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Pharmacological targeting of constitutively active truncated androgen receptor by nigericin and suppression of hormone-refractory prostate cancer cell growth

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Running Title: blockade of truncated androgen receptor

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Abbreviations:

AR, androgen receptor; HRPC, hormone-refractory prostate cancer; PSA, prostate specific antigen; tAR, truncated AR; DHT, dihydrotestosterone; ARE, androgen response elements; NTD, amino-terminal domain; DBD, DNA binding domain; LBD, ligand-binding domain; SCADS, Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Priority Area “Cancer” from the Ministry of Education, Culture, Sports, Science and Technology, Japan; CSS, charcoal-stripped serum; PCR, polymerase chain reaction; CMV, cytomegalovirus; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; qRT-PCR, quantitative reverse transcription-PCR; siRNA, small interfering RNA; UTR, untranslated region.

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Abstract

In prostate cancer, blockade of androgen receptor (AR) signaling confers a therapeutic benefit. Nevertheless, this standard therapy allows relapse of hormone-refractory prostate cancer (HRPC) with a poor prognosis. HRPC cells often express variant ARs, such as point-mutated alleles and splicing isoforms, resulting in androgen-independent cell growth and resistance to antiandrogen (e.g., flutamide). However, a pharmacological strategy to block such aberrant ARs remains to be established. Here we established a reporter system that monitors AR-mediated activation of a *prostate specific antigen* (*PSA*) promoter. Our chemical library screening revealed that the antibiotic nigericin inhibits AR-mediated activation of the *PSA* promoter and PSA production in prostate cancer cells. Nigericin suppressed the androgen-dependent LNCaP cell growth, even though the cells expressed a flutamide-resistant mutant AR. These effects were caused by AR suppression at the mRNA and post-translational levels. In HRPC 22Rv1 cells, which express the full-length AR and the constitutively active, truncated ARs (tARs) lacking the carboxy-terminal ligand-binding domain, siRNA-mediated knockdown of both AR isoforms efficiently suppressed the androgen-independent cell growth, whereas knockdown of the full-length AR alone had no significant effect. Importantly, nigericin was able to mimic the knockdown of both AR isoforms: it reduced the expression of the full-length and the truncated ARs, and induced G1 cell cycle arrest and apoptosis of 22Rv1 cells. These observations suggest that nigericin-like compounds that suppress AR expression at the mRNA level could be applied as new-type therapeutic agents that inhibit a broad spectrum of AR variants in HRPC.

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Introduction

Prostate cancer is the most frequently diagnosed, non-dermatologic cancer in men in the USA. Recently, the number of prostate cancer patients has also been increasing greatly in Japan. Androgen receptor (AR) signaling plays a central role in prostate cancer cell growth and survival (Heinlein and Chang, 2004), and androgen ablation therapy has been the standard therapy for advanced and metastatic prostate cancer (Chen et al., 2008). Disease in most patients who undergo androgen ablation, however, progresses within 2 years from androgen-dependent status to hormone-refractory prostate cancer (HRPC). Although the recurrent tumors are often resistant to standard AR-targeting agents, which deprive androgens or block androgen-AR interaction, AR-mediated signaling still plays a key role in the development and maintenance of HRPC in most cases (Chen et al., 2004; Grossmann et al., 2001; Taplin and Balk, 2004). Therefore, additional new therapeutic agents or approaches that target the AR signaling are needed to cure HRPC.

AR is a member of the steroid hormone receptor superfamily and mediates androgen-dependent transcription (Lubahn et al., 1988). In its inactive state, AR is associated with cellular chaperones in the cytoplasm. After binding to androgens, including testosterone and dihydrotestosterone (DHT), AR translocates into the nucleus, where it interacts with androgen response elements (ARE) in the promoter/enhancer regions of AR target genes, such as *prostate specific antigen* (PSA), and regulates their expression. AR is composed of three major domains: an amino-terminal domain (NTD), a central DNA binding domain (DBD), and a carboxy-terminal ligand-binding domain (LBD) (Dehm and Tindall, 2007). The NTD and LBD possess separate transcriptional activation subdomains, termed AF-1 and AF-2. HRPC cells frequently express mutated ARs that are resistant to antiandrogens, such as flutamide. Moreover, these ARs are often activated constitutively or in an androgen-independent manner and therefore contribute to androgen-independent growth and survival of HRPC cells (Chen et al., 2004; Guo et al., 2006; Heinlein and Chang, 2004; Mellinghoff et al., 2004; Xu et al., 2009).

Recently, truncated forms of AR (tAR) that lack the LBD have been reported to be androgen-independent, constitutively active ARs (Dehm et al., 2008; Guo et al., 2009). These observations suggest that agents that modulate aberrant ARs could act as selective agents for HRPC treatment.

A chemical biological approach provides a powerful way to identify new antitumor agents and novel therapeutic targets for cancer (Collins and Workman, 2006; Shoemaker et al., 2002). Here, utilizing a chemical library consisting of compounds that are clinically used or known to target specific signaling pathways or cellular molecules, we screened compounds that block the AR-mediated signaling in HRPC cells. We identified nigericin, an antibiotic derived from *Streptomyces hygroscopicus*, as a novel AR inhibitor, and examined its effect on androgen-dependent and -independent growth of prostate cancer cells.

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Materials and Methods

Chemicals

The SCADS inhibitor kits (Kawada et al., 2006) were kindly provided by a Grant-in-Aid for Scientific Research on Priority Area “Cancer” from the Ministry of Education, Culture, Sports, Science and Technology, Japan. DHT and MG-132 were purchased from Sigma (St. Louis. MO, USA). Nigericin (Steinrauf et al., 1968) was purchased from LKT Lab., Inc. (St. Paul, MN, USA). 17-AAG was purchased from InvivoGen (San Diego CA, USA). Thapsigargin and actinomycin D were purchased from Nacalai tesque (Kyoto, Japan).

Cell lines and cell culture

Human prostate cancer cell lines, LNCaP [AR(T877A)-positive, androgen-dependent] (van Bokhoven et al., 2003), 22Rv1 [AR(H874Y, exon 3 duplicated)- and tAR-positive, androgen-independent] (Dehm et al., 2008; van Bokhoven et al., 2003), PC-3 and DU145 (AR-negative, androgen-independent) (van Bokhoven et al., 2003), were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml of kanamycin. The cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air. To examine the effects of androgen on the cells, we cultured LNCaP cells in phenol red-free RPMI1640 (Invitrogen, Tokyo, Japan) containing 5% charcoal-stripped serum (CSS) (HyClone, South Logan, UT, USA) for 2 days or 22Rv1 cells in the medium for 3 days and then DHT was added to the medium.

Cell cycle, proliferation, and apoptosis assays

Cell cycle analysis was performed using flow cytometry as described previously (Mashima et al., 1995). After each treatment, viable cells were counted by Sysmex (Hyogo, Japan). Apoptotic cells were determined and counted as described previously (Yang et al., 2003). Data represent the mean ± standard deviation (SD).

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Vector construction

The basic frame of two luciferase reporter constructs, PSA-Luc and PSA enhancer/promoter-Luc, is the pGL3-vector (Promega, Madison, WI, USA). Entire PSA enhancer-promoter region (-5824 to -1) (Wang et al., 2005) was amplified by polymerase chain reaction (PCR) with KOD Plus polymerase (Toyobo, Co. Ltd., Osaka, Japan) and LNCaP genomic DNA as a template. The PCR product was cloned into a pGL3-basic vector (firefly luciferase reporter vector) to generate PSA-Luc. The PSA enhancer region (-5292 to -3709) directly linked with its promoter region (-677 to +1) was amplified by PCR, using a pDRIVE-PSA-hPSA vector (InvivoGen) as a template. The PCR product was cloned into the pGL3-Basic vector to generate the PSA enhancer-promoter-Luc. phRL-CMV, the *Renilla* luciferase reporter vector with cytomegalovirus (CMV) promoter (CMV-*R*Luc), was purchased from Promega and was used as a control in reporter assays. The full-length cDNA for human AR was kindly provided by Drs. Shigeaki Kato and Hirochika Kitagawa (University of Tokyo, Tokyo, Japan) or was amplified by PCR and cloned into a pLPCX retroviral vector to generate pLPCX-AR. The *AR* promoter (-5400/+580)-luciferase construct in the pGL3 basic vector was kindly provided by Dr. Donald J Tindall (Mayo Clinic, Rochester, MN, USA).

Transient transfection and luciferase reporter assay

Transient transfections were performed using a Lipofectamine 2000 reagent (Invitrogen). Cells were transfected with PSA-Luc + CMV-*R*Luc or PSA enhancer/promoter-Luc + CMV-*R*Luc. After 24 h, the transfected cells were seeded into 96-well opaque plates at $4x10^4$ cells/well in 10% CSS medium. The next day, we added test compounds to the cells at various concentrations (0.2 – 20 μM). After 1 h, DHT (1 nM final concentration) was added and incubation was continued for another 24 h. Luciferase activities were measured using the Dual-Glo™ Luciferase Assay System (Promega). Firefly luciferase activity, which reflects the

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PSA promoter activity, was normalized by an internal control, CMV-driven *Renilla* luciferase activity.

Subcellular fractionation and western blot analysis

To obtain cell lysates, we washed cells in ice-cold phosphate-buffered saline (PBS) and lysed them in NP-40 lysis buffer [150 mM NaCl, 1.0% NP-40, 50 mM Tris HCl (pH 8.0)] supplemented with 1x Protease Inhibitor Cocktail (Sigma). After centrifugation at 14,000x *g* for 15 min at 4^{o}C, the supernatant was subjected to SDS-PAGE. Subcellular fractions were obtained using a ProteoExtract Subcellular Proteome Extraction kit (Calbiochem, San Diego, CA, USA). Each extract derived from the same number of the cells was subjected to SDS-PAGE. Following SDS-PAGE, separated proteins were transferred to PVDF membranes. After a 30-min incubation with Tris-buffered saline containing 0.1% Tween 20 and 5% skim milk, the membranes were incubated for 2 hr at room temperature or overnight at 4^{o}C with the following primary antibodies: anti-AR (441) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-PSA (C-19) (Santa Cruz Biotechnology, Inc.), anti-poly(ADP-ribose) polymerase (PARP) (BD Pharmingen, Franklin Lakes, NJ, USA), anti-β-actin (Sigma), anti-heat shock protein (Hsp) 90 (Stressgen, Victoria, BC Canada), anti-tubulin (Sigma), anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA, USA) and anti-cleaved PARP (Cell Signaling Technology). After extensive washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody and visualized using an ECL kit (GE Healthcare, Buckinghamshire, UK).

Quantitative reverse transcription-PCR (qRT-PCR) analysis

Total RNAs were extracted from cells using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was reverse transcribed into cDNA and the reverse transcribed product was used for PCR to amplify DNA fragments for *AR*, *PSA*, and a housekeeping gene, *β-actin* with a

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SuperScript™ III Platinum® Two-Step qRT-PCR Kit with ROX (Invitrogen) according to the protocols provided by the manufacturer. The primer sequences for the amplifications were 5'-GGGTCAAGAACTCCTCTGGTTCA-3' and 5'-GTAGCACACTGGGGACCACCTGC[FAM]AC-3' for *PSA*. For the amplification of *AR* and *β-actin*, we used Certified LuXTM Primer sets (Invitrogen). PCR reactions were conducted in the 96-well spectrofluorometric thermal cycler ABI PRISM 7700 (Applied Biosystems, CA, USA). For the qRT-PCR to determine AR mRNA stability, PCR reactions were conducted in LightCycler 480 Probe Master (Roche, Mannheim, Germany). The primer sequences for the amplifications were 5'-GCCTTGCTCTCTAGCCTCAA-3' and 5'-GGTCGTCCACGTGTAAGTTG-3' for *AR* and 5'-CCCGTCCTTGACTCCCTAGT-3' and 5'-GTGATCGGTGCTGGTTCC-3' for *GAPDH*, respectively.

Small interfering RNA (siRNA) treatment

The stealth siRNA oligonucleotides to AR were synthesized by Invitrogen (Carlsbad, CA). The targeting sequences of siRNA for AR were 5'-CCCTTTCAAGGGAGGTTACACCAAA-3'(exon 1-1), 5'-CCGAATGCAAAGGTTCTCTGCTAGA-3'(exon 1-2), 5'-GACTCCTTTGCAGCCTTGCTCTCTA-3'(exon 4) and 5'-TCTCAAGAGTTTGGATGGCTCCAAA-3'(exon 6). As a control, a negative universal control siRNA (medium #2) (Invitrogen) was used. siRNAs were transiently introduced into the cells with Lipofectamine 2000 as described previously (Mashima et al., 2005).

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Results

The PSA reporter screening system identifies a set of AR signaling inhibitors

To screen inhibitors of AR-dependent signaling in prostate cancer cells, we first established a reporter system for the AR-dependent transcription. Because PSA is a well-known transcriptional target of AR (Huang et al., 1999; Wang et al., 2005), we cloned the *PSA* enhancer and promoter region that contains androgen responsive elements (AREs) and constructed reporter constructs, PSA-Luc and PSA enhancer-promoter-Luc (Figure 1A). We also measured the CMV promoter-dependent transcription as a negative control by using CMV-*R*Luc. We chose prostate cancer 22Rv1 cells for the initial screening, since this cell line expresses the full-length AR [AR(H874Y)] and possesses functional AR-mediated signaling (Attardi et al., 2004; Sramkoski et al., 1999). Although 22Rv1 cells also express tAR lacking the ligand-binding domain and the tAR constitutively activates transcription of some AR target genes at the basal level (Dehm et al., 2008), our GeneChip microarray analysis revealed that androgen treatment still greatly activates AR target genes transcription in the cells (unpublished data). As shown in Figure 1B, when the cells were transiently transfected with the reporter constructs and were subsequently treated with DHT, selective induction of *PSA* promoter-dependent transcription was observed in a dose-dependent manner. Moreover, this transcriptional activation was significantly suppressed by knockdown of AR using a specific siRNA (Supplementary Figure S1 and Figure 5C). These results indicate that our reporter system clearly detects the AR-dependent transcription in the prostate cancer cells.

To verify mediators of the AR-dependent transcription and identify novel inhibitors of the AR signaling, we utilized this reporter system to screen a chemical library. The SCADS inhibitor kit consists of 190 compounds including well-known antitumor agents and signaling molecule inhibitors. 22Rv1 cells transfected with the reporter constructs were treated with each compound with or without DHT for 24 h (see *Materials and Methods*). As a result of our screening, we identified a set of compounds that efficiently suppressed the AR-dependent

transcription (Figure 1C). The compounds included several inhibitors of signaling molecules that are known to be involved in AR regulation, such as an Hsp 90 inhibitor, and Ca^{2+} ionophores (Fang et al., 1996; Gong et al., 1995; Solit et al., 2002; Vanaja et al., 2002; Wang et al., 2007). These compounds significantly suppressed the androgen-dependent induction of *PSA* promoter activity (Figure 2A), while they did not affect the CMV promoter activity, verifying the selective inhibition of the AR-dependent transcription.

Nigericin inhibits androgen-dependent PSA expression and LNCaP cell growth

Among the compounds that we identified for the first time as AR signaling inhibitors, we focused on nigericin (Steinrauf et al., 1968), an antibiotic derived from *Streptomyces hygroscopicus*, since it also inhibited the androgen-dependent growth of prostate cancer cells (see below). As shown in Figure 2A and B, 20 nM or higher concentrations of nigericin strongly suppressed the androgen-dependent induction of *PSA* promoter activity but not the control CMV promoter activity in 22Rv1 cells. Nigericin did not directly inhibit the firefly or *Renilla* luciferase activities *in vitro* (data not shown).

Next, we examined the effect of nigericin on the androgen-dependent expression of endogenous PSA in prostate cancer LNCaP cells, which express the androgen-responsive full-length AR. We used LNCaP cells, instead of 22Rv1, because the latter expressed an undetectably low level of endogenous PSA protein (data not shown). As shown in Figure 3A and B, nigericin suppressed DHT-dependent PSA production in time- and dose-dependent manners. The AR in LNCaP cells has a point mutation of T877A, which confers flutamide resistance. Thus, nigericin was effective against flutamide-resistant AR. It was noteworthy that nigericin also affected the AR protein level: while DHT increases the AR amount by enhancing the protein's stability (Marcelli et al., 1994), nigericin decreased the AR protein level even in the presence of DHT (Figure 3B, *middle panel*). Longer exposure to nigericin further reduced the level of the AR protein (Supplementary Figure S2). These effects were also observed when

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nigericin was added to the cells after treatment with DHT (data not shown), indicating that the inhibitory effects of the compound would not be caused by interference of androgen incorporation into the cells. LNCaP cells show androgen-dependent growth. Nigericin at 10 or 100 nM effectively suppressed the DHT-dependent growth of LNCaP cells (Figure 3C and D), while its growth inhibitory effect was not significantly observed in DHT-depleted LNCaP cells (Figure 3E). These results indicate that nigericin suppresses the AR-dependent transcription and inhibits androgen-dependent growth of prostate cancer cells.

Nigericin suppresses AR signaling at the mRNA and post-translational levels

To examine the molecular mechanisms of nigericin-mediated AR inhibition, we analyzed the *AR* mRNA expression level in LNCaP cells during nigericin treatment. qRT-PCR analysis confirmed that nigericin suppressed *PSA* expression in a dose-dependent manner (Figure 4A). As a control for normalization, nigericin did not affect *β-actin* expression (data not shown). It is important to note that nigericin also reduced *AR* mRNA expression in a dose-dependent manner (Figure 4B). This result coincided with nigericin-mediated downregulation of the AR protein level (Figure 3B, *middle panel*). These results suggest that nigericin could suppress the AR expression at the mRNA level possibly through the inhibition of *AR* promoter/enhancer-dependent transcription or affecting the AR mRNA stability.

So, we further analyzed the effect of nigericin on the AR mRNA level under the actinomycin D treatment where mRNA transcriptions were suppressed. As shown in Figure 4C, we found that nigericin significantly decreases the AR mRNA stability. On the other hand, we also examined the effect of nigericin on the *AR* gene promoter activity, and found that nigericin did not affect the promoter activity (data not shown). These results indicate that nigericin reduces the AR mRNA level mainly through decreasing the AR mRNA stability. To verify these observations, we examined the effect of nigericin on exogenously expressed AR. Namely, we transiently introduced the CMV promoter-driven full-length *AR* expression vector into

AR-negative prostate cancer PC-3 cells (PC-3/AR; Figure 4D, *left panel*). In this expression vector, the *AR* cDNA (open reading frame) lacks the 3' untranslated region (UTR), which would be important for the mRNA stability (Faber et al., 1991). As a result, nigericin treatment for 24 h did not decrease the protein level of the exogenous AR nor its upregulation by DHT (Figure 4D, *right panel*). Consistent with these observations, nigericin could not efficiently inhibit the DHT-induced PSA promoter activity in PC-3/AR cells (Figure 4E). By contrast, thapsigargin, 17-AAG, and MG132, repressed both the protein amounts of the exogenous AR and the DHT-induced *PSA* promoter activity. These data support the idea that nigericin blocked the AR signaling mainly by destabilizing the *AR* mRNA.

Since nigericin slightly reduced the exogenous AR-dependent *PSA* promoter activation without affecting AR protein level (Figure 4D and E), some post-translational mechanism could also be involved in the compound's action. One possibility is that nigericin might promote the AR protein degradation, which could not be detected at 24 h after the drug treatment. However, even after 72-h exposure to nigericin, the level of the exogenous AR protein was not affected (Supplementary Figure S3A). Again, the *PSA* promoter activity was partially reduced under these conditions (Supplementary Figure S3B). Together, these results indicate that nigericin suppresses the AR signaling by destabilization of the *AR* mRNA and an additional post-translational mechanism, which would not involve the AR protein degradation.

Nigericin suppresses constitutively active tAR expression and HRPC cell growth

Recent studies have reported that HPRC cells frequently express tARs, which lack the ligand-binding domain and contribute to androgen-independent cell growth (Dehm et al., 2008; Guo et al., 2009). 22Rv1 cells express tAR(s) of approximately 80 kDa as well as full-length AR (Figure 4D, *left panel* and 5A). Consistent with the previous observation, DHT translocated the full-length AR from cytoplasm to the nucleus in both LNCaP and 22Rv1 cells (Figure 5B). By contrast, nearly half of the tAR pool constitutively resided in the nucleus even

without DHT stimulation, and DHT had no detectable effect on it. Previous studies have suggested the role of tAR in prostate cancer cell growth (Dehm et al., 2008; Guo et al., 2009). However, the specificity of RNAi used for the studies could still be open to question since the each result was obtained from experiments with only 1 siRNA and the growth inhibition by RNAi treatment is often observed as its off-target effect. Therefore, to validate the role of tAR in 22Rv1 cell growth further, we designed two types of siRNAs that knockdown both full-length AR and tAR (siEx1-1 and 1-2) or only full-length AR (siEx4 and 6) (Figure 5A). These siRNAs efficiently reduced the levels of target proteins (Figure 5C) and remained effective for at least 7 days (Supplementary Figure S4A). As shown in Figure 5D, *left panel*, 22Rv1 cells were able to proliferate even without DHT. Knockdown of both full-length and truncated ARs by siEx1-1 or 1-2 efficiently suppressed this androgen-independent cell growth whereas knockdown of the full-length AR alone by siEx4 or 6 had no significant effect. Knockdown of the full-length AR *per se* proved functional since it inhibited androgen-dependent growth of 22Rv1 cells (Figure 5D, *right panel* and Supplementary Figure S4B). Together, these observations verify that tAR plays an essential role in androgen-independent growth of 22Rv1 cells, and suggest that inhibition of both full-length and truncated ARs is an effective way to treat tAR-positive HRPC.

Next, we examined the effect of nigericin on tAR expression and 22Rv1 cell growth. We found that nigericin significantly suppressed the tAR expression as well as the full-length AR expression in a dose-dependent manner (Figure 6A and B). This effect of nigericin was similar to those of AR siRNAs, siEx1-1 and 1-2 (Figure 5C). Indeed, nigericin inhibited the androgen-independent growth of 22Rv1 cells in a dose-dependent manner (Figure 6C). Flow cytometry analysis revealed that nigericin treatment induced significant G1 cell cycle arrest at the concentrations of 10 and 100 nM (Figure 6D). Moreover, at the higher concentration (100 nM), cells underwent apoptotic cell death (Figure 6E). Nigericin-treated cells consistently exhibited the cleavage (i.e., activation) of pro-apoptotic caspase-3 as well as PARP cleavage,

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both hallmarks of apoptosis (Figure 6A). To test the specificity of these effects, we compared the difference of its cytotoxicity among prostate cancer cell lines. We found that nigericin showed selective growth inhibitory effect on tAR- or AR-dependent cell lines, 22Rv1 and LNCaP, while it was less toxic to AR-negative DU145 cells (Supplementary Figure S5). These results indicate that suppressing constitutively active tAR with chemicals, such as nigericin, could be an effective strategy to overcome tAR-positive HRPC.

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Discussion

In most HRPC, AR is constitutively activated even without androgen stimulation. In addition to tAR expression, several mechanisms are involved in such hormone-refractory AR signaling: AR overexpression, mutations in the AR LBD, and the AR phosphorylation by oncogenic kinases such as c-Src (Grossmann et al., 2001; Guo et al., 2006; Heinlein and Chang, 2004). Clinically used antiandrogens (e.g., flutamide and bicalutamide) mainly suppress AR signaling by directly interacting with its LBD. Therefore, these agents are no more effective against the variant ARs that possess mutations in the LBD and remain active in the absence of androgen. In this study, we found that nigericin reduces *AR* mRNA expression. It reduced the expression not only of the full-length but also of truncated ARs. Taking advantage of this mode of action, nigericin was able to block flutamide-resistant AR [AR(T877A) and AR(H874Y)] (Tan et al., 1997; van Bokhoven et al., 2003; Veldscholte et al., 1990) in LNCaP and 22Rv1 cells, respectively, and constitutively active tAR in 22Rv1 cells. These observations suggest that nigericin-like compounds that suppress AR expression at the mRNA level could be a new class of therapeutic agents that effectively inhibit a broad spectrum of AR variants in HRPC.

Nigericin was originally identified as a K^+/H^+ antiporter (Shavit et al., 1968). In mammalian cells, intracellular pH level is maintained by the Na^+/H^+ antiporter that excludes H^+ from the cells in exchange for Na^+. A previous report has shown that nigericin inhibits DNA synthesis of cancer cells by increasing intracellular pH and causing acidification of cytoplasm (Margolis et al., 1989; Rotin et al., 1987). However, it is still unclear whether the AR inhibition by nigericin could be mediated through the intracellular acidification caused by the agent. We have shown that nigericin selectively suppresses the androgen-dependent growth of LNCaP cells at 10 to 100 nM (Figure 3C), while nigericin was reported to affect intracellular pH level at much higher concentrations (1 to 10 μM) (Margolis et al., 1989; Rotin et al., 1987). These data indicate that the compound could inhibit AR through an intracellular pH-independent manner. Nigericin suppresses androgen-dependent LNCaP cell growth, while it does not show any

marked cytotoxicity to the cells in the androgen-depleted conditions (Figure 3E). In addition, nigericin showed selective growth inhibitory effect on tAR- or AR-dependent cell lines, but not to AR-negative DU145 cells (Supplementary Figure S5). These results further suggest that the agent does not cause nonspecific cytotoxicity to the cells through modifying intracellular pH but it could selectively suppress prostate cancer growth through the AR inhibition.

Our data indicate that nigericin reduces the AR expression level through destabilizing the *AR* mRNA. The endogenous *AR* mRNA contains very long 3'-UTR (approximately 6.8 kb) that could regulate the mRNA stability (Faber et al., 1991), and nigericin could decrease the *AR* mRNA stability by affecting this untranslated region. Consistent with this idea, nigericin did not suppress the expression of exogenous AR that does not contain the long 3'-UTR (Figure 4D and Supplementary Figure S3A).

In this study, we identified nigericin as a novel AR signaling inhibitor. Since nigericin reduces the AR mRNA level, a broad range of AR variants in HRPC would be susceptible to the functional blockade by this compound. Recently, it was reported that intratumoral androgen production plays a role in prostate tumor progression (Hofland et al., 2010). Since Nigericin is also known as a suppressor of steroidogenesis (Cheng et al., 1993), its effect on the intratumoral androgen production should be tested further. Further analysis on the mechanisms of AR inhibition by the compound will provide new clues to the development of AR-targeting agents.

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References

Attardi BJ, Burgenson J, Hild SA and Reel JR (2004) Steroid hormonal regulation of growth, prostate specific antigen secretion, and transcription mediated by the mutated androgen receptor in CWR22Rv1 human prostate carcinoma cells. *Mol Cell Endocrinol* **222**:121-132.

Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG and Sawyers CL (2004) Molecular determinants of resistance to antiandrogen therapy. *Nat Med* **10**:33-39.

Chen Y, Sawyers CL and Scher HI (2008) Targeting the androgen receptor pathway in prostate cancer. *Curr Opin Pharmacol* **8**:440-448.

Cheng B, Horst IA and Kowal J (1993) Nigericin inhibits adrenocorticotropic hormone- and dibutyryl-cAMP-stimulated steroidogenesis of cultured mouse adrenocortical tumor (Y1) cells. *Horm Metab Res* **25**:391-392.

Collins I and Workman P (2006) New approaches to molecular cancer therapeutics. *Nat Chem Biol* **2**:689-700.

Dehm SM, Schmidt LJ, Heemers HV, Vessella RL and Tindall DJ (2008) Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. *Cancer Res* **68**:5469-5477.

Dehm SM and Tindall DJ (2007) Androgen receptor structural and functional elements: role and regulation in prostate cancer. *Mol Endocrinol* **21**:2855-2863.

Faber PW, van Rooij HC, van der Korput HA, Baarends WM, Brinkmann AO, Grootegoed JA and Trapman J (1991) Characterization of the human androgen receptor transcription unit. *J Biol Chem* **266**:10743-10749.

Fang Y, Fliss AE, Robins DM and Caplan AJ (1996) Hsp90 regulates androgen receptor hormone binding affinity in vivo. *J Biol Chem* **271**:28697-28702.

Gong Y, Blok LJ, Perry JE, Lindzey JK and Tindall DJ (1995) Calcium regulation of androgen receptor expression in the human prostate cancer cell line LNCaP. *Endocrinology* **136**:2172-2178.

Grossmann ME, Huang H and Tindall DJ (2001) Androgen receptor signaling in androgen-refractory prostate cancer. *J Natl Cancer Inst* **93**:1687-1697.

Guo Z, Dai B, Jiang T, Xu K, Xie Y, Kim O, Nesheiwat I, Kong X, Melamed J, Handratta VD, Njar VC, Brodie AM, Yu LR, Veenstra TD, Chen H and Qiu Y (2006) Regulation of androgen receptor activity by tyrosine phosphorylation. *Cancer Cell* **10**:309-319.

Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, Chen H, Kong X, Melamed J, Tepper CG, Kung HJ, Brodie AM, Edwards J and Qiu Y (2009) A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. *Cancer Res* **69**:2305-2313.

Heinlein CA and Chang C (2004) Androgen receptor in prostate cancer. *Endocr Rev* **25**:276-308.

Hofland J, van Weerden WM, Dits NF, Steenbergen J, van Leenders GJ, Jenster G, Schroder FH and de Jong FH (2010) Evidence of limited contributions for intratumoral steroidogenesis in prostate cancer. *Cancer Res* **70**:1256-1264.

Huang W, Shostak Y, Tarr P, Sawyers C and Carey M (1999) Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. *J Biol Chem* **274**:25756-25768.

Kawada M, Inoue H, Masuda T and Ikeda D (2006) Insulin-like growth factor I secreted from prostate stromal cells mediates tumor-stromal cell interactions of prostate cancer. *Cancer Res* **66**:4419-4425.

Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS and Wilson EM (1988) Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science* **240**:327-330.

Marcelli M, Zoppi S, Wilson CM, Griffin JE and McPhaul MJ (1994) Amino acid substitutions in the hormone-binding domain of the human androgen receptor alter the stability of the hormone receptor complex. *J Clin Invest* **94**:1642-1650.

Margolis LB, Novikova IY, Rozovskaya IA and Skulachev VP (1989) K+/H+-antiporter nigericin arrests DNA synthesis in Ehrlich ascites carcinoma cells. *Proc Natl Acad Sci U S A* **86**:6626-6629.

Mashima T, Naito M, Kataoka S, Kawai H and Tsuruo T (1995) Aspartate-based inhibitor of interleukin-1 beta-converting enzyme prevents antitumor agent-induced apoptosis in human myeloid leukemia U937 cells. *Biochem Biophys Res Commun* **209**:907-915.

Mashima T, Oh-hara T, Sato S, Mochizuki M, Sugimoto Y, Yamazaki K, Hamada J, Tada M, Moriuchi T, Ishikawa Y, Kato Y, Tomoda H, Yamori T and Tsuruo T (2005) p53-defective tumors with a functional apoptosome-mediated pathway: a new therapeutic target. *J Natl Cancer Inst* **97**:765-777.

Mellinghoff IK, Vivanco I, Kwon A, Tran C, Wongvipat J and Sawyers CL (2004) HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. *Cancer Cell* **6**:517-527.

Rotin D, Wan P, Grinstein S and Tannock I (1987) Cytotoxicity of compounds that interfere with the regulation of intracellular pH: a potential new class of anticancer drugs. *Cancer Res* **47**:1497-1504.

Shavit N, Dilley RA and San Pietro A (1968) Ion translocation in isolated chloroplasts. Uncoupling of photophosphorylation and translocation of K+ and H+ ions induced by Nigericin. *Biochemistry* **7**:2356-2363.

Shoemaker RH, Scudiero DA, Melillo G, Currens MJ, Monks AP, Rabow AA, Covell DG and Sausville EA (2002) Application of high-throughput, molecular-targeted screening to anticancer drug discovery. *Curr Top Med Chem* **2**:229-246.

Solit DB, Zheng FF, Drobnjak M, Munster PN, Higgins B, Verbel D, Heller G, Tong W, Cordon-Cardo C, Agus DB, Scher HI and Rosen N (2002) 17-Allylamino-17-demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. *Clin Cancer Res* **8**:986-993.

Sramkoski RM, Pretlow TG, 2nd, Giaconia JM, Pretlow TP, Schwartz S, Sy MS, Marengo SR, Rhim JS, Zhang D and Jacobberger JW (1999) A new human prostate carcinoma cell line, 22Rv1. *In Vitro Cell Dev Biol Anim* **35**:403-409.

Steinrauf LK, Pinkerton M and Chamberlin JW (1968) The structure of nigericin. *Biochem Biophys Res Commun* **33**:29-31.

Tan J, Sharief Y, Hamil KG, Gregory CW, Zang DY, Sar M, Gumerlock PH, deVere White RW, Pretlow TG, Harris SE, Wilson EM, Mohler JL and French FS (1997) Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. *Mol Endocrinol* **11**:450-459.

Taplin ME and Balk SP (2004) Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *J Cell Biochem* **91**:483-490.

van Bokhoven A, Varella-Garcia M, Korch C, Johannes WU, Smith EE, Miller HL, Nordeen SK, Miller GJ and Lucia MS (2003) Molecular characterization of human prostate carcinoma cell lines. *Prostate* **57**:205-225.

Vanaja DK, Mitchell SH, Toft DO and Young CY (2002) Effect of geldanamycin on androgen receptor function and stability. *Cell Stress Chaperones* **7**:55-64.

Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO and Mulder E (1990) A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem Biophys Res Commun* **173**:534-540.

Wang Q, Carroll JS and Brown M (2005) Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. *Mol Cell* **19**:631-642.

Wang Y, Kreisberg JI and Ghosh PM (2007) Cross-talk between the androgen receptor and the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer. *Curr Cancer Drug Targets* **7**:591-604.

Xu K, Shimelis H, Linn DE, Jiang R, Yang X, Sun F, Guo Z, Chen H, Li W, Chen H, Kong X, Melamed J, Fang S, Xiao Z, Veenstra TD and Qiu Y (2009) Regulation of androgen receptor transcriptional activity and specificity by RNF6-induced ubiquitination. *Cancer Cell* **15**:270-282.

Yang L, Mashima T, Sato S, Mochizuki M, Sakamoto H, Yamori T, Oh-Hara T and Tsuruo T (2003) Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide. *Cancer Res* **63**:831-837.

Footnotes

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Legends for figures

Figure 1. Chemical library screening for AR signaling inhibitors

(A) Schematic view of luciferase reporter constructs used to screen androgen receptor (AR) signaling inhibitors. PSA-Luc contains whole *prostate specific antigen* (PSA) enhancer-promoter region (-5824 to -1) upstream of firefly luciferase (Luc) gene in a pGL3-basic vector. PSA enhancer-promoter-Luc contains PSA enhancer region (-5292 to -3709) directly linked with its promoter region (-677 to -1) upstream of Luc. CMV-*R*Luc contains a cytomegalovirus (CMV) promoter upstream of the *Renilla* luciferase (Rluc) gene. PSA enhancer and promoter regions contain six to two androgen responsive elements (AREs), respectively (Huang et al., 1999; Shang et al., 2002; Wang et al., 2005) **(B)** Induction of PSA enhancer/promoter activity of the reporter constructs by androgen treatment. 22Rv1 cells were transiently transfected with a pGL3-basic (Luc construct without a promoter: mock-Luc), PSA-Luc or PSA enhancer-promoter-Luc together with CMV-*R*Luc and were treated with the indicated concentrations of dihydrotestosterone (DHT) for 24 h. Firefly luciferase activity, which reflects the PSA promoter activity, was normalized by an internal control, CMV-driven *Renilla* luciferase activity. **(C)** Dose-dependent inhibition of promoter activities by chemical compounds. 22Rv1 cells transfected with PSA-Luc and CMV-*R*Luc were treated with 0.2, 2 and 20 μM of agents in the presence of 1 nM DHT for 24 h. Leptomycin B cells were treated with 0.02, 0.2 and 2 μM of the agent. Promoter activities were measured, as described in *Materials and Methods*. Relative promoter activities in the drug-treated samples compared with untreated controls were calculated and indicated as a heat map.

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Figure 2 Suppression of androgen-dependent PSA promoter activation by nigericin in 22Rv1 cells

(A) 22Rv1 cells were transiently transfected with PSA-Luc and CMV-*R*Luc and were treated with the indicated concentrations of thapsigargin, 17-AAG or nigericin in the absence (*white bar*) or the presence (*black bar*) of 1 nM DHT for 24 h, as described in *Materials and Methods*. **(B)** 22Rv1 cells were transfected with PSA enhancer-promoter-Luc and CMV-*R*Luc and were treated with the indicated concentrations of nigericin in the absence (*white bar*) or the presence (*black bar*) of 1 nM DHT for 24 h. Each promoter-dependent transcription is shown as relative promoter-dependent luciferase activity. *Error bars* show standard deviations.

Figure 3. Suppression of androgen-dependent PSA production and cell growth by nigericin in LNCaP cells

(A) Time course analysis of the inhibition of androgen-dependent PSA induction by nigericin. LNCaP cells were treated with 0 or 10 nM DHT for the indicated time periods in the absence or presence of 100 nM nigericin. **(B)** Dose-dependent inhibition of androgen-dependent PSA induction by nigericin. LNCaP cells were treated with or without 10 nM DHT for 24 h in the absence or presence of the indicated concentrations of nigericin. Relative PSA and AR protein levels in each sample were determined by densitometry. **(C)** Inhibition of androgen-dependent cell growth by nigericin. LNCaP cells were treated with 0 (*closed circle*), 1 (*open circle*), 10 (*closed triangle*) and 100 nM (*open triangle*) of nigericin in the presence of 10 nM DHT for the indicated time periods. Cell numbers were determined with a Sysmex counter. **(D)** LNCaP cells were treated with DHT and nigericin as in (C) for 5 days. Changes in cell morphology were

shown. **(E)** Effect of nigericin on LNCaP cell growth in the absence or the presence of androgen. LNCaP cells were treated with 0 and 10 nM of nigericin in the presence or the absence of 10 nM DHT for 3 days. The increase ratios of cell number relative to day 0 were shown. Statistical significance was tested using the two-sided Student *t* test (**, $P<0.01$).

Figure 4. Nigericin inhibits AR at the mRNA level.

(A), (B) Inhibition of androgen-dependent *PSA* mRNA induction by nigericin coincides with *AR* mRNA inhibition. LNCaP cells were treated with or without 10 nM DHT for 24 h in the absence or presence of the indicated concentrations of nigericin. Expression of *PSA* (A) and *AR* (B) mRNA levels normalized by *β-actin* mRNA levels were determined by quantitative reverse transcription-PCR analysis, as described in *Materials and Methods*. Statistical significance was tested using the two-sided Student *t* test (**, $P<0.01$). (**C**) Effect of nigericin on *AR* mRNA stability. LNCaP cells in 5% CSS medium with or without 10 nM DHT were treated with actinomycin D (10 μg/ml), minus or plus nigericin (100 nM). Equal amounts of RNA at indicated time points were analyzed for the abundance of the *AR* mRNA (normalized by that of *GAPDH* mRNA) by qRT-PCR analysis. *Open circle*, control; *closed circle*, DHT; *closed triangle*, DHT + nigericin. Statistical significance (DHT treatment versus DHT + nigericin treatment at 4 h and 8 h) was tested using the two-sided Student *t* test (**, $P<0.01$). **(D)** *Left panel*: Expression level of exogenous AR in PC-3 cells relative to those of endogenous ARs in LNCaP and 22Rv1 cells was determined. *Right panel*: Effect of nigericin and other compounds that suppress AR signaling on the exogenous AR expression. PC-3 cells transfected with pLPCX-AR were treated with the indicated concentrations of nigericin and other agents in the

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absence or presence of 10 nM DHT for 24 h. AR protein expression as well as β-actin expression as a control was determined by western blot analysis. Relative AR expression level in each sample was determined by densitometry. **(E)** PC-3 cells were transfected with pLPCX-AR together with PSA-Luc and CMV-*R*Luc and were treated with the indicated concentrations of nigericin and other agents in the absence or presence of 10 nM DHT for 24 h. Relative PSA promoter–dependent transcription normalized by CMV promoter activity level is shown. *Error bars* show standard deviations. Statistical significance (DHT + each drug versus DHT alone) was tested using the two-sided Student *t* test (*, $P<0.05$).

Figure 5. Essential role of truncated AR (tAR) in androgen-independent 22Rv1 cell growth.

(A) Schematic view of full-length and truncated AR. TAD, transactivation domain; DBD, DNA binding domain; NLS, nuclear localization signal; LBD, ligand-binding domain; AF-1 and AF-2, activation function 1 and 2 (transcriptional activation subdomains). The target sites of 4 siRNAs used in this study are shown. **(B)** Subcellular localization of full-length and truncated ARs. LNCaP and 22Rv1 cells were left untreated or were treated with the indicated concentrations of DHT for 24 h. Cytoplasmic (C) and nuclear (N) fractions were prepared, and subjected to western blot analysis with the indicated primary antibodies. Blots with anti-Hsp90 and PARP antibodies demonstrate the purity of their respective fractions. **(C)** Selective knockdown of different AR forms by specific siRNAs. 22Rv1 cells were treated with each siRNA for 72 h in the absence or presence of 1 nM DHT. The expressions of full-length and truncated ARs were examined. **(D)** Effect of AR knockdown on 22Rv1 cell growth. 22Rv1 cells were treated with

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control siRNA (*open circle*), siEx1-1 (*open triangle*), siEx1-2 (*closed triangle*), siEX4 (*open square*) or siEx6 (*closed square*) for the indicated time periods in the absence or presence of 1 nM DHT. Cell numbers were determined with a Sysmex counter. Statistical significance between samples was tested using the two-sided Student *t* test (**, $P<0.01$).

Figure 6. Simultaneous inhibition of full-length and truncated ARs by nigericin blocks androgen-independent growth of 22Rv1 cells

(A) Effect of nigericin treatment on AR levels and apoptosis markers' expression. 22Rv1 cells were treated with the indicated concentrations of nigericin in the absence of androgens for 72 h. The expressions of full-length and truncated ARs as well as markers of apoptosis induction, cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase-3 were examined. **(B)** Quantitative analysis of AR protein levels after nigericin treatment. Full-length (*open bar*) and truncated (*closed bar*) AR levels in (A) were determined by densitometry. *Error bars* show standard deviations. Statistical significance between the drug-treated versus control (untreated) was tested using the two-sided Student *t* test (*, $P<0.05$; **, $P<0.01$). **(C)** Effect of nigericin treatment on 22Rv1 cell growth. 22Rv1 cells were treated with 0 (*closed circle*), 1 (*open circle*), 10 (*closed triangle*) and 100 (*open triangle*) nM nigericin for the indicated time periods. Cell numbers were determined. **(D)** Effect of nigericin treatment on cell cycle distribution. Cells were treated with nigericin as in (A). Cell cycle distribution was analyzed by flow cytometry. *Gray bar*, G_1; *white bar*, S; *black bar*, G_2/M. **(E)** Nigericin treatment induces apoptosis. Cells were treated with nigericin as in (A). Apoptosis induction was determined as described in

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Materials and Methods. Error bars show standard deviations. Statistical significance (drug-treated versus untreated) was tested using the two-sided Student *t* test (*, $P<0.05$).

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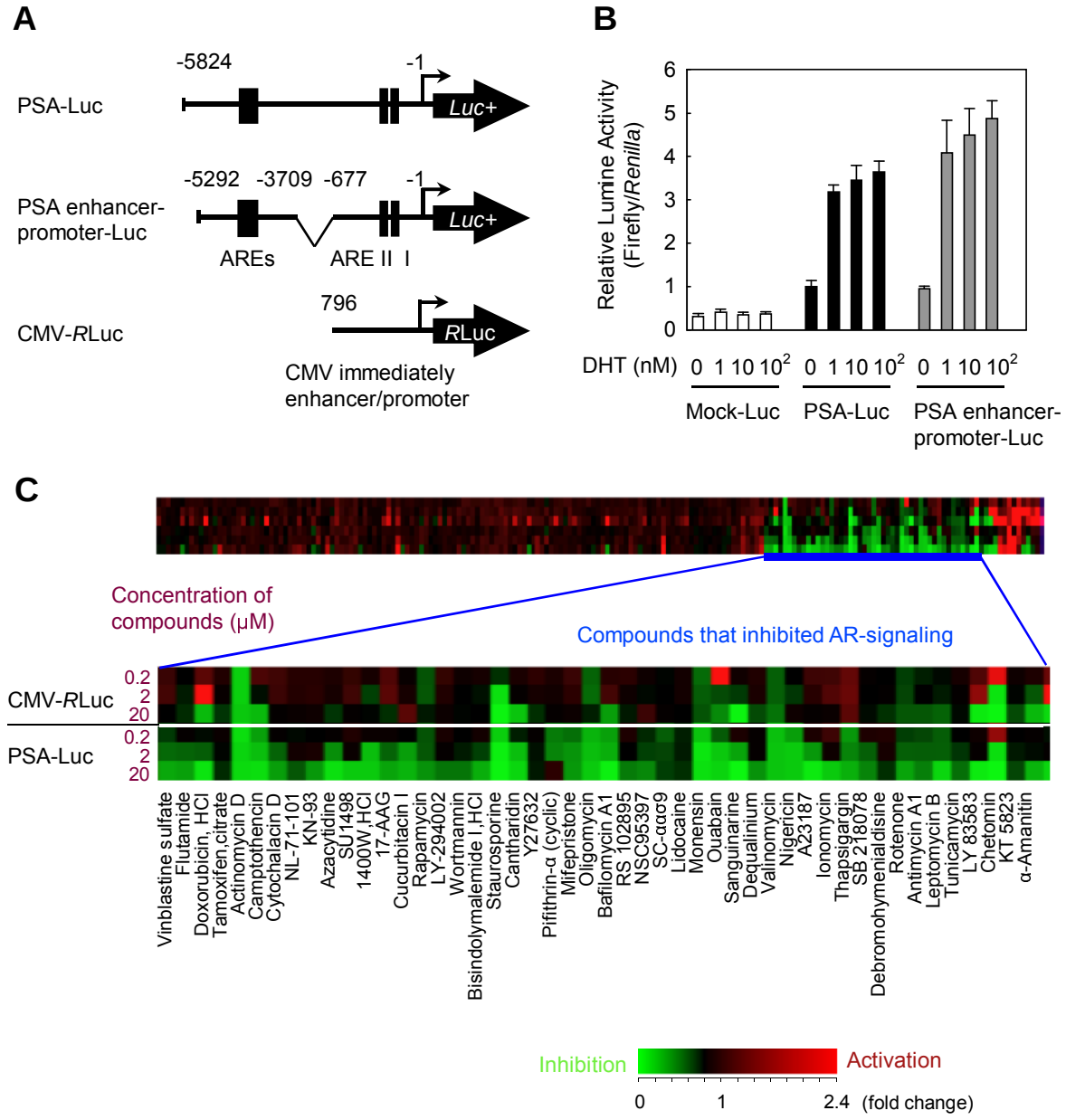

Figure 1

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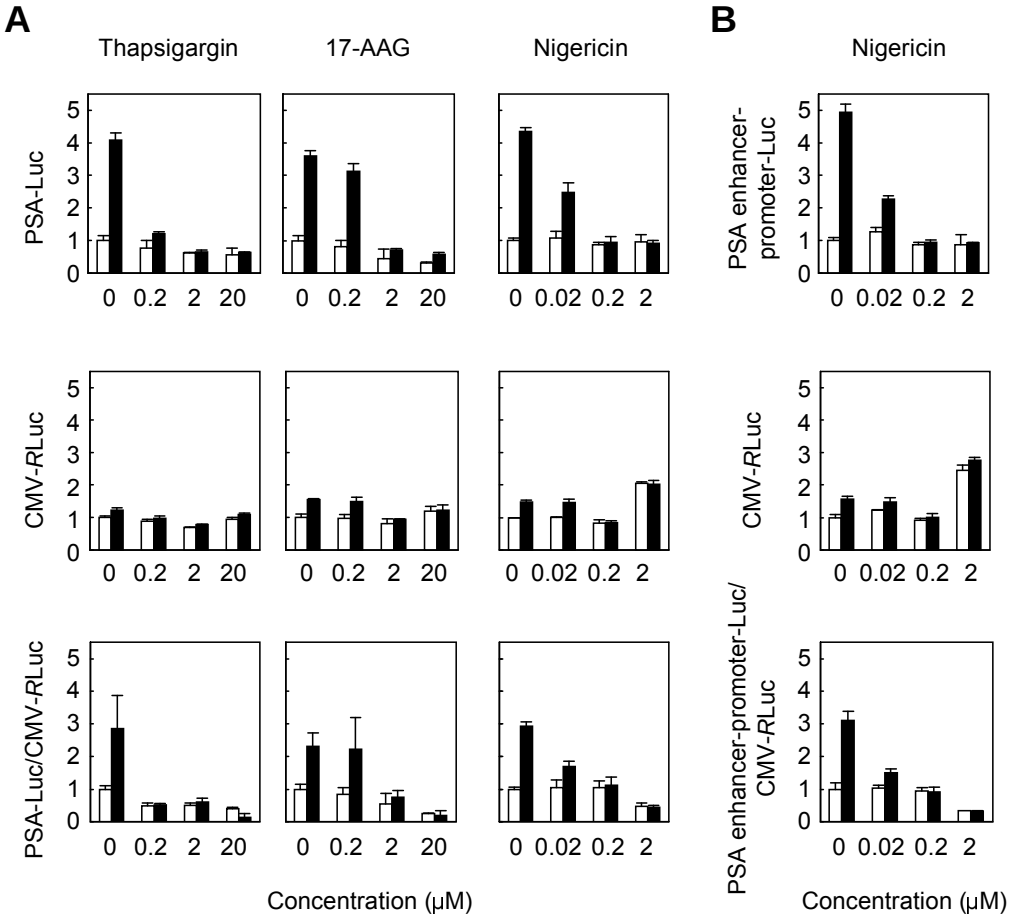

Figure 2

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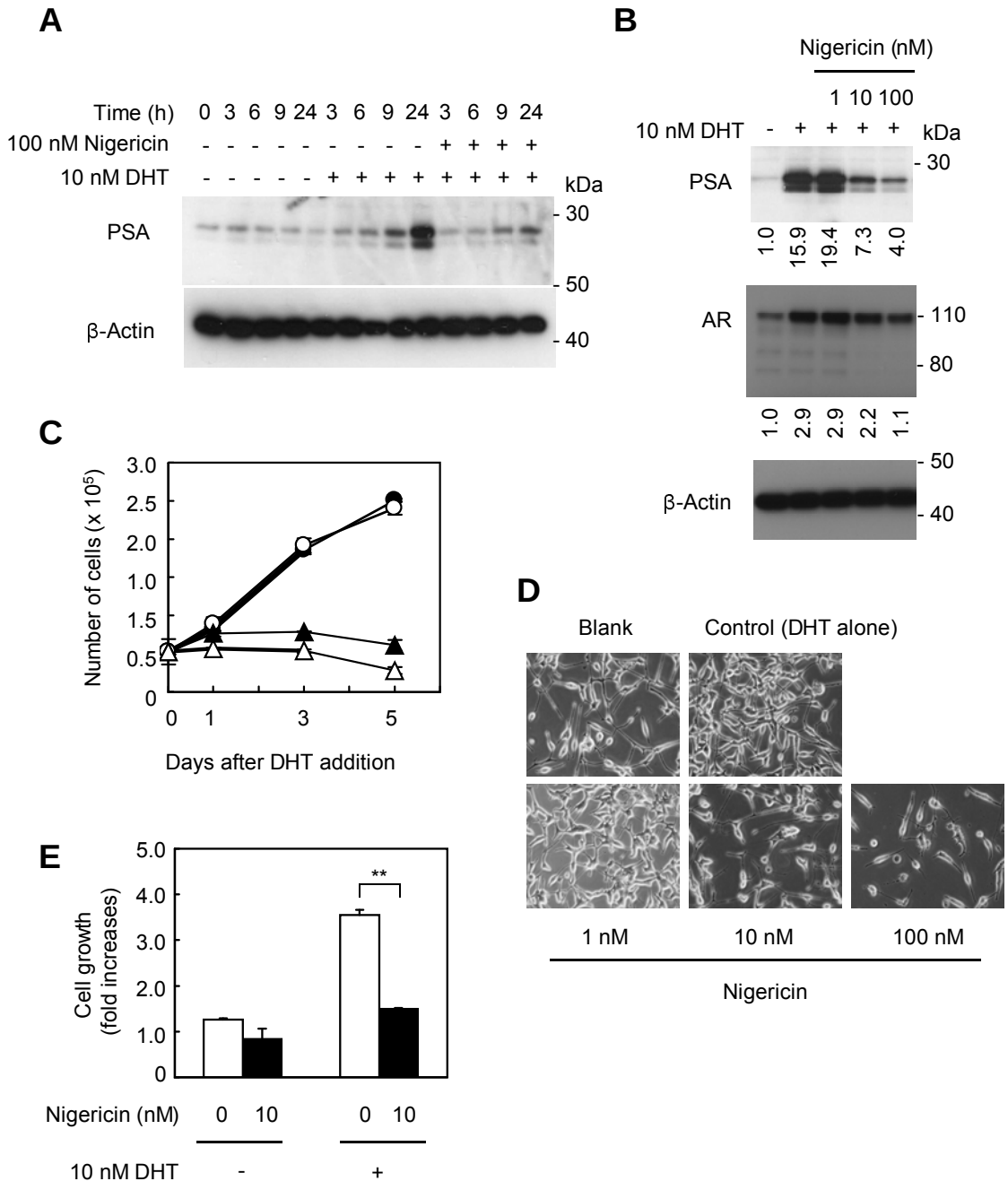

Figure 3

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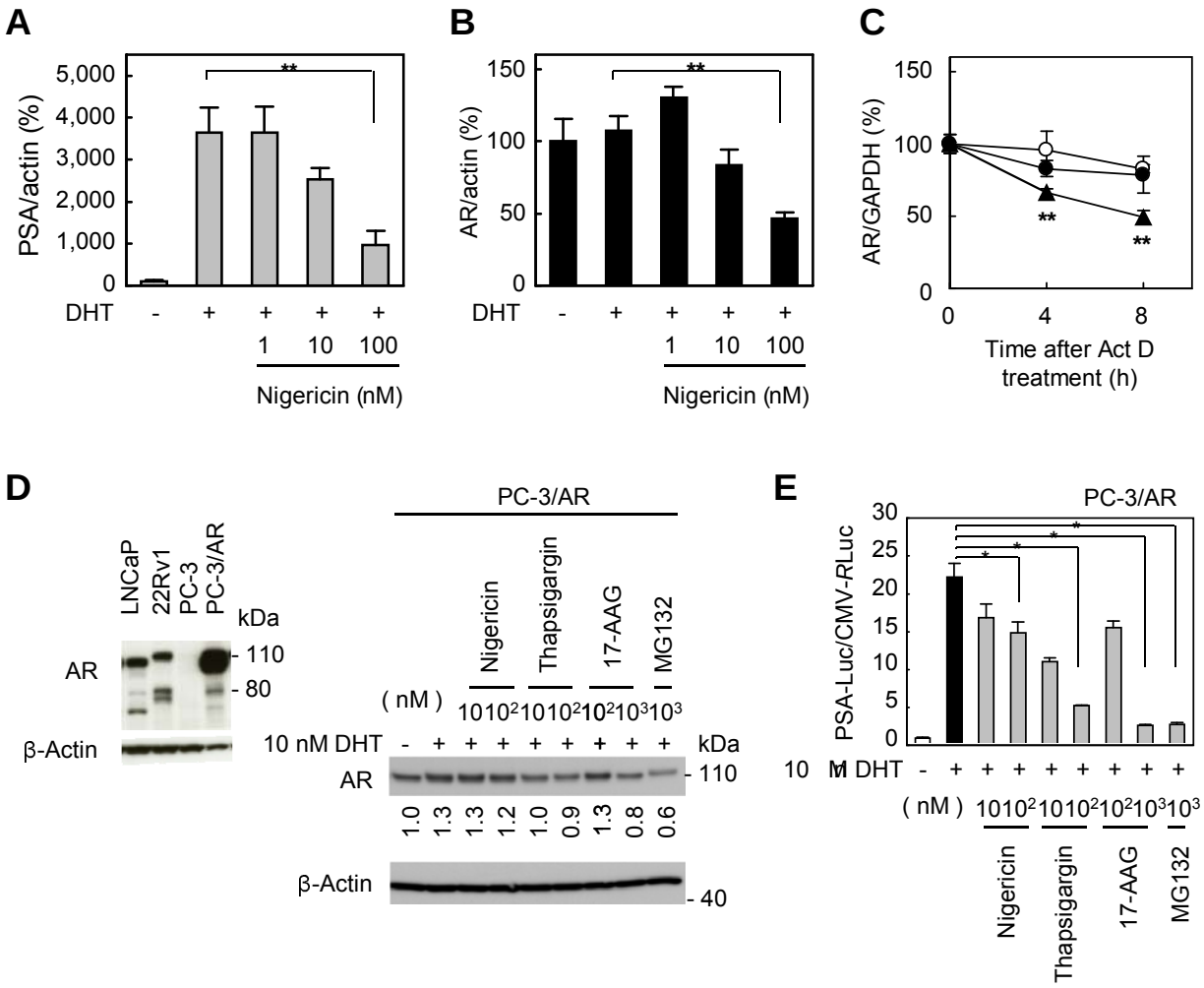

Figure 4

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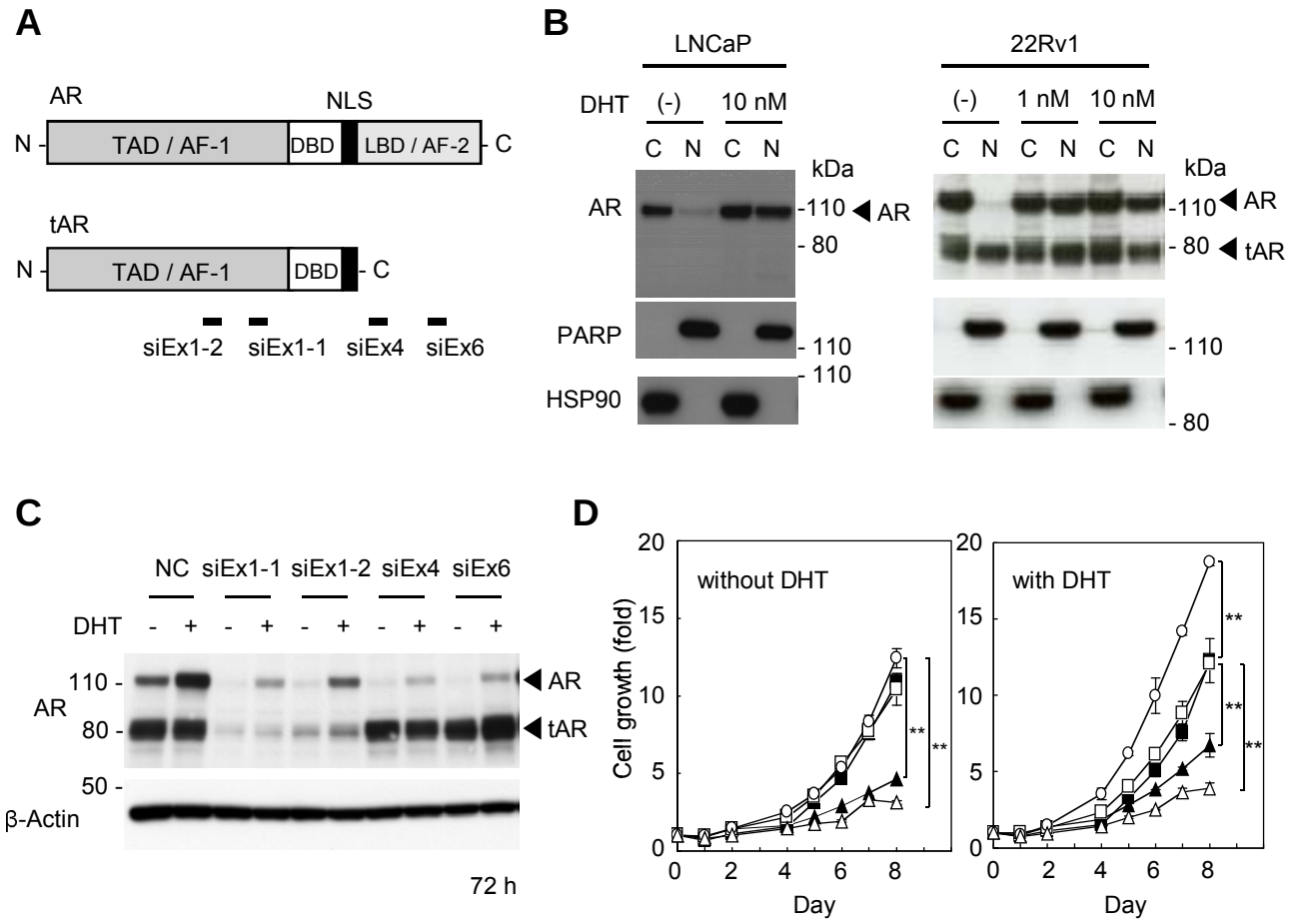

Figure 5

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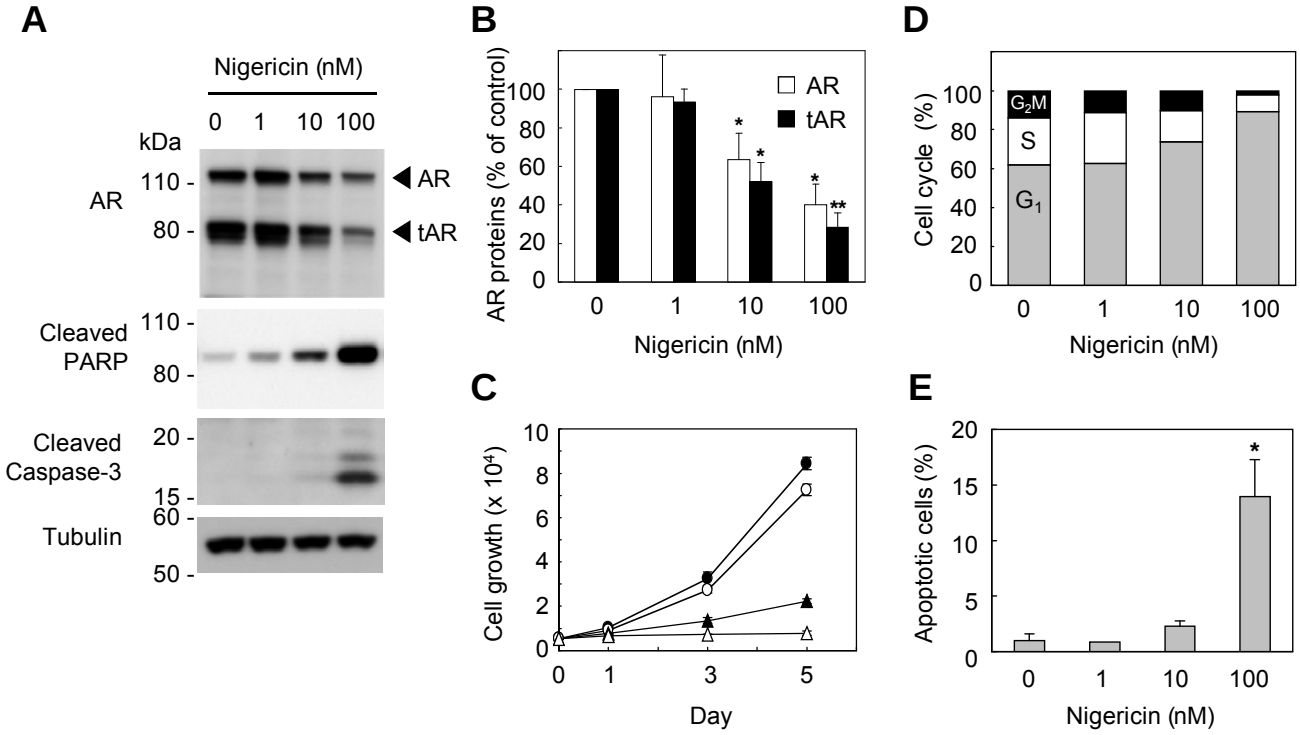

Figure 6