

Molecular Pharmacological Approach Reveals Potential New Strategies to Suppress Androgen Receptor Signaling in Prostate Cancer

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PharmSight on Mashima T, et al. Pharmacological targeting of constitutively active truncated androgen receptor by nigericin and suppression of hormone-refractory prostate cancer cell growth. *Mol Pharmacol* 2010;78:846-54.

Abstract

Androgen receptor (AR) plays a pivotal role in growth and survival of prostate cancer cells. Currently, androgen deprivation therapy is a standard approach to manage advanced and metastatic prostate cancer. In most patients, however, relapse eventually occurs and so far, only few treatment options are available to manage recurrence. Previous studies have demonstrated that, in hormone-refractory prostate cancer (HRPC), various mechanisms affect AR including overexpression, mutations and alternative splicing. These alterations can lead to androgen-independent AR activation or resistance to antiandrogen-mediated AR inhibition. In many cases, HRPC growth is still dependent on AR and therefore, AR inhibition by alternative methods would be a rational strategy to treat HRPC. Our recent work indicated that simultaneous inhibition of multiple forms of AR via small interfering RNA (siRNA)-mediated knockdown or by treatment with nigericin, an antibiotic derived from *Streptomyces hygroscopicus*, resulted in efficient growth suppression of HRPC. These data indicated that the inhibition of AR expression at the mRNA level could be an effective strategy to block a wide range of AR mutants or variants that are expressed in HRPC. Our chemical screening additionally identified new inhibitors of the AR-mediated signaling, such as SU1498 that is an inhibitor of vascular endothelial growth factor receptor 2 and 5-azacytidine that inhibits DNA methyltransferase. Collectively, these results provide novel potential

strategies to suppress androgen receptor signaling in prostate cancer cells.

Keywords: Androgen receptor; Hormone refractory prostate cancer (HRPC); Splicing variant; Nigericin; SU1498; 5-azacytidine

Unusual AR activation as a therapeutic target of hormone-refractory prostate cancer (HRPC)

Prostate cancer growth is initially dependent on androgen and androgen receptor (AR)-mediated signaling. Therefore, chemical or surgical androgen blockade is effective in nearly 90% of patients with metastatic prostate cancer (1). However, after a couple of years' androgen ablation therapy, the tumor becomes HRPC. In HRPC, various alterations in AR have been reported that result in androgen-independent AR activation or resistance to antiandrogen-mediated AR inhibition (Figure 1). For example, in some HRPCs, mutations or amplification in the AR gene occur (2). The mutated ARs recognize nonspecific AR ligands (e.g. glucocorticoids or progesterone) as 'agonists' and are activated even in the absence of androgen (3). Androgen-independent activation of AR in HRPC is also caused by other signaling pathways such as tyrosine phosphorylation or interleukin-6 (IL-6)-janus kinase (JAK) /signal transducer and activator of transcription (STAT) 3 pathway (2, 4), while it was shown that increased AR expression is the most common mechanism of AR activation in HRPC (5). Recent reports further revealed that local androgen levels increase by up-regulation of intratumoral de novo steroidogenesis and are involved in progression of castration-resistant prostate cancer (6). These observations indicate that AR still plays a critical

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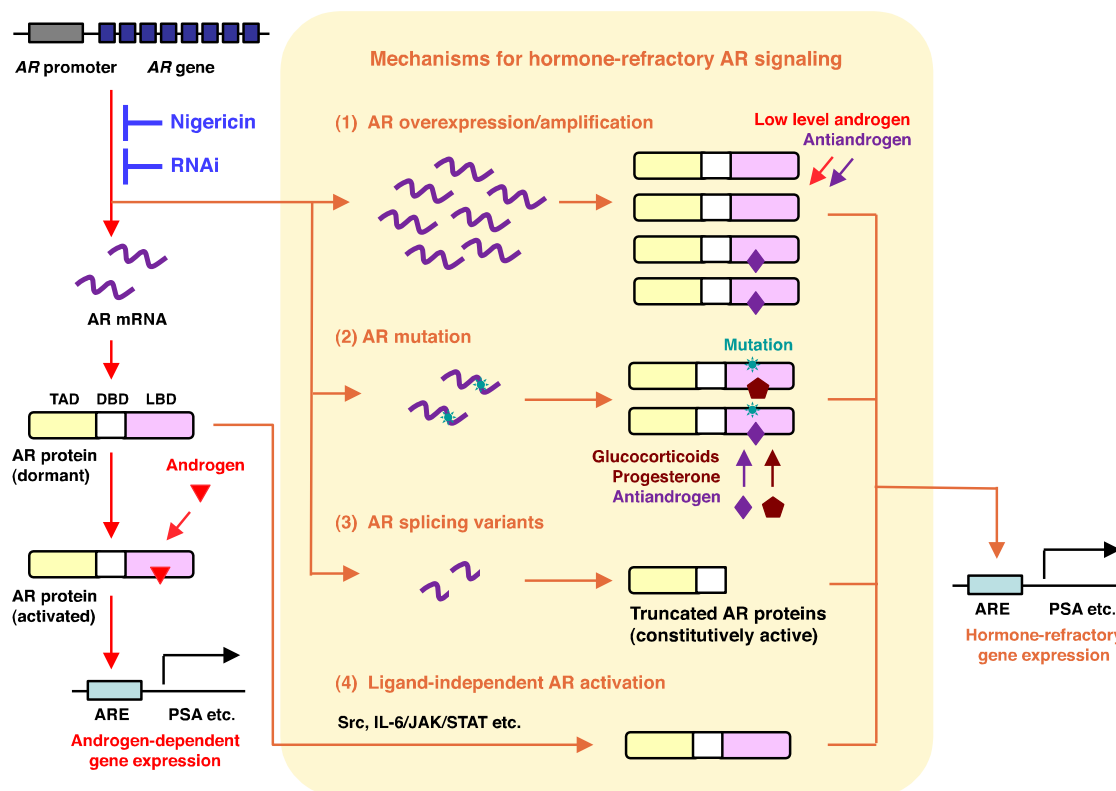


Figure 1. Mechanisms of androgen receptor (AR) activation in hormone-refractory prostate cancer (HRPC) and AR inhibition by nigericin or RNA interference (RNAi). In hormone-sensitive prostate cancer, androgen binds and activates AR. The activated AR binds to androgen responsive elements (AREs) on promoters of AR target genes [such as prostate specific antigen (PSA)] and promotes their expressions. By contrast, in HRPC, various changes in AR protein structure, expression level and regulation cause androgen-independent AR activation or resistance to antiandrogen-mediated AR inhibition. Nigericin- or RNAi-mediated inhibition of AR mRNA expression and inhibit androgen-independent HRPC growth (12). TAD, transactivation domain; DBD, DNA binding domain; LBD, ligand-binding domain.

role in the growth and survival of HRPC and is a rational molecular target. Consistently, a recently developed AR antagonist, MDV3100, which suppresses androgen from binding to the AR and inhibits AR nuclear translocation, seems to show a promising effect on HRPC (7, 8).

AR splicing variants as new mechanisms of HRPC

AR protein is composed of three domains: an amino-terminal transactivation domain (TAD), a DNA binding domain (DBD), and a carboxy-terminal ligand-binding domain (LBD) (9). Among the domains, LBD is not only important as a 'receptor' site for its specific ligand, androgen, but it also acts as the main binding site for antiandrogen agents. Recently, alternative splicing of AR was found to occur in HRPC that results in generation of truncated forms of AR (tARs) lacking the LBD (10, 11). The tARs are basically constitutively active, while they

are usually co-expressed with full-length AR in prostate cancer cells including clinical samples. We and others have demonstrated that, in 22Rv1 cells that express both the full length and the truncated ARs, inhibition of the full-length AR alone was not enough to suppress the cell proliferation, suggesting that the tAR-mediated signaling would be independent of the full length AR (10, 12). By contrast, some recent reports have shown that there is a greater diversity in the AR splicing variants and, at least in some forms of tAR, they promote castration resistance by increasing expression of the full-length AR or by acting through the full-length AR (13, 14). These observations indicate that the AR-mediated signaling could be more complex than has been expected particularly in the cells that express the tARs. Therefore, the antiandrogens, which bind and target the AR protein on the LBD, would not always be effective in suppressing the HRPC, and development of additional strategies could further be necessary to improve the treatment outcome.

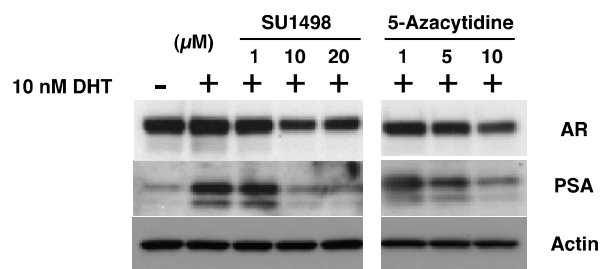


Figure 2. Suppression of androgen-dependent PSA induction by SU1498 or 5-azacytidine. Human prostate cancer LNCaP cells were treated with or without 10 nM dihydrotestosterone (DHT) for 24 h in the absence or the presence of indicated concentrations of the agents. The expressions of androgen receptor (AR), prostate specific antigen (PSA) and actin were analyzed by western blot analysis as described previously (12).

Inhibition of AR expression as an effective way to conquer a wide range of HRPC

Although various forms of AR are involved in HRPC growth and survival, we speculate that regulation mechanisms of the AR expression would not be so diverse in HRPC cells. From this viewpoint, agents that interfere with the AR expression would be effective in a broader spectrum of HRPC than classical types of agents that directly bind and suppress the AR protein or inhibit androgen production. We have tried to screen new class of AR-inhibitory agent, and, as a result, we identified the antibiotic nigericin, as a compound that inhibits the AR expression (12). Nigericin blocked AR-mediated prostate specific antigen (PSA) promoter activation and inhibited the androgen-dependent growth of prostate cancer LNCaP cells at relatively low concentrations (10-100 nM). These effects were caused by the suppression of AR expression at the mRNA level.

We further tested the effect of the compound on various forms of AR and found that nigericin inhibited the expression of both the full length and the constitutively active truncated ARs. Consistently, it efficiently suppressed androgen-independent growth and induced apoptosis in the HRPC 22Rv1 cells, which express both the full-length and the truncated ARs. Our results indicate that nigericin-like compounds that inhibit AR expression at the mRNA levels would be able to suppress the growth of HRPC that expresses wide variety of AR variants (Figure 1). There are two possible ways to suppress the AR expression at the mRNA level: inhibition of AR promoter/enhancer-dependent transcription and destabilization of the

AR mRNAs. We showed that nigericin destabilized the AR mRNA without affecting AR promoter/enhancer-dependent transcription (12). The AR mRNA possesses long 3'-untranslated region, which could regulate the mRNA stability (15). Therefore, nigericin may affect such regulation mechanisms of the mRNA stability. In fact, expression level of AR exogene that lacked the long 3'-untranslated region was not affected by nigericin (12). Several other compounds have also been reported that suppress the AR expression in prostate cancer cells (16-19). Although the inhibition mechanisms are still not completely clear, these types of agents would potentially be candidate lead compounds to develop a new class of drug that targets the AR expression in HRPC.

Potential new targets to suppress AR signaling in prostate cancer

By using our PSA reporter system (12) and the SCADS inhibitor kit, consisting of 190 well known anticancer drugs and signaling molecule inhibitors (20), we screened compounds that inhibit AR signaling. As a result, we identified several classes of compounds that inhibit AR signaling (Table 1). The screening result includes modifiers of the molecules that have previously been reported to play a role in the AR-mediated signaling, such as calcium signaling-related agents (21), heat shock protein 90 (Hsp90) inhibitor (22), proteasome inhibitors (23), and the inhibitor of JAK/STAT3 (24). These results indicate that our screening worked expectedly. In addition to these agents, we identified the following two compounds that we believe to be new AR inhibitors.

(1) SU1498

SU1498 is a selective inhibitor of vascular endothelial growth factor receptor 2 (VEGFR2, also known as Flk-1 and KDR) kinase activity. The IC_{50} in the Flk-1 kinase inhibition is 0.7 μ M, while the IC_{50} in epidermal growth factor receptor (EGFR) or HER-2 kinase inhibition is more than 100 μ M (25). We found that this compound suppressed androgen-induced PSA induction at 10 μ M or higher dosage (Figure 2). It is still not established whether the VEGF-VEGFR pathway would play any role in AR signal regulation (26), but, at least, our results suggest that the target of this compound could be involved in the AR regulation of prostate cancer cells.

Table 1. AR signaling inhibitors identified in this study. Human prostate cancer 22Rv1 cells were transfected with PSA-Luc + CMV-RLuc and were subsequently treated with agents in the presence of 1 nM DHT for 24 h. Luciferase reporter assay was performed as described previously (12). Relative promoter activities in the drug-treated samples compared with untreated controls were measured and calculated. The compounds that suppress DHT-dependent PSA promoter activation with an IC₅₀ value smaller than 20 μ M are listed in this table. +++, IC₅₀ smaller than 0.2 μ M; ++, IC₅₀ smaller than 2 μ M; +, IC₅₀ smaller than 20 μ M

Category	Target	Compound	Inhibition of androgen-dependent PSA promoter activation
Ionophore, ion channel	Na ionophore	Monensin	+++
	Na/K ATPase	Ouabain	+++
	V-ATPase	Bafilomycin A1	+++
Calcium signaling	Ca ionophore	A23187	++
	Ca ionophore	Ionomycin	+
	Ca-ATPase	Thapsigargin	+++
	CAMKII	KN93	+
Protein kinase/ phosphatase	PI3K	LY294002	+
	PKC, PKA, PKG, MLCK	Staurosporine	+++
	Chk 1	SB 218078	+
	JAK/STAT	Cucurbitacin I	++
	PP2A	Cantharidin	++
Anticancer drug	DNA	Doxorubicin, HCl	++
	DNA topoisomerase I	Camptothecin	+++
	Tubulin	Vinblastine sulfate	++
Cell cycle	Cdc25	NSC95397	+
Cytoskeleton	Actin filament	Cytocharasin D	++
Epigenetic regulation	DNA methyltransferase	5-Azacytidine	+
Growth factor receptor	VEGFR2/Fik-1	SU1498	+
Nuclear export chaperone	CRM1	Leptomycin B	++
Heat shock protein	HSP90	17-AAG	++
NO regulation	iNOS	1400W, HCl	++
Proteasome	Proteasome	MG-132	+++
	Proteasome	Lactacystin	+

(2) 5-Azacytidine

Epigenetic modifications play a key role in malignant progression of cancer, and agents that target the epigenetic changes have become a topic in cancer research (27). In prostate cancer, the agents or related drugs enhance the responsiveness of prostate cancer cells to antiandrogen or some antitumor agents in preclinical models (28,29), while it is still not sure whether these agents would modify the AR signaling pathways. 5-azacytidine is

an inhibitor of DNA methylation. We found that this compound inhibited the androgen-induced PSA induction of LNCaP cells (Figure 2). These data suggest that some AR regulators could epigenetically be modified in prostate cancer cells.

Chemical biological approach is a powerful way to uncover novel molecular targets of cancer. As described above, our screening suggested some molecular pathways as potential targets to inhibit the AR-dependent prostate cancer cell growth. Additional studies will be required to clarify the

mechanisms under which these compounds inhibit the AR signaling.

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Conflicts of Interest

No potential conflicts of interest to disclose.

References

- Chen Y, Sawyers CL, Scher HI. Targeting the androgen receptor pathway in prostate cancer. *Curr Opin Pharmacol* 2008;8:440-8.
- Grossmann ME, Huang H, Tindall DJ. Androgen receptor signaling in androgen-refractory prostate cancer. *J Natl Cancer Inst* 2001;93:1687-97.
- Zhao XY, Malloy PJ, Krishnan AV et al. Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. *Nat Med* 2000;6:703-6.
- Guo Z, Dai B, Jiang T et al. Regulation of androgen receptor activity by tyrosine phosphorylation. *Cancer Cell* 2006;10:309-19.
- Chen CD, Welsbie DS, Tran C et al. Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 2004;10:33-9.
- Montgomery RB, Mostaghel EA, Vessella R et al. Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. *Cancer Res* 2008;68:4447-54.
- Tran C, Ouk S, Clegg NJ et al. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* 2009;324:787-90.
- Scher HI, Beer TM, Higano CS et al. Antitumour activity of MDV3100 in castration-resistant prostate cancer: a phase 1-2 study. *Lancet* 2010;375:1437-46.
- Dehm SM, Tindall DJ. Androgen receptor structural and functional elements: role and regulation in prostate cancer. *Mol Endocrinol* 2007;21:2855-63.
- Dehm SM, Schmidt LJ, Heemers HV et al. Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. *Cancer Res* 2008;68:5469-77.
- Guo Z, Yang X, Sun F et al. A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. *Cancer Res* 2009;69:2305-13.
- Mashima T, Okabe S, Seimiya H. Pharmacological targeting of constitutively active truncated androgen receptor by nigericin and suppression of hormone-refractory prostate cancer cell growth. *Mol Pharmacol* 2010;78:846-54.
- Watson PA, Chen YF, Balbas MD et al. Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. *Proc Natl Acad Sci USA* 2010;107:16759-65.
- Sun S, Sprenger CC, Vessella RL et al. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. *J Clin Invest* 2010;120:2715-30.
- Faber PW, van Rooij HC, van der Korput HA et al. Characterization of the human androgen receptor transcription unit. *J Biol Chem* 1991;266:10743-9.
- Vander Griend DJ, Antony L, Dalrymple SL et al. Amino acid containing thapsigargin analogues deplete androgen receptor protein via synthesis inhibition and induce the death of prostate cancer cells. *Mol Cancer Ther* 2009;8:1340-9.
- Stan SD, Singh SV. Transcriptional repression and inhibition of nuclear translocation of androgen receptor by diallyl trisulfide in human prostate cancer cells. *Clin Cancer Res* 2009;15:4895-4903.
- Kim SH, Singh SV. D,L-Sulforaphane causes transcriptional repression of androgen receptor in human prostate cancer cells. *Mol Cancer Ther* 2009;8:1946-54.
- Chun JY, Nadiminty N, Lee SO et al. Mechanisms of selenium down-regulation of androgen receptor signaling in prostate cancer. *Mol Cancer Ther* 2006;5:913-8.
- Kawada M, Inoue H, Masuda T et al. Insulin-like growth factor I secreted from prostate stromal cells mediates tumor-stromal cell interactions of prostate cancer. *Cancer Res* 2006;66:4419-25.
- Gong Y, Blok LJ, Perry JE et al. Calcium regulation of androgen receptor expression in the human prostate cancer cell line LNCaP. *Endocrinology* 1995;136:2172-8.
- Fang Y, Fliss AE, Robins DM et al. Hsp90 regulates androgen receptor hormone binding affinity in vivo. *J Biol Chem* 1996;271:28697-702.
- Kang Z, Pirsanen A, Janne OA et al. Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex. *J Biol Chem* 2002;277:48366-71.
- Chen T, Wang LH, Farrar WL. Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCaP prostate cancer cells. *Cancer Res* 2000;60:2132-5.
- Boguslawski G, McGlynn PW, Harvey KA et al. SU1498, an inhibitor of vascular endothelial growth factor receptor 2, causes accumulation of phosphorylated ERK kinases and inhibits their activity in vivo and in vitro. *J Biol Chem* 2004;279:5716-24.

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26. Aragon-Ching JB, Dahut WL. VEGF inhibitors and prostate cancer therapy. *Curr Mol Pharmacol* 2009;2:161-8.
27. Cang S, Lu Q, Ma Y et al. Clinical advances in hypomethylating agents targeting epigenetic pathways. *Curr Cancer Drug Targets* 2010;10:539-45.
28. Festuccia C, Gravina GL, D'Alessandro AM et al. Azacitidine improves antitumor effects of docetaxel and cisplatin in aggressive prostate cancer models. *Endocr Relat Cancer* 2009;16:401-13.
29. Gravina GL, Marampon F, Di Staso M et al. 5-Azacitidine restores and amplifies the bicalutamide response on preclinical models of androgen receptor expressing or deficient prostate tumors. *Prostate* 2010;70:1166-78.