Cancer Letters 385 (2017) 108–116

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Article

Combination of sorafenib and enzalutamide as a potential new approach for the treatment of castration-resistant prostate cancer

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ARTICLE INFO

Article history:
Received 28 July 2016
Received in revised form
19 October 2016
Accepted 22 October 2016

Keywords:
Sorafenib
Enzalutamide
Castration-resistant prostate cancer
Androgen receptor
Tyrosine kinase

ABSTRACT

Enzalutamide, a novel androgen receptor (AR) antagonist, prolongs overall survival of patients with castration-resistant prostate cancer (CRPC); however, patients eventually progress with enzalutamide resistance. We studied the efficacy of sorafenib combined with enzalutamide in a CRPC model and explored a potential strategy to improve enzalutamide efficacy in vitro and in LNCaP xenografts. The results indicated that enzalutamide combined with sorafenib potently decreased cell proliferation and induced apoptosis in the prostate cancer cell lineLNCaP. In castrate-resistant LNCaP xenografts, the combination of enzalutamide with sorafenib significantly suppressed tumor growth compared with eachsingle agent. Western blots and immunohistochemical staining assay showed that the expression of AR was down-regulated, and the extracellular signal-regulated kinase (ERK) signaling pathway was inhibited after combination treatment, suggesting a synergistic inhibitory effect on the AR and ERK pathways. These results demonstrated that sorafenib therapy improved the efficacy of enzalutamide in the CRPC model, indicating a promising therapeutic strategy for clinical CRPC patients.

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Introduction

Prostate cancer (PCa) accounts for 27% of incident cases in men in America and is the second primary cause of male cancer-related mortality after lung cancer in Western countries [1]. Prostate cell survival relies mainly on the androgen receptor (AR) [2,3] during the development of normal prostate and prostate cancer. The primary treatment of localized prostate cancer is androgen deprivation therapy (ADT) or radiation therapy (RT) [4]. In a randomized study with an unselected cohort of 1205 patients with localized prostate cancer, the addition of RT to ADT improved overall survival [5]. Although these therapies initially lead to disease regression, advanced prostate cancer ultimately progresses to castration-resistant prostate cancer (CRPC) that is unresponsive to previous therapies [6]. The evolution of prostate cancer from an androgen-dependent state to CRPC marks its lethal progression [7]. Patients with metastatic CRPC were limited to docetaxel treatment for numerous years [8,9]. However, further therapeutics of metastatic CRPC include enzalutamideas well as additional sipuleucel-T, docetaxel, cabozantinib, and alpharadin [10,11].

AR also plays a critical role in prostate carcinogenesis and progression to CRPC [12–14]. Enzalutamide is a specific AR antagonistand blocks nuclear translocation and DNA binding to androgen response elements [15–17]. Given that enzalutamide also targets the AR ligand binding domain, point mutations in this domain can lead to resistance [18]. Enzalutamide resistance to CRPC represents a major clinical problem. These mechanisms may involve ligand-

Abbreviations: PCa, prostate cancer; CRPC, castration-resistant prostate cancer; ADT, androgen deprivation therapy; AR, androgen receptor; Enz, enzalutamide; Sor, sorafenib; DHT, dihydrotestosterone; ERK, extracellular signal-related kinase; HSP, heat-shock proteins; MEK, mitogen-activated protein/ERK kinase; PSA, prostate specific antigen; RAS, rat sarcoma oncogene; TUNEL, terminal deoxynucleotidyltransferase-mediate ddUTP nick end-labeling; WB, western blot; SDS-PAGE, sodium salt (SDS)-Polyacrylamide gel electrophoresis.

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http://dx.doi.org/10.1016/j.canlet.2016.10.036
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dependent AR hypersensitivity and ligand-independent AR activation pathways. Various in vitro studies have suggested that multiple growth factors, cytokines and kinase pathways, especially the tyrosine kinases, increase AR signaling, thereby promoting progression to CRPC in a ligand-independent manner [7]. The crosstalk between AR and key growth factors and kinases in prostate cancer is an active research area [19–21]. Gene expression induced by growth factors is dependent on the expression of AR and is inhibited by bicalutamide which is an AR antagonist [22,23].

Sorafenib, a tyrosine kinase inhibitor, has also been investigated in PhaseII clinical trials in progressive CRPC patients [24]. As sorafenib inhibits multiple molecular targets, the effects of sorafenib in different tumor types may be regulated by a variety of mechanisms. Sorafenib reduces ERK phosphorylation and sequentially inhibits ERK signalingin several cell lines across multiple tumor types [25]. In addition to inhibiting Raf serine/threonine kinases, sorafenib also targets platelet-derived growth factor receptor-β (PDGFR-β) tyrosine kinases and the proangiogenic vascular endothelial growth factor receptor (VEGFR) in vitro [26]. A phase II trial of sorafenib combined with bicalutamide in patients with chemotherapy-naïve CRPC regardless of the presence metastasesindicated that 47% of patients have either a PSA response or stable disease ≥6 months [27]. A proportion of CRPC patients cannot benefit from the trials, so further investigations of combined targeted therapies of cell signaling inhibitors with drugs targeting AR signaling are warranted.

Enzalutamide, a second generation of AR antagonist, has an 8-fold increased affinity for its target compared with bicalutamide. Enzalutamide prevents AR nuclear translocation, coactivator recruitment, and DNA binding, and induces cell apoptosis. This study was carried out to explore the efficacy of enzalutamide combined with sorafenib in CRPC models. Moreover, the molecular mechanisms of the combination effect of enzalutamide and sorafenib were explored.

Materials and methods

Drugs

Sorafenib was purchased from Bayer, Inc. in the form of powder. Enzalutamide was synthesized by Jiangsu Key Laboratory of Drug Discovery for Metabolic Disease, China Pharmaceutical University (Nanjing, China) according to the chemical structure [16]. Sorafenib and enzalutamide were dissolved in DMSO to produce 100 mM stock solutions and stored at −20 °C. The stock solutions were diluted with RPMI1640 medium to the desired concentrations for in vitro studies or chow containing sorafenib or sorafenib in combination with enzalutamide for in vivo studies.

Cell lines and animals

The prostate cancer cell line LNCaP, a gift from Shanghai Medical College Fudan University (Shanghai, China) in 2015, was maintained in RPMI 1640 (Invitrogen Gibco, USA) containing 10% (vol/vol) fetal bovine serum at 37 °C in 5% CO_2 in air. LNCaP cells were cultured less than 10 passages during this study and were routinely (last tested May 2016) monitored by STR to confirm the authenticity of the cells. Male Balb/c nude mice (6 week old; 14–16 g) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China).

MTT assay

LNCaP cells were seed in a 96-well plate with 10000 cells (100 μL cells suspension) in each well and cultured in an incubator for 24 h. Then, the cells were treated with various concentrations of sorafenib or enzalutamide alone or in combination for 72 h. The cell viability of was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue]. Finally, 5 mg/mL MTT was added to each well and incubated for 4 h at 37 °C in 5% CO_2. Absorbance at 570 nm was measured using a Thermo Scientific Multiskan MK3 Reader. The viability of the cells was normalized to vehicle control (0.1% DMSO). Synergy between sorafenib and enzalutamide was evaluated according to the cell viability by the Chou–Talalay combination index method [28] as described previously [29]. Compusyn Version 1.0 was used to calculate the combination index (CI) values using the non-constant Ratio Combination Design [30]. Drug combinations that yielded CI values <1 were considered to be synergistic [31,32].

Western blots

LNCaP cells (1×10^6 cells) were seeded every well in 6-well plates and incubated for 24 h to allow for adherence. Cells were treated with indicated concentrations for 24 h, and then whole cell lysates were obtained for Western blots. Briefly, cells lysates were prepared by washing the cells twice with PBS followed by lysis in radio immunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics, Germany) and 10 mM Na_3VO_4. For xenograft tissues, small pieces from snap-frozen tumor tissues were prepared and homogenized with glass homogenate in RIPA lysis buffer. A Thermo Scientific Pierce BCA protein Assay Kit was used to measure the protein concentration. Then, 30 μg of every sample was loaded on 8–12% sodium salt (SDS)-Polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, USA) for protein detection. After blocking with 5% nonfat milk, PVDF membranes were incubated with primary antibodies overnight at 4 °C. Bound antibodies were detected with horseradish peroxidase secondary antibodies (Santa Cruz Biotechnology, USA) and visualized by enhanced chemiluminescence reagent (Thermo Scientific, USA).

Mouse xenograft model

Castrate-resistant LNCaP xenografts were established as described previously [33]. Briefly, six million LNCaP cells (100 μL in 50% Matrigel (BD Biosciences) and 50% growth media) were injected subcutaneously in the flank of BALB/c nude mice. Once tumors reached a volume of 300–500 mm^3, the mice were castrated and randomly divided into 4 groups of 6 animals each. When the PSA rose to pre-castration levels, the mice were treated with sorafenib (30 mg/kg), enzalutamide (10 mg/kg), sorafenib (30 mg/kg) plus enzalutamide (10 mg/kg), or vehicle alone (0.5% sodium carboxymethyl cellulose) daily by oral gavage. The sorafenib dose was chosen based on previous studies of sorafenib in LNCap and PC3 xenografts models [25,34,35], and enzalutamide was used at a concentration used in the enzalutamide-resistant LNCaP/AR and castrate-resistant LNCaP models [33,36]. Tumor size was measured twice a week with calipers and tumor volumes were calculated with the formula volume = length × $width^2/2$. Animals were sacrificed when tumors formed skin abscesses and the tumor volume reached ≥10% of body weight in the treatment group. Animals were sacrificed after 3 weeks of drug treatment and the resected tumors were divided for 10% neutral-buffered formalin fixation (for immunohistochemistry) and snap-frozen tissue samples (for Western blot analysis). Experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of center for new drug evaluation and research, China Pharmaceutical University (Nanjing, China).

FITC-AnnexinV/PI and TUNEL staining

LNCaP cells (1×10^6 cells) were seeded every well in 6-well plates and incubated for 24 h. When cells were adherent, the medium was replaced with new medium contained drugs for 24 h, and groups of the drugs were as follows: control, 6.25 μM Enz, 12.5 μM Enz, 5 μM Sor, 5 μM Sor + 6.25 μM Enz, 5 μM Sor + 12.5 μM Enz. Then, every well was washed using PBS, and cells were fixed with 4% paraformaldehyde for 15 min. After washing cells with PBS 3 times, the mixture buffer containing Annexin V-FITC and propidium iodide was prepared. Each slide was covered with 30 μL of mixture buffer and incubated for 30 min at 37 °C away from light. The slides were observed with a fluorescence microscopewith Annexin V displaying green fluorescence and PI displaying red fluorescence. FITC-AnnexinV/PI double staining distinguishes early and late apoptotic cells.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was used to detect apoptosis in prostate cancer cells after drugs treatment. The paraffin-embedded sections of subcutaneous tumor tissues were baked at 60 °C for 30 min and were dewaxed with xylene at 50 °C for 30 min. Then, sections were rehydrated with gradient alcohol followed by permeabilization with proteinase K for 20 min at 37 °C. Then, sections were washed with PBST for 3 thrice and soaked in 3% H_2O_2 to block endogenous peroxidases. TUNEL reaction mixture buffer was prepared with terminal deoxynucleotidyl transferase (TdT) enzyme and fluorescein-labeled dUTP. Then, sections were incubated with the mixture buffer for 1 h at 37 °C. The converter-POD was added on the section and incubated for 30 min at 37 °C in the dark. Finally, sections were incubated with DAB for approximately 3 min, and the staining effect was observed with a fluorescence microscope.

Immunohistochemical staining

The paraffin-embedded sections were cut from xenograft tumor tissues to a thickness of 4 μm. After dewaxing and rehydration, the sections were soaked in sodium citrate buffer for heat-induced epitope retrieval. Next, the sections were incubated with 10% goat serum for 1 h to block the nonspecific binding sites. Then, sections were incubated with anti-Ki67 antibody (1:500, Abcam) and anti-androgen receptor antibody (1:50, Abcam) overnight at 4 °C, followed by incubation with horseradish peroxidase secondary antibodies for 1 h. The sections were developed using a Diaminobenzidine Substrate Kit (TIANGEN, China) and counterstained with hematoxylin. Positive stained cells were scored and counted

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