Pharmacological Targeting of Constitutively Active Truncated Androgen Receptor by Nigericin and Suppression of Hormone-Refractory Prostate Cancer Cell Growth[S]

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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 lacking the carboxyl-terminal ligand-binding domain, small interfering RNA-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. It is noteworthy that nigericin was able to mimic the knockdown of both AR isoforms: it reduced the expression of the full-length and the truncated ARs, and it induced G_1 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.

Introduction

Prostate cancer is the most frequently diagnosed, nondermatological cancer in men in the United States. In recent times, the number of patients with prostate cancer 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 (Grossmann et al., 2001; Chen et al., 2004; 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),

ABBREVIATIONS: AR, androgen receptor; HRPC, hormone-refractory prostate cancer; PSA, prostate-specific antigen; tAR, truncated androgen receptor; DHT, dihydrotestosterone; ARE, androgen response element; DBD, DNA binding domain; LBD, ligand-binding domain; SCADS, Screening Committee of Anticancer Drugs; CSS, charcoal-stripped serum; PCR, polymerase chain reaction; PARP, poly(ADP-ribose) polymerase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; siRNA, small interfering RNA; UTR, untranslated region; 17-AAG, 17-demethoxy-17-(2-propenylamino)geldanamycin; MG-132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; PAGE, polyacrylamide gel electrophoresis; Hsp90, 90-kDa heat shock protein; PC-3/AR, androgen receptor-transfected prostate cancer PC-3 cells.

AR translocates into the nucleus, in which it interacts with androgen response elements (AREs) in the promoter/enhancer regions of AR target genes, such as *PSA*, and regulates their expression. AR is composed of three major domains: an amino-terminal domain, a central DNA binding domain, and a carboxyl-terminal ligand-binding domain (LBD) (Dehm and Tindall, 2007). The amino-terminal domain and LBD possess separate transcriptional activation subdomains, termed activation function-1 and activation function-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; Heinlein and Chang, 2004; Mellinghoff et al., 2004; Guo et al., 2006; Xu et al., 2009). 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 (Shoemaker et al., 2002; Collins and Workman, 2006). Here, using 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.

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 (Tokyo, Japan). DHT and *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG-132) were purchased from Sigma-Aldrich (St. Louis. MO). Nigericin (Steinrauf et al., 1968) was purchased from LKT Lab., Inc. (St. Paul, MN). 17-Demethoxy-17-(2-propenylamino)geldanamycin (17-AAG) was purchased from InvivoGen (San Diego, CA). 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] (van Bokhoven et al., 2003; Dehm et al., 2008), PC-3, and DU145 (AR-negative, androgen-independent) (van Bokhoven et al., 2003) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml 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 RPMI 1640 medium (Invitrogen, Tokyo, Japan) containing 5% charcoal-stripped serum (CSS) (HyClone Laboratories, Logan, UT) 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 ± S.D.

Vector Construction. The basic frame of two luciferase reporter constructs, PSA-Luc and PSA enhancer/promoter-Luc, is the pGL3-vector (Promega, Madison, WI). 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 reniformis* luciferase reporter vector with 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).

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 4×10^4 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 *PSA* promoter activity, was normalized by an internal control, CMV-driven *R. reniformis* luciferase activity.

Subcellular Fractionation and Western Blot Analysis. To obtain cell lysates, we washed cells in ice-cold phosphate-buffered saline and lysed them in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, and 50 mM Tris-HCl, pH 8.0) supplemented with $1\times$ Protease Inhibitor Cocktail (Sigma-Aldrich). After centrifugation at 14,000g for 15 min at 4°C, the supernatant was subjected to SDS-PAGE. Subcellular fractions were obtained using a ProteoExtract Subcellular Proteome Extraction kit (Calbiochem, San Diego, CA). Each extract derived from the same number of the cells was subjected to SDS-PAGE. After SDS-PAGE, separated proteins were transferred to polyvinylidene difluoride membranes. After a 30-min incubation with Tris-buffered saline containing 0.1% Tween 20 and 5% fat-free milk, the membranes were incubated for 2 h at room temperature or overnight at 4°C with the following primary antibodies: anti-AR (441) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-PSA (C-19) (Santa Cruz Biotechnology), anti-poly(ADP-ribose) polymerase (PARP) (BD Pharmingen, San Diego CA), anti-β-actin (Sigma-Aldirch), anti-90-kDa heat shock protein (Hsp90) (Stressgen, Montreal, QC Canada), anti-tubulin (Sigma-Aldrich), anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), 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, Chalfont St. Giles, Buckinghamshire, UK).

Quantitative Reverse Transcription-PCR Analysis. Total RNAs were extracted from cells using an RNeasy Mini kit (QIAGEN, Valencia, CA). 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 SuperScript III Platinum Two-Step quantitative reverse transcription-PCR (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 LuX Primer sets (Invitrogen). PCRs were conducted in the 96-well spectrofluorometric thermal cycler ABI PRISM 7700 (Applied Biosystems, Foster City, CA). For the qRT-PCR to determine AR mRNA stability, PCRs were conducted in LightCycler 480 Probe Master (Roche Diagnostics, Mannheim, Germany). The primer sequences for the amplifications were 5′-GCCTTGCTCTCTAGCCTCAA-3′ and 5′-GGTCGTCCACGTGTA-AGTTG-3′ for *AR* and 5′-CCCGTCCTTGACTCCCTAGT-3′ and 5′-GTGATCGGTGCTGGTTCC-3′ for *GAPDH*, respectively.

Small Interfering RNA Treatment. The stealth small interfering RNA (siRNA) oligonucleotides to AR were synthesized by Invitrogen. The targeting sequences of siRNA for AR were 5′-CCCTT-TCAAGGGAGGTTACACCAAA-3′ (exon 1-1), 5′-CCGAATGCAAAG-GTTCTCTGCTAGA-3′ (exon 1-2), 5′-GACTCCTTTGCAGCCTT-GCTCTCTA-3′ (exon 4), and 5′-TCTCAAGAGTTTGG ATGGCTCCAAA-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).

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 AREs and constructed reporter constructs PSA-Luc and PSA enhancer-promoter-Luc (Fig. 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, because this cell line expresses the full-length AR [AR(H874Y)] and possesses functional AR-mediated signaling (Sramkoski et al., 1999; Attardi et al., 2004). 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 Fig. 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 (Supplemental Fig. S1 and Fig. 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 used this reporter system to screen a chemical library. The SCADS
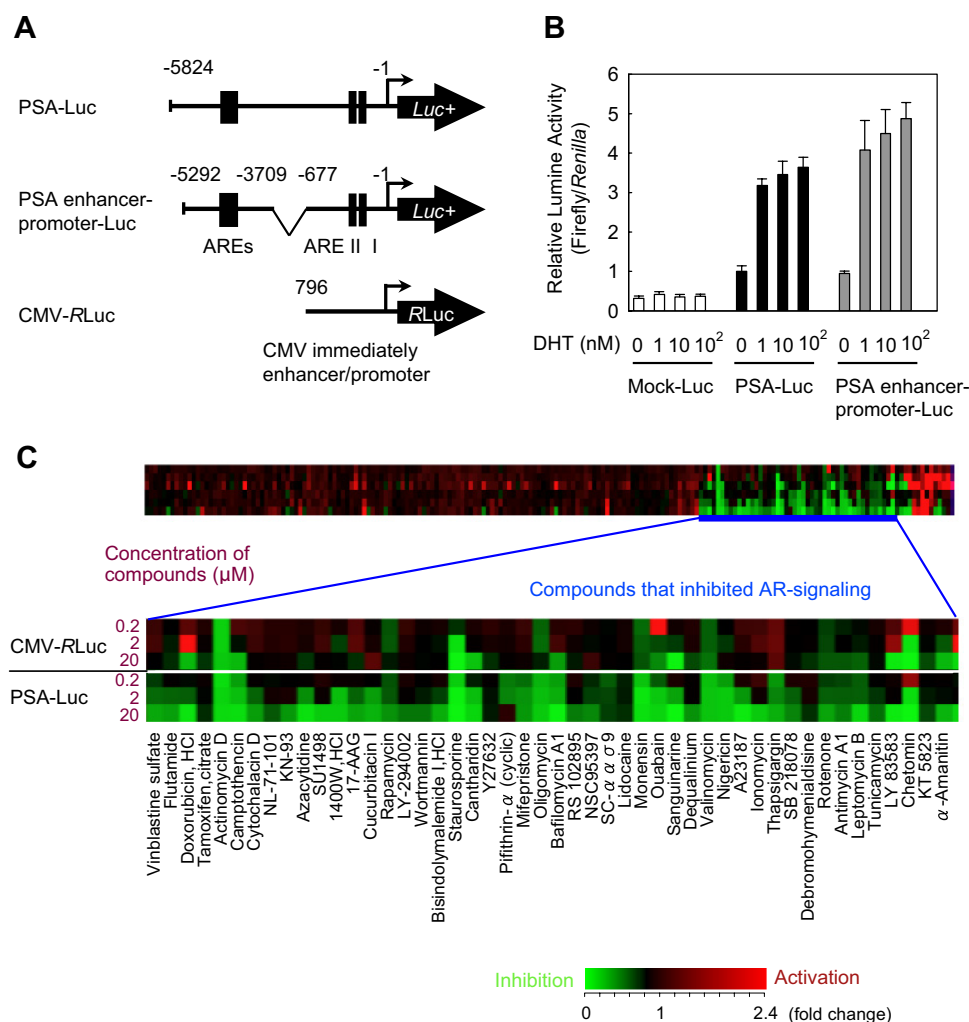
Fig. 1. Chemical library screening for AR signaling inhibitors. A, schematic view of luciferase reporter constructs used to screen AR signaling inhibitors. PSA-Luc contains whole *PSA* enhancer-promoter region (-5824 to -1) upstream of firefly luciferase (Luc) gene in a pGL3-basic vector. PSA enhancer-promoter-Luc contains *PSA* enhancer region (-5292 to -3709) directly linked with its promoter region (-677 to -1) upstream of Luc. CMV-*R*Luc contains a CMV promoter upstream of the *R. reniformis* luciferase (Rluc) gene. *PSA* enhancer and promoter regions contain six to two AREs, respectively (Huang et al., 1999; 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 DHT for 24 h. Firefly luciferase activity, which reflects the *PSA* promoter activity, was normalized by an internal control, CMV-driven *R. reniformis* 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 concentration 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 concentration of the agent. Promoter activities were measured, as described under *Materials and Methods*. Relative promoter activities in the drug-treated samples compared with untreated controls were calculated and indicated as a heat map.

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 (Fig. 1C). The compounds included several inhibitors of signaling molecules that are known to be involved in AR regulation, such as an Hsp90 inhibitor, and Ca^{2+} ionophores (Gong et al., 1995; Fang et al., 1996; Solit et al., 2002; Vanaja et al., 2002; Wang et al., 2007). These compounds significantly suppressed the androgen-dependent induction of *PSA* promoter activity (Fig. 2A), whereas they did not affect the CMV promoter activity, verifying the selective inhibition of the AR-dependent transcription.
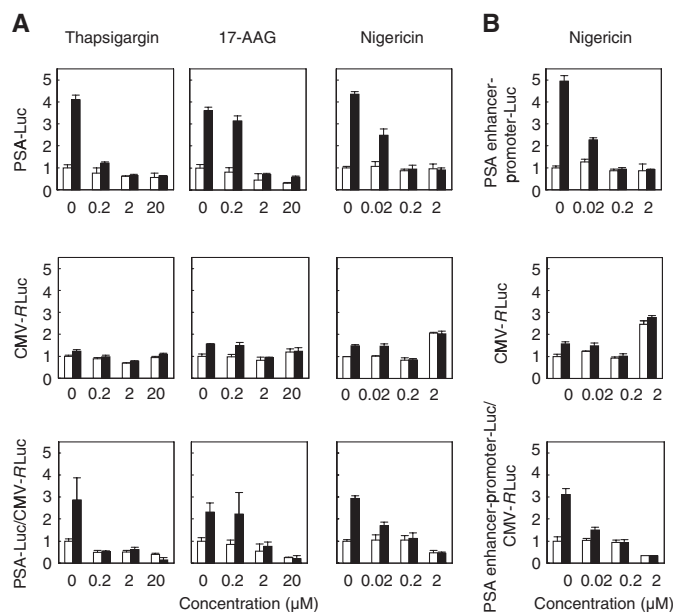
Nigericin Inhibits Androgen-Dependent PSA Expression and LNCaP Cell Growth. Among the compounds that we identified for the first time as AR signaling inhibitors, we focused on nigericin (Steinrauf et al., 1968), an antibiotic derived from *S. hygroscopicus*, because it also inhibited the androgen-dependent growth of prostate cancer cells (see below). As shown in Fig. 2, A 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 *R. reniformis* 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 Fig. 3, A 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: whereas 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 (Fig. 3B, middle). Longer exposure to nigericin further reduced the level of the AR protein (Supplemental Fig. S2). These effects were also observed when 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 (Fig. 3, C and D), whereas its growth-inhibitory effect was not significantly observed in DHT-depleted LNCaP cells (Fig. 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 (Fig. 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 (Fig. 4B). This result coincided with nigericin-mediated down-regulation of the AR protein level (Fig. 3B, middle). 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.

Therefore, we further analyzed the effect of nigericin on the AR mRNA level under the actinomycin D treatment in which mRNA transcriptions were suppressed. As shown in Fig. 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 by transiently introducing the CMV promoter-driven full-length *AR* expression vector into AR-negative prostate cancer PC-3 cells (PC-3/AR; Fig. 4D, left). 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 decreased neither the protein level of the exogenous AR nor its up-regulation by DHT (Fig. 4D, right). Consistent with these observations, nigericin could not efficiently inhibit the DHT-induced *PSA* promoter activity in PC-3/AR cells (Fig. 4E). By contrast, thapsigargin, 17-AAG, and MG-132 repressed both

Fig. 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 (□) or the presence (■) of 1 nM DHT for 24 h, as described under *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 (□) or the presence (■) of 1 nM DHT for 24 h. Each promoter-dependent transcription is shown as relative promoter-dependent luciferase activity. Error bars show standard deviations.

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.

Because nigericin slightly reduced the exogenous AR-dependent *PSA* promoter activation without affecting AR protein level (Fig. 4, D 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 (Supplemental Fig. S3A). Again, the *PSA* promoter activity was partially reduced under these conditions (Supplemental Fig. 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 HRPC 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 (Figs. 4D, left, and 5A). Consistent with the previous observation, DHT translocated the full-length AR from cytoplasm to the nucleus in both LNCaP and 22Rv1 cells (Fig. 5B). By contrast, nearly half of the tAR pool con-

stitutively 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 RNA interference used for the studies could still be open to question because each result was obtained from experiments with only one siRNA, and the growth inhibition by RNA interference 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 knock down both full-length AR and tAR (siEx1-1 and 1-2) or only full-length AR (siEx4 and 6) (Fig. 5A). These siRNAs efficiently reduced the levels of target proteins (Fig. 5C) and remained effective for at least 7 days (Supplemental Fig. S4A). As shown in Fig. 5D, left, 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 because it inhibited androgen-dependent growth of 22Rv1 cells (Fig. 5D, right, and Supplemental Fig. 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 signifi-
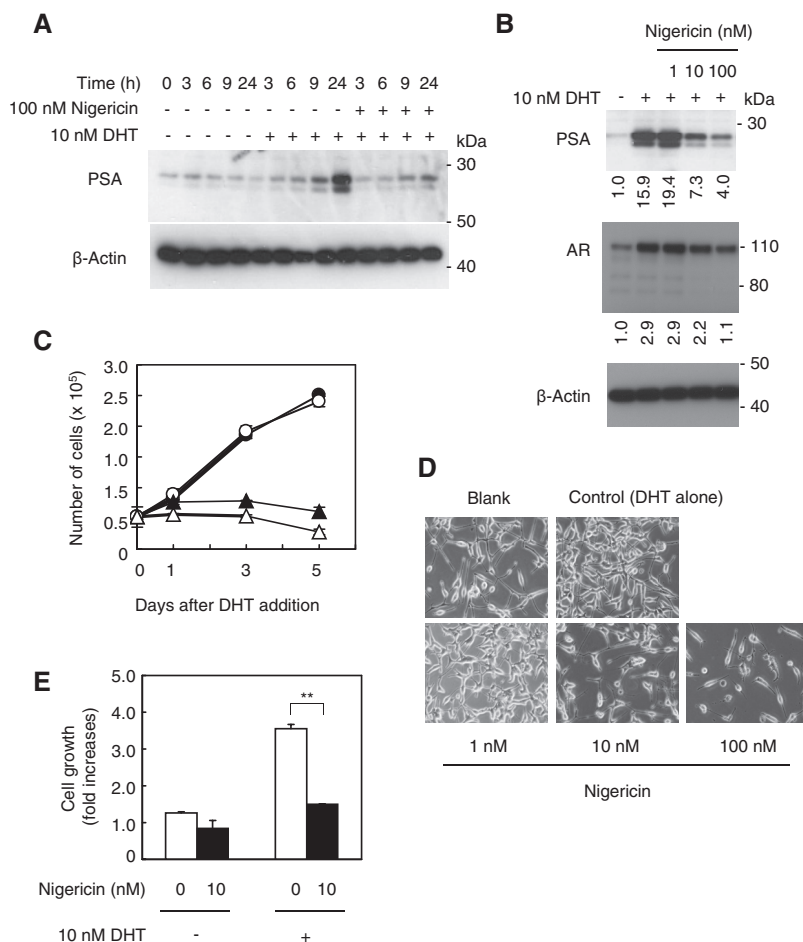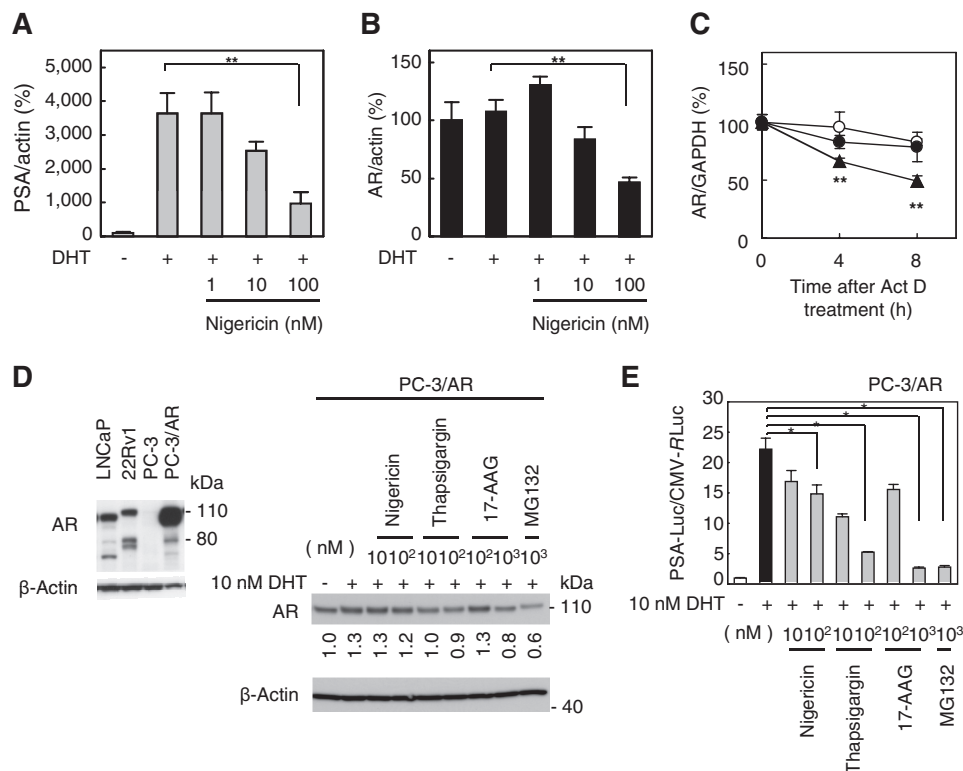

Fig. 3. Suppression of androgen-dependent PSA production and cell growth by nigericin in LNCaP cells. A, time course analysis of the inhibition of androgen-dependent PSA induction by nigericin. LNCaP cells were treated with 0 or 10 nM DHT for the indicated time periods in the absence or presence of 100 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 (●), 1 (○), 10 (▲), and 100 nM (△) 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 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's *t* test (∗∗, *P* < 0.01).

cantly suppressed the tAR expression and the full-length AR expression in a dose-dependent manner (Fig. 6, A and B). This effect of nigericin was similar to those of AR siRNAs siEx1-1 and 1-2 (Fig. 5C). Indeed, nigericin inhibited the androgen-independent growth of 22Rv1 cells in a dose-dependent manner (Fig. 6C). Flow cytometry analysis revealed that nigericin treatment induced significant G_1 cell cycle arrest at the concentrations of 10 and 100 nM (Fig. 6D). Moreover, at the higher concentration (100 nM), cells underwent apoptotic cell death (Fig. 6E). Nigericin-treated cells consistently exhibited the cleavage (i.e., activation) of pro-apoptotic caspase-3 and PARP cleavage, both hallmarks of apoptosis (Fig. 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, whereas it was less toxic to AR-negative DU145 cells (Supplemental Fig. S5). These results indicate that suppressing constitutively active tAR with chemicals, such as nigericin, could be an effective strategy to overcome tAR-positive HRPC.

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; Heinlein and Chang, 2004; Guo et al., 2006). 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)] (Veldscholte et al., 1990; Tan et al., 1997; van Bokhoven et al., 2003) 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

Fig. 4. Nigericin inhibits AR at the mRNA level. A and 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 under *Materials and Methods*. Statistical significance was tested using the two-sided Student's 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. ○, control; ●, DHT; ▲, DHT + nigericin. Statistical significance (DHT treatment versus DHT + nigericin treatment at 4 and 8 h) was tested using the two-sided Student's t test (∗∗, $P < 0.01$). D, left, expression level of exogenous AR in PC-3 cells relative to those of endogenous ARs in LNCaP and 22Rv1 cells was determined. Right, 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 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's t test (∗, $P < 0.05$).
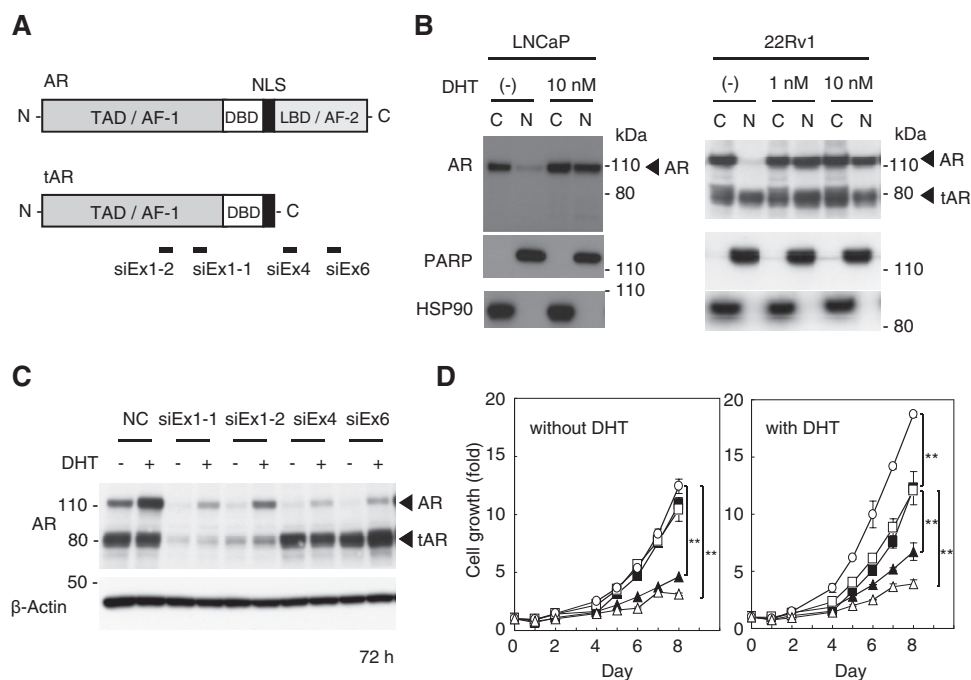
Fig. 5. Essential role of tAR in androgen-independent 22Rv1 cell growth. A, schematic view of full-length and truncated AR. TAD, transactivation domain; DBD, DNA binding domain; NLS, nuclear localization signal; AF-1 and AF-2, activation function 1 and 2, respectively (transcriptional activation subdomains). The target sites of four 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 control siRNA (○), siEx1–1 (△), siEx1–2 (▲), siEX4 (□), or siEx6 (■) 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's t test (∗∗, $P < 0.01$).

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
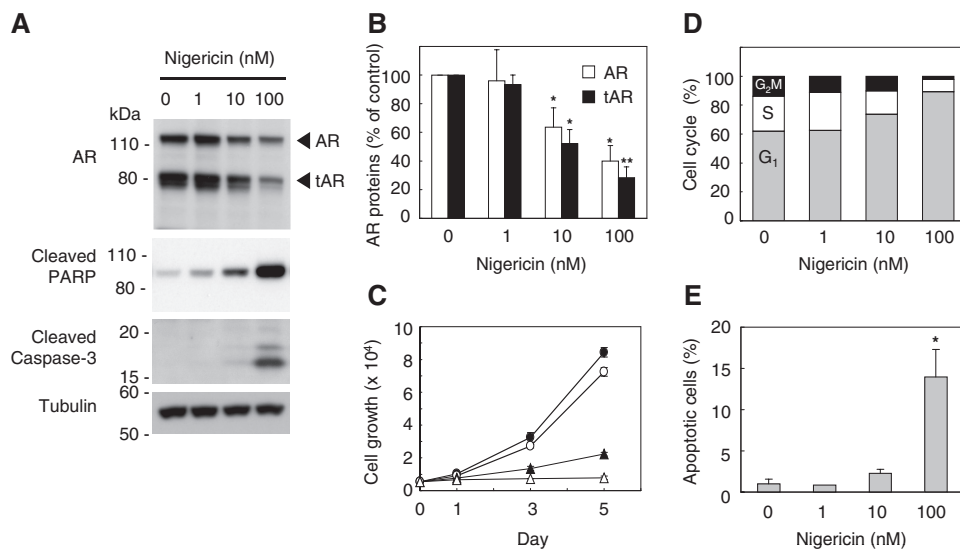
Fig. 6. Simultaneous inhibition of full-length and truncated ARs by nigericin blocks androgen-independent growth of 22Rv1 cells. A, effect of nigericin treatment on AR levels and apoptosis markers' expression. 22Rv1 cells were treated with the indicated concentrations of nigericin in the absence of androgens for 72 h. The expressions of full-length and truncated ARs and markers of apoptosis induction, cleaved PARP, and cleaved caspase-3 were examined. B, quantitative analysis of AR protein levels after nigericin treatment. Full-length (□) and truncated (■) 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's t test (∗, $P < 0.05$; ∗∗, $P < 0.01$). C. effect of nigericin treatment on 22Rv1 cell growth. 22Rv1 cells were treated with 0 (●), 1 (○), 10 (▲), and 100 (△) 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. ▦, G_1; □, S; ■, G_2/M. E, nigericin treatment induces apoptosis. Cells were treated with nigericin as in A. Apoptosis induction was determined as described in *Materials and Methods*. Error bars show standard deviations. Statistical significance (drug-treated versus untreated) was tested using the two-sided Student's t test (∗, $P < 0.05$).

shown that nigericin inhibits DNA synthesis of cancer cells by increasing intracellular pH and causing acidification of cytoplasm (Rotin et al., 1987; Margolis et al., 1989). 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 (Fig. 3C), whereas nigericin was reported to affect intracellular pH level at much higher concentrations (1–10 μM) (Rotin et al., 1987; Margolis et al., 1989). These data indicate that the compound could inhibit AR through an intracellular pH-independent manner. Nigericin suppresses androgen-dependent LNCaP cell growth, whereas it does not show any marked cytotoxicity to the cells in the androgen-depleted conditions (Fig. 3E). In addition, nigericin showed a selective growth-inhibitory effect on tAR- or AR-dependent cell lines but not on AR-negative DU145 cells (Supplemental Fig. 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 kilobases) 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 (Fig. 4D and Supplemental Fig. S3A).

In this study, we identified nigericin as a novel AR signaling inhibitor. Because nigericin reduces the AR mRNA level, a broad range of AR variants in HRPC would be susceptible to the functional blockade by this compound. It has been reported that intratumoral androgen production plays a role in prostate tumor progression (Hofland et al., 2010). Because 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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