Fisher Scientific, Bremen, Germany). Mass calibration was performed for both ESI polarities before the analysis using the standard Thermo Calmix solution with addition of compounds to cover the low mass range, and the signals of 83.0604 *m/z* (2× ACN + H) and 91.0037 m/*z* (2× formate-H) were selected as lock masses for the positive and negative modes, respectively, during each analytical run. The *m/z* for oxidized glutathione detected in negative ion mode was 611.1454, and the retention time was 17.3 min.

Data extraction

Data extraction was carried out by using Sieve 1.3 (Thermo Fisher Scientific, Loughborough, UK). The extraction ion chromatograms were pasted into an Excel macro written in house, and library was searched against a database of accurate masses for compounds in the Human Metabolome Data Base, KEGG and Metlin.

Results and discussion

Effect of SKi on AR expression

We show here that the treatment of androgen-sensitive LNCaP and androgen-independent LNCaP-AI cells with SK1 inhibitor SKi (10 μM, 24–48 h) induced a substantial reduction in AR expression (Figure 1A–C). This reduction in AR expression results in abrogated AR signalling as the PSA level is also reduced (Figure 1A). Two forms of the AR were detected with anti-AR antibody. These are full-length AR, which has a molecular mass of 100 kDa, and a second smaller form with a molecular mass of 87 kDa, which has been shown to lack the first 187 amino acids resulting from proteolysis (Wilson and McPhaul, 1994; Gregory *et al.*, 2001). The change in AR protein expression was associated with a reduction in AR mRNA as assessed by quantitative PCR (Figure 1B), indicating that SKi affects *AR* gene expression. It is, therefore, possible that the reduction in AR protein expression reduces AR-dependent transcriptional regulation of the AR gene. However, SKi appears to reduce AR protein expression principally via a post-translational-dependent mechanism as the response persisted in cells treated with 5 μg·mL^{-1} of cycloheximide, which inhibits *de novo* protein synthesis, as assessed by the reduction in cyclin D1 expression (Figure 1C).

We next investigated the mechanism by which SKi induces a reduction in AR expression. Under the conditions used here, we have established that SKi substantially induces the proteasomal degradation of SK1a and SK1b in LNCaP cells and SK1a in LNCaP-AI cells (Loveridge *et al.*, 2010). However, the reduction in AR expression does not involve the proteasomal degradation of SK1 as MG132 (a proteasomal inhibitor) failed to reverse the effect of SKi on AR levels but did reverse the SKi-induced degradation of c-Myc (Figure 1D).

Treatment of LNCaP cells with SKi increases C22:0-ceramide levels, while decreasing S1P levels in LNCaP cells (Loveridge *et al.*, 2010). Similarly, the two SK1 inhibitors (*S*)-FTY720 vinylphosphonate and FTY720 (Tonelli *et al.*, 2010) also reduced AR expression (Figure 1A), demonstrating that two structurally unrelated SK1 inhibitors (compared with SKi) induce the down-regulation of AR expression. SKi also promoted a decrease in AR expression in LNCaP-AI cells (Figure 1A), which we have shown are resistant to apoptosis in response to SKi (Loveridge *et al.*, 2010), suggesting that the reduction in AR expression is not contingent on apoptosis. Indeed, treatment of LNCaP cells with the caspase-3/7 inhibitor Ac-DEVD-CHO (100 μM) failed to reverse the effect of SKi (10 μM) on AR expression in LNCaP cells (Figure 1E). Caspase-2 has been recently shown to have an important function in apoptosis (Bouchier-Hayes 2010) and can catalyse proteolytic degradation of SK1 in response to genotoxic stress (Heffernan-Stroud *et al.*, 2011). However, pre-treatment of LNCaP cells with the caspase-2 inhibitor ICH-1 (20–40 μM) failed to reverse the effect of SKi (10 μM) on AR expression (Figure 1E). In addition, we observed that the pan-caspase inhibitor (Z-VAD-FMK, 10 μM) had no effect on the SKi-induced degradation of AR (data not shown). These results are consistent with the fact that AR does not contain consensus caspase cleavage sites.

Pre-treatment with the cathepsin B inhibitor CA074Me (10 μM) also failed to reverse the effect of SKi treatment (10 μM, 48 h) on AR expression in LNCaP cells (Figure 1F). Data obtained with CA 074 Me are confirmed using chloroquine (a lysosomal inhibitor), which had no effect on the SKi-induced degradation of AR (data not shown). Therefore, AR degradation is not part of an autophagic process that could be potentially induced by SKi. This is confirmed by the finding that SKi inhibits rather than activates autophagy, as reflected by the accumulation of the non-lipidated inactive form I of LC3 (autophagy marker protein) in LNCaP and

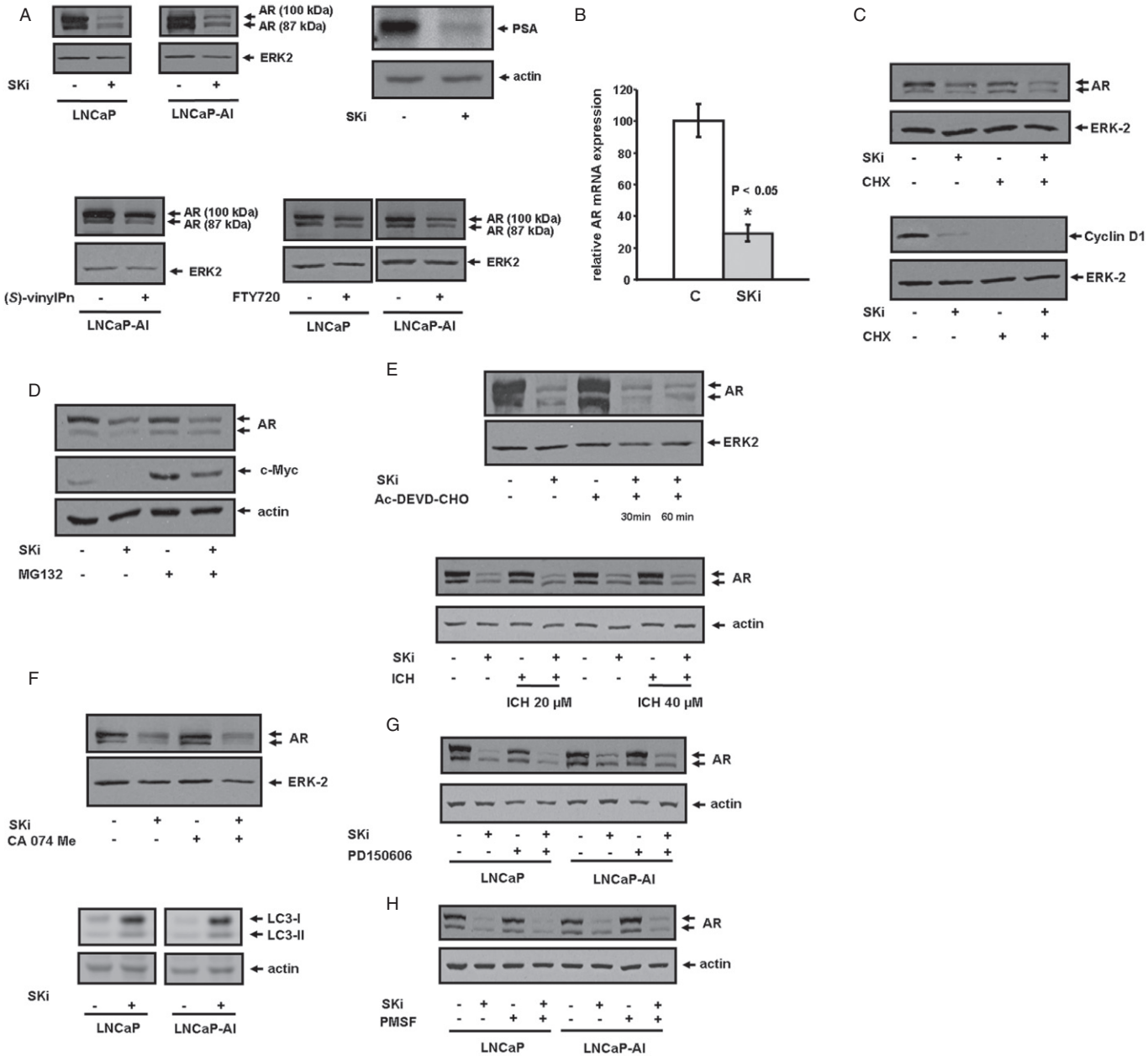

Figure 1

Effect of SKi on AR expression in LNCaP and LNCaP-AI cells. (A) LNCaP or LNCaP-AI cells were treated with SKi (10 μM, 48 h) or (*S*)-FTY720 vinylphosphonate (10 μM, 48 h, (*S*)-vinylPn) or FTY720 (10 μM, 24 h) or with vehicle (DMSO, 0.1% v/v) alone, and AR levels were measured by Western blot analysis with anti-AR antibody. Also shown is the effect of SKi (10 μM, 48 h) on PSA levels, detected with anti-PSA antibody. (B) Quantitative Real Time-PCR analysis of AR mRNA levels in LNCaP cells treated for 24 h with SKi (10 μM). GAPDH was used as a reference gene. Data are expressed as relative AR mRNA expression, normalized to GAPDH mRNA levels. Bars represent mean of triplicate values ± SE * $P < 0.05$ (Student's *t*-test) compared with control. (C) LNCaP cells were pre-treated for 30 min with cycloheximide (CHX) (5 $\mu g \cdot mL^{-1}$) before addition of SKi (10 μM, 24 h), and AR and cyclin D1 expression levels were measured by Western blotting. (D) LNCaP cells were pre-treated for 30 min with MG132 (10 μM) and then SKi (10 μM) for 8 h or with vehicle (DMSO, 0.1% v/v) alone and AR and c-Myc levels measured by Western blot analysis. (E–H) LNCaP or LNCaP-AI cells were pre-treated with (E) AC-DEVD-CHO (100 μM for 30 or 60 min, LNCaP) or ICH (20 or 40 μM for 60 min, LNCaP); (F) CA074Me (10 μM for 30 min, LNCaP). Also shown is the effect of SKi (10 μM, 24 h) on LC3-I and LC3-II levels; (G) PD150606 (100 μM for 4 h); (H) PMSF (1 mM for 4 h) prior to treatment with SKi (10 μM, 18–48 h). In (A), (C) and (D-H) blots were immunostained with AR or LC3 or cyclin D1 or c-Myc antibodies and then stripped and re-probed with anti-ERK-2 or anti-actin antibodies to ensure comparable protein loading. Results are representative of at least three separate experiments.

LNCaP-AI cells (Figure 1F). Since AR contains a calpain cleavage site and is a substrate for calpain-catalysed proteolysis (Libertini *et al.*, 2007), we tested the effect of calpain inhibitor on the degradation of AR in response to SKi. As shown in Figure 1G, pre-treatment of LNCaP cells with the calpain 1/2 inhibitor PD150606 (100 μM) did not prevent SKi (10 μM) from reducing AR expression in LNCaP or LNCaP-AI cells. AR has also been reported to be a substrate for serine protease-catalysed cleavage (de Boer *et al.*, 1987). However, the serine protease inhibitor PMSF did not reverse the decrease in AR expression in response to SKi (Figure 1H).

Ceramide and AR expression

The effect of SKi on AR levels does not involve the proteasomal degradation of SK1 but instead might involve the inhibition of SK1 catalytic activity and disruption of the sphingolipid rheostat, leading to a ceramide-dependent reduction in AR expression. Indeed, treatment of LNCaP cells with C2-ceramide (50 μM) reduced AR protein expression (Figure 2A). However, unlike that observed with SKi, the C2-ceramide-induced AR down-regulation was prevented by pre-treatment of cells with the proteasomal inhibitor MG132 (Figure 2A), suggesting that the proteasome is involved in the response to C2-ceramide. However, a role for endogenous ceramide from the salvage pathway in the action of SKi on AR expression is unlikely given that the ceramide synthase inhibitor fumonisin B1 (100 μM) did not reverse the SKi-induced reduction in AR protein expression (Figure 2B). In contrast, fumonisin B1 was effective in partially preventing the SKi-induced proteasomal degradation of SK1 (Loveridge *et al.*, 2010). Therefore, disruption of the endogenous ceramide formation by SKi does not appear to affect the level of AR protein and does not involve the proteasomal degradation route. These findings suggest that the effects of SKi on AR expression precede activation of the proteasome, which is dependent on endogenous ceramide formation from the salvage pathway. Therefore, the C2-ceramide-dependent modulation of AR expression is likely to be differently spatially and temporally controlled compared with the effects of SKi on endogenous ceramide formation and distally the proteasomal degradation of SK1. These findings suggest a role for the reduction in intracellular S1P on the regulation of androgen receptor expression in response to SKi.

It is known that S1P derived from SK1 can be released from cells or partitions into the plasma membrane to bind to proximal S1P receptors to induce a cell response termed 'inside-out' signaling (Takabe *et al.*, 2008), and this might include modulation of AR expression. Indeed, we have previously shown that LNCaP cells express $S1P_2$ and $S1P_3$ receptors (Tonelli *et al.*, 2010). However, we excluded this possibility because exogenous S1P (10 μM) did not rescue the SKi-induced down-regulation of AR (Figure 2C). As the treatment of LNCaP cells with SKi also results in increased intracellular levels of dihydrosphingosine and various dihydroceramide species (Loveridge *et al.*, 2010), we tested the effect of these sphingolipids on AR expression. However, C2-dihydroceramide (50 μM), a cell-permeable analogue of endogenous dihydroceramide, and dihydrosphingosine (25 μM) were unable to induce down-regulation AR expression in LNCaP cells (Figure 2A).

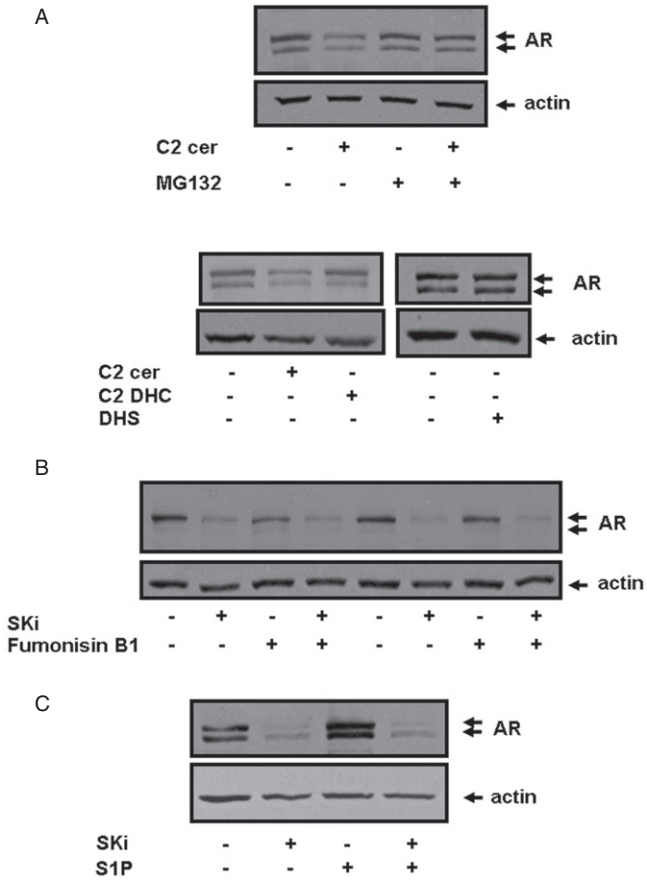

Figure 2

Effect of ceramide metabolites, ceramide synthesis inhibition or exogenous S1P on SKi-induced down-regulation of AR in LNCaP cells. (A) LNCaP cells were pre-treated with or without MG132 (10 μM for 30 min) prior to addition of C2 ceramide (C2-Cer, 50 μM) for 8 h. Cells were also treated with C2-dihydroceramide (C2-DHC, 50 μM), C2 ceramide (C2-Cer, 50 μM) or dihydrosphingosine (DHS, 25 μM) for 24 h. (B) LNCaP cells were pre-treated with fumonisin B1 (FB1) (100 μM, 1 h) prior to treatment with SKi (10 μM, 24 h). (C) LNCaP cells were treated with or without S1P (10 μM) and SKi (10 μM) for 48 h. Blots were immunostained with anti-AR antibody and then stripped and re-probed with anti-ERK-2 or anti-actin antibodies to ensure comparable protein loading. Results are representative of two to three separate experiments.

Role of SK2

SKi inhibits both SK1 and SK2 activity. Therefore, we investigated whether SK2 has a role in regulating AR expression. For this purpose, we used the selective SK2 inhibitor (*R*)-FTY720 methyl ether. Treatment of LNCaP cells with (*R*)-FTY720 methyl ether (10 μM) failed to decrease AR expression (Figure 3A), although a substantial decrease in AR expression was produced in response to a higher concentration of (*R*)-FTY720 methyl ether (50 μM) (Figure 3A). However, (*R*)-FTY720 methyl ether (10 μM, 24 h) induces a complete down-regulation of ectopic SK2 expression (Figure 3B), suggesting that higher concentrations of this inhibitor decrease AR expression via an 'off-target' effect. (*R*)-FTY720 methyl ether induces down-regulation of ectopically expressed SK2

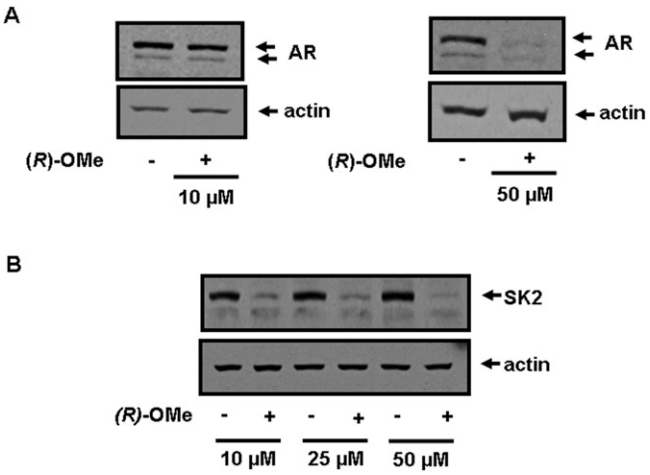

Figure 3

Effect of (*R*)-FTY720-OMe on AR expression in LNCaP cells. LNCaP cells were treated with (*R*)-FTY720-OMe (R-OMe, 10–50 μM, 24 h), and the effect of this inhibitor on (A) AR expression or (B) myc tagged SK2 expression was assayed by Western blotting analysis. Blots were immunostained with anti-AR or anti-myc antibodies and then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments.

in HEK 293 cells via an unidentified mechanism that does not involve its degradation by the proteasome (Lim *et al.*, 2011b). These findings suggest that the major effect of SKi on AR expression might be via inhibition of SK1 activity.

Role of reactive oxygen species (ROS) in regulating AR expression

Knock down of SK1 using shRNA increased levels of ROS in doxorubicin-treated carcinoma cells, resulting in increased DNA damage and apoptosis (Huwiler *et al.*, 2011). Interestingly, Fajardo *et al.* (2012) demonstrated that oxidative stress-induced AR down-regulation in several prostate cancer cell lines. Therefore, we tested whether the effect of SKi on AR expression is mediated by a ROS-dependent pathway. For this purpose, we pre-treated LNCaP cells with the ROS scavenger NAC before treating the cells with SKi. SKi (10 μM) induced down-regulation of AR, and this effect was reversed by NAC (10 mM) (Figure 4A). Further evidence that SKi induces oxidative stress and that this is linked with AR regulation was demonstrated by the finding that SKi increases oxidized glutathione levels. The ratio of GSSG/GSH levels in SKi-treated versus control cells was 19.7 compared with cells treated with SKi and NAC (10 mM), which was 5.65 ($n = 3$; *P*-values for the increase in GSSG levels in SKi/control and SKi and NAC/control were ≤0.03 and ≤0.009 respectively). The GSSG/GSH ratio in cells treated with SKi and NAC compared with SKi alone was 0.29 ($P \leq 0.014$, $n = 3$). Therefore, in addition to rescuing AR expression (Figure 4A), NAC also inhibited the increase in oxidized glutathione levels in response to SKi, thereby confirming that this compound abrogates the oxidative stress response to SKi. The NOX inhibitor DPI did not reverse the effect of SKi on AR expression in LNCaP cells

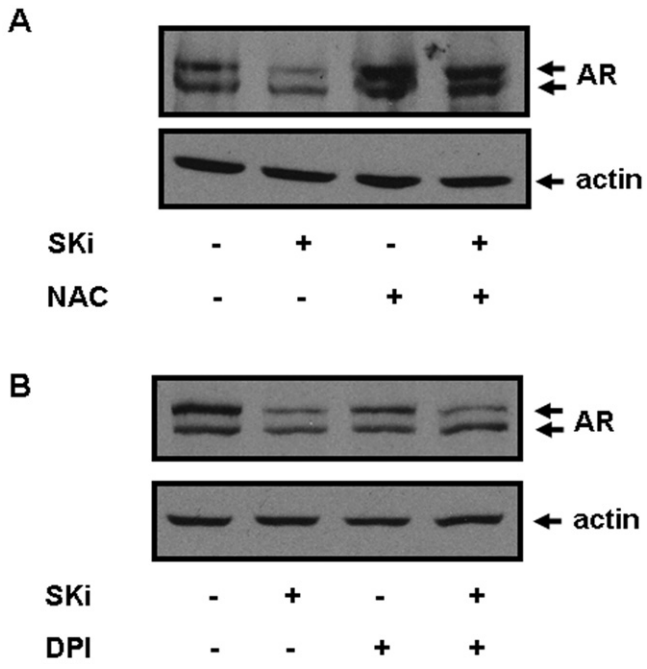

Figure 4

Effect of ROS scavenging and DPI on the SKi-induced reduction in AR expression. LNCaP cells were pre-treated for 1 h with (A) NAC (10 mM) or (B) DPI (10 μM) prior to treatment with SKi (10 μM, 18–24 h). Blots were immunostained with anti-AR antibody and then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments.

(Figure 4B). Therefore, it is possible that SKi might disrupt oxidative phosphorylation in the mitochondria to produce ROS.

Role of p53 in regulation of AR expression

Alimirah *et al.* (2007) demonstrated that p53 negatively regulates AR expression. p53 expression is regulated by Mdm2, an E3 ligase that catalyses polyubiquitination and proteasomal degradation of p53. Indeed, we demonstrate here that the proteasomal inhibitor MG132 (10 μM) increases p53 expression in both LNCaP and LNCaP-AI cells (Figure 5). Moreover, the pretreatment of LNCaP-AI cells with SKi (10 μM) induces an increase in p53 expression (Figure 5). SKi reduces AR expression in both LNCaP and LNCaP-AI cells but fails to increase p53 expression in LNCaP cells (Figure 5). Therefore, it is possible that p53-mediated oxidative stress is not involved in the regulation of AR expression by SK1.

Conclusion

We have demonstrated that the SK1 inhibitor 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole reduces AR expression via a p53-independent oxidative stress-mediated response in androgen-sensitive prostate cancer cells. The effect of this SK1 inhibitor is also recapitulated in androgen-independent prostate cancer cells. These findings are important because treatment options for androgen-independent prostate cancer are limited, and removal of constitutively

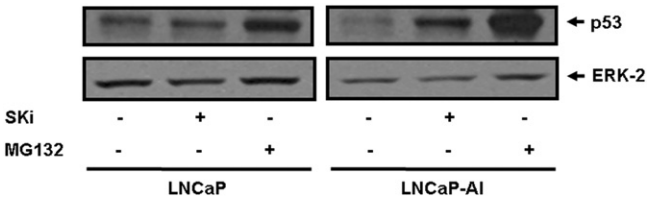

Figure 5
Effect of SKi on p53 expression. LNCaP and LNCaP-AI cells were treated with MG132 (10 μM) or SKi (10 μM) for 48 h. Blots were immunostained with anti-p53 antibody and then stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. Results are representative of three separate experiments.

active AR from this cancer would severely reduce the growth of these tumours. The results of this study indicate that novel SK1 inhibitors may add therapeutic potential in promoting the removal of the AR receptor from prostate cancer cells, resulting in an increased efficacy, which is likely to be superior to inhibitors that simply reversibly inhibit AR signaling.

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Conflict of interest

Authors have no conflict of interest.

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