Therapeutic Discovery

Cotargeting Stress-Activated Hsp27 and Autophagy as a Combinatorial Strategy to Amplify Endoplasmic Reticular Stress in Prostate Cancer

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Abstract

Hsp27 is a stress-activated multifunctional chaperone that inhibits treatment-induced apoptosis and causes treatment resistance in prostate and other cancers. We previously showed that targeted suppression of Hsp27 sensitizes cancer cells to hormone and chemotherapy. However, mechanisms by which Hsp27 confers cell treatment resistance are incompletely defined. Here, we report that Hsp27 protects human prostate cancer cells against proteotoxic stress induced by proteasome inhibition, and that Hsp27 silencing using siRNA or antisense (OGX-427) induced both apoptosis and autophagy through mechanisms involving reduced proteasome activity and induction of endoplasmic reticulum (ER) stress. We found that autophagy activation protected against ER stress-induced cell death, whereas inhibition of autophagy activation following Hsp27 silencing using either pharmacologic inhibitors or atg3 silencing enhanced cell death. Importantly, cotargeting Hsp27 and autophagy by combining OGX-427 with the autophagy inhibitor, chloroquine, significantly delayed PC-3 prostate tumor growth in vivo. These findings identify autophagy as a cytoprotective, stress-induced adaptive pathway, activated following disruption of protein homeostasis and ER stress induced by Hsp27 silencing. Combinatorial cotargeting cytoprotective Hsp27 and autophagy illustrates potential benefits of blocking activation of adaptive pathways to improve treatment outcomes in cancer. Mol Cancer Ther; 11(8); 1661–71. ©2012 AACR.

Introduction

Many strategies used to kill cancer cells induce stress responses that promote the emergence of a treatment-resistant phenotype. In prostate cancer, androgen ablation induces remission in most patients but also progression to castration-resistant prostate cancer (CRPC; ref. 1). Although docetaxel chemotherapy (2) and, more recently, AR pathway inhibitors such as abiraterone (3) and MDV-3100 prolong survival by several months, treatment resistance frequently emerges, highlighting the need for additional therapies targeting the molecular basis of CRPC and treatment resistance.

Development of CRPC is attributed to reactivation of the androgen receptor (AR) axis (4), alternative growth factor pathways (5), stress-induced survival genes (6), and cytoprotective chaperone networks (7). Molecular chaperones such as Hsps help cells cope with stress-induced misfolded proteins and play prominent roles in cellular signaling and transcriptional regulatory networks. In particular, Hsp27 is a stress-activated chaperone highly expressed in CRPC and other cancers that inhibits treatment-induced apoptosis (8–10). Hsp27 is induced by anti-AR and chemotherapy, inhibiting apoptosis by regulating components of both stress- and receptor-induced apoptotic pathways (11–14). We previously reported that Hsp27 silencing induces apoptosis and enhances anticancer drug sensitivity in prostate and bladder cancer cells (7, 15, 16). The Hsp27 inhibitor, OGX-427, delays progression and enhances activity of chemotherapy in CRPC and other cancers (7, 15, 16), and is currently in multicenter phase II studies of CRPC and metastatic bladder cancer (ClinicalTrials.gov identifier, NCT01454089 and NCT01120470).

Hsps are particularly important in regulating misfolded protein and endoplasmic reticulum (ER) stress responses, an emerging area of interest in cancer progression and treatment resistance (17). In cancer, ER stress and misfolded protein levels are elevated because of mutated genes and stressed microenvironments (18); moreover, many anticancer agents induce ER stress (19). ER stress activates a complex intracellular signaling pathway, called the unfolded protein response (UPR),...
tailored to reestablish protein homeostasis (proteostasis) by inhibiting protein translation and promoting ER-associated protein degradation (ERAD) by stimulating the ubiquitin–proteasome system (UPS) and autophagy to reduce levels of misfolded proteins (20). Autophagy is an evolutionarily conserved bulk degradation system that facilitates clearance of stress-induced misfolded or aggregated proteins, as well as organelles (21). Classically, autophagy is activated in starvation or oxidative stress and generally considered an adaptive survival mechanism (22); however, when ER stress and unfolded protein burden overwhelsms the degradation capacity of the proteasome or autophagy, then cell death can occur.

Although many studies link UPS inhibition and ER stress to autophagy induction (23–26), whether treatment-induced autophagy is cytoprotective to facilitate development of acquired treatment resistance, or alternatively mediates treatment-induced cell death, remain controversial. Because Hsp27 has been identified as a cytoprotective chaperone linked to treatment resistance and cell survival (7, 11, 27, 28) as well as ER stress and UPS activity (12, 29), we set out to explore its role in treatment-induced ER stress and protein homeostasis in prostate cancer.

Materials and Methods

Prostate cancer cell lines and reagents

LNCaP and C4-2 cells were kindly provided by Dr. Leland W. K. Chung (Cedars Sinai, Los Angeles, CA) tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer Ix platform in July 2009. PC-3 cells were purchased from the American Type Culture Collection (2008, ATCC authentication by isoenzymes analysis). LNCaP and C4-2 cells were maintained in RPMI-1640 media (Invitrogen Life Technologies). PC-3 cells were maintained in RPMI-1640 media (Invitrogen Life Technologies) containing 5% heat-inactivated FBS (Invitrogen Life Technologies). LNCaP and C4-2 cells were kindly provided by Dr. Leland W. K. Chung (Cedars Sinai, Los Angeles, CA) tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer Ix platform in July 2009. PC-3 cells were purchased from the American Type Culture Collection (2008, ATCC authentication by isoenzymes analysis). LNCaP and C4-2 cells were maintained in RPMI-1640 media (Invitrogen Life Technologies) containing 5% heat-inactivated FBS (Invitrogen Life Technologies). PC-3 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen Life Technologies) containing 5% FBS (Invitrogen Life Technologies). A scrambled (ScrB) siRNA for HSP27 (5'-CCAGUGAAUAAUGCUUACGUG3'; Dharmacon) and a control (ScrC) siRNA for Atg3 (5'-CCUGGAAUAAUGCUUACGUG3'; Dharmacon) were used as a control for RNA interference experiments.

Western blotting analysis

Total proteins were extracted using radioimmunoprecipitation assay buffer (50 mmol/L Tris, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 100 mmol/L NaCl, Roche complete protease inhibitor cocktail) and submitted to Western blot as we described previously (27).

Proteasome activity

Peptidase activity of the proteasome was measured by mixing tissue homogenate with 20 μmol/L fluorogenic peptide Suc-LLVT-AMC (succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; Calbiochem) as we previously described (30).

Analysis of Xbp-1 splicing

Total RNA was isolated using TRIzol reagent according to the manufacturer instructions (Life, Technology). cDNA was synthesized from 2 μg of RNA using the Superscript II First-Strand Synthesis Kit (Invitrogen) and amplified with a pair of primers corresponding to nucleotides 285–308 (forward; 5'- AACAGAGTACGCTCAGCTG-3') and -735–758 (reverse; 5'-TCTTCTGGTAGACCTCTGGGAG-3') of XBP-1 cDNA. β-Actin (forward: GAACCTTGGACCAAGATG; reverse: AGCTCTGTTGGCTGACA) was used as endogenous control. PCR products were analyzed on 2.5% agarose gel.

In vitro cell growth assay

Cells were plated in 12-well plate, allowed to attach for 24 hours, and treated with indicated concentrations of siRNA for 1 day or ASO for 2 days. Cell growth was then assessed using crystal violet assay as described previously (31). Absorbance was determined with a microculture plate reader (Becton Dickinson Labware) at 560 nm, and the percentage of cell growth was calculated relative to vehicle-treated cells. Each assay was carried out in triplicate.

Cell-cycle analysis

Cell-cycle populations were analyzed by propidium iodide–staining using a FACSCAN flow cytometer (Becton-Dickinson & Co.) as previously described (28).
Immunofluorescence staining
Cells were fixed with methanol containing 3% acetone and immunofluorescence was carried out as previously described (27, 28, 30) using anti-LC3 antibody (1:250; Cell Signaling Technology). Puncta from 100 to 150 cells were counted from 3 independent experiments for quantitative analysis as described previously (32). Cells displaying more than 15 brightly fluorescent LC3 puncta were counted as positive. Photomicrographs were taken at 40× magnification using Zeiss Axioplan II fluorescence microscope.

Immunohistochemistry
Immunohistochemistry was carried out on formalin-fixed, paraffin-embedded 4-μm sections of tumor samples. Immunohistochemical staining was conducted using Hsp27 for target expression, Ki-67 for cell proliferation, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) for apoptosis antibody in the Ventana autostainer Discover XT (Ventana Medical System) with enzyme-labeled biotin-streptavidin system- and solvent-resistant 3,3′-diaminobenzidine Map kit. All comparisons of staining intensities were made at ×200 magnifications.

Assessment of in vivo tumor growth
For in vivo xenograft studies, PC-3 cells were inoculated subcutaneously in the flank of 6- to 8-week-old male athymic nude mice (Harlan Sprague Dawley, Inc.) via a 27-gauge needle under isoflurane anesthesia. Each experimental group consisted of 8 mice. Tumor volume measurements were carried out once weekly and calculated by the formula length × width × depth × 0.5236. Data points were expressed as average tumor volume ± SEM. All animal procedures were carried out according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

Statistical analysis
Differences between the 2 groups were compared using Student t test and Mann–Whitney U test. All statistical calculations were carried out using Statview 5.0 software (Abacus Concepts, Inc.), and P values less than 0.05 were considered significant.

Results
Hsp27 attenuates MG132-induced ER stress and apoptosis
Although Hsp27 has been reported to confer resistance to proteasome inhibition in lymphoma cells (33), the underlying mechanisms remain undefined. Here, we showed that the proteasome inhibitor MG132 induces upregulation of Hsp27 and pHsp27 in the PC-3 cells (Fig. 1A). PC-3 cells stably overexpressing Hsp27 showed similar cell growth and apoptotic rates compared with parental or empty vector under basal, unstressed conditions (Supplementary Fig. S1); moreover PC-3 and LNCaP cells overexpressing Hsp27 acquire resistance to MG132-induced apoptosis as shown by decreased sub-G0 population (Fig. 1B, Supplementary Fig. S2A), increased cleaved-PARP and cleaved-caspase3 expression (Fig. 1C) and decreased cell growth rates (Fig. 1D and Supplementary Fig. S2B). Because MG132 is known to induce ER stress by inhibiting the UPS (34), we next investigated role of Hsp27 on MG132-induced ER stress response and activation of the UPR. PC-3Hsp27 and PC-3Empty were treated with indicated concentrations of MG132 and expression of UPR-related proteins analyzed. Hsp27 overexpression ameliorated MG132-induced ER stress and UPR activation of GRP78, ATF4, CHOP, and cleaved-ATF6 compared with empty vector (Fig. 1C). Interestingly this effect correlated with increased proteasome activity and reduced accumulation of ubiquitinated proteins in PC-3Hsp27 compared with PC-3Empty or PC-3parental cells, consistent with prior reports (refs. 12, 29; Fig. 1E). We also observed that the Hsp90 inhibitor, 17-AAG, also induced ER stress and upregulation of Hsp27 in LNCaP cells (Supplementary Fig. S2C) and that Hsp27 overexpression also conferred resistance to Hsp90 inhibitor–induced apoptosis (Supplementary Fig. S2D). These data suggested that Hsp27 functions to help maintain protein homeostasis under ER stress condition by increasing UPS activity and enhancing clearance of ubiquitinated proteins.

Hsp27 inhibition reduces UPS activity and induces ER stress
To further define the role of Hsp27 in ER stress and UPR activation, levels of UPR-related proteins were analyzed by Western blot after Hsp27 knockdown using siRNA or the antisense drug, OGX-427. We found that Hsp27 knockdown in PC-3 cells using siRNA (Fig. 2A, left panel) or OGX-427 (Fig. 2A, right panel) leads to upregulation of GRP78, phospho-eIF2α, ATF4, CHOP, and cleaved-ATF6 protein levels; similar effects were seen in LNCaP cells (Supplementary Fig. S3). In addition, spliced-XBP1 was detected (Fig. 2B). Spliced-XBP1 is a transcription factor that binds to UPR-responsive promoter elements in a subset of genes to stimulate expression of chaperones as a mechanism to cope with the unfolded protein load (35). Moreover, we observed that siRNA-Hsp27 (Fig. 2C, left panel) or OGX-427 (Fig. 2C, right panel) decreased proteasome activity and led to accumulation of ubiquitinated proteins. These results indicated that Hsp27 knockdown decreases proteasome activity, induces ER stress, and activates the UPR.
Hsp27 knockdown induces autophagy in PC-3 cells

Recent reports have defined mechanistic links between the UPS and autophagy–lysosome systems in proteostasis (36, 37). Because inhibition of the proteasome can activate autophagy (23), we explored whether Hsp27 inhibition induces autophagy using a variety of assays. LC3-II expression, which is a microtubule-associated protein light chain 3 and a marker of autophagy, was induced in a time-dependent manner after Hsp27 knockdown using siRNA (Fig. 3A, left panel), or OGX-427 (Fig. 3A, right panel). Interestingly, we observed that induction of autophagy correlates strongly with the extent of Hsp27 knockdown, which is achieved more potently with siRNA compared with OGX-427. Next, autophagic flux was analyzed using LC3 turnover assay (38). Because bafilomycin (Fig. 3B, upper panel) blocks the fusion of autophagosome and lysosome, LC3-II degradation in autolysosomes is also blocked and results in LC3-II accumulation. Hsp27 knockdown using siRNA (Fig. 3B, lower left panel) or OGX-427 (Fig. 3B, lower right panel) increased autophagic flux with higher LC3-II levels after bafilomycin treatment. To further evaluate changes in autophagic flux after Hsp27 knockdown, we quantified green fluorescence puncta of endogenous LC3 in PC-3 cells. As expected, Hsp27 knockdown significantly increased LC3 puncta compared with controls (Fig. 3C). Collectively, these data indicated that Hsp27 knockdown...
inhibits proteasome activity and results in increased autophagic flux in PC-3 cells.

**Cotargeting Hsp27 and autophagy enhances prostate cancer cell death**

Recent studies indicated that autophagy activation in cancer cells is context dependent and can be either cytoprotective or a mediator of apoptosis (23–24, 39, 40). Because Hsp27 knockdown induces ER stress and autophagy, we next examined effects of inhibition of autophagy on apoptotic rates after OGX-427 treatment in prostate cancer cells. First, we confirmed that OGX-427 induces autophagy in other prostate cancer cell lines by measuring autophagic flux in LNCaP and C4-2 prostate cancer cell lines (Fig. 4A). Next, we evaluated the effect of autophagy inhibition using 3-MA or chloroquine after OGX-427-induced ER stress in these prostate cancer cell lines. The 3-MA inhibits the activity of class III PI3 kinases required for autophagosome formation, whereas chloroquine is an antimalaria drug that inhibits autophagosome–lysosome fusion and lysosomal acidification, resulting in inhibition of autophagy and LC3-II degradation. As shown in Fig. 4B, either chloroquine or 3-MA significantly enhanced the cell-growth inhibitory effects of OGX-427 in prostate cancer cell lines. Also, combined OGX-427 plus chloroquine, compared with chloroquine or OGX-427 monotherapy, significantly increased apoptotic rates as measured by cleaved PARP (Fig. 4C, left panel and Supplementary Fig. S4) and flow cytometric analysis (Fig. 4C, right panel) in PC-3 cells. Similar effects on apoptosis and cell growth were observed in PC3 cells after Hsp27 knockdown using siHsp27 (Supplementary Fig. S5). To further
define the specific role of autophagy in Hsp27 inhibition-induced ER stress and apoptosis, we used siRNA to silence Atg3, a key autophagy gene essential for autophagosome formation (41). Consistent with the chloroquine and 3-MA results, combined siRNA Atg3 plus OGX-427, compared with siRNA Atg3 or OGX-427 mono-therapy, increased cleaved PARP (Fig. 4D, left panel) and sub-G0/G1 population (Fig. 4D, right panel). These results indicated that inhibition of stress-induced activation of autophagy increases Hsp27 knockdown-induced apoptosis and suggested that combining OGX-427 with autophagy inhibitors may enhance antitumor effects in prostate cancer.

Combined OGX-427 plus chloroquine treatment delays PC-3 xenograft growth in vivo

PC-3 tumor-bearing mice were randomly assigned to groups treated with ScrB + PBS, ScrB + CQ, OGX-427 + PBS, or OGX-427 + CQ when PC-3 tumors reached 100 mm³. Mean tumor volume at baseline was similar in all groups. All treatments were carried out for 7 weeks. Combination therapy of OGX-427 plus chloroquine significantly reduced the rate of PC-3 tumor growth (Fig. 5A) compared with all other groups (P = 0.0065, 0.017, and 0.038 against ScrB + PBS, ScrB + CQ and OGX-427 + PBS, respectively). Combination treatment of OGX-427 plus chloroquine also suppressed Ki-67 expression compared with other groups (Fig. 5B), although heterogeneous immunostaining of Ki-67 suppression was apparent among individual mice (data not shown). Furthermore, OGX-427 plus chloroquine–treated tumors had higher apoptotic rates as shown by increased TUNEL staining compared with other groups. These studies indicated that cotargeting Hsp27 and adaptive activation of autophagy significantly delays growth and increases apoptotic rates of PC-3 xenografts.
Discussion

Survival proteins and signaling pathways upregulated following anticancer treatment that function to inhibit cell death are of special interest in acquired treatment resistance. In particular, molecular chaperones play an important role in many stress-activated cell signaling and transcriptional regulatory networks (17, 42). In addition, they play key roles in protein homeostasis by reducing accumulation of misfolded proteins induced by different stresses, such as heat, irradiation, oxidative stress, or anticancer therapy. Hsp27, for instance, is a stress-activated molecular chaperone commonly detected in many cancers (8–10), in which it confers thermostolerance and cytoprotection by regulating steroid hormone response (43), Akt signaling (44), and NF-κB or stat3 transactivation (12, 13). Hsp27 also directly inhibits components of both stress- and receptor-induced apoptotic pathways (28).

In addition, Hsp27 reduces burden of misfolded or ubiquitinated proteins by stabilizing client protein complexes (45) and enhancing proteasome activity (29). For example, Hsp27 is associated with activated UPS and rapid degradation of UPS substrates IκBα and p27kip1 by the 26S proteasome (12, 46). Stress-induced
increases in Hsp27 after hormone- or chemotherapy inhibit treatment-induced cell death, render cells more resistant to therapy, and accelerate progression (7, 12, 13, 27–29).

As an important regulator of cell survival and treatment stress, Hsp27 is now recognized as therapeutic target in cancer. In this regard, selective inhibition of Hsp27 expression using (47, 48) or antisense oligonucleotide (7, 15, 16) siRNA-based therapy has been shown to suppress tumor growth and sensitize cancer cells to chemo- and radiotherapy. On the basis of these preclinical studies, OGX-427, a second-generation antisense inhibitor of Hsp27, has recently advanced into multicenter phase II studies of CRPC and metastatic bladder (ClinicalTrials.gov identifier, NCT01454089 and NCT01120470). Defining molecular mechanisms by which Hsp27 regulates cancer cell survival will provide insights into context-dependent Hsp27 action and better guide rational combination strategies that cotarget adaptive responses mediating treatment resistance.

This study set out to define the effects of Hsp27 inhibition on ER stress and proteostasis and to evaluate biologic significance of autophagy activation under conditions of Hsp27 inhibition. We confirm that Hsp27 reduces proteasome inhibitor–induced ER stress and accumulation of misfolded/ubiquitinated protein levels,
Through mechanisms involving increased UPS activity and/or stabilization client–protein complexes (29, 45). On the other hand, Hsp27 inhibition suppresses UPS activity and leads to increased levels of ubiquitinated protein with induction of ER stress and the UPR. Interestingly, Hsp27 knockdown affects the 3 transmembrane ER stress sensors, namely PERK (increase of p-eIF2-a, ATF-4), IRE1 (cleaved XBP1), and ATP-6 (cleaved ATF-6). These data link treatment-induced increases in Hsp27 levels to enhanced ERAD and maintenance of proteostasis. We also show, for the first time, that Hsp27 knockdown induces autophagy, and that combined inhibition of Hsp27 and autophagy further disrupts proteostasis with increased apoptosis in prostate cancer cells. These data suggest that improved anticancer effects can be achieved when ER stress and unfolded protein burden overwhelms the degradation capacity of the UPS or autophagy.

Although UPS and autophagy had been considered distinct and separate systems for protein clearance, recent studies suggest that they are linked especially under conditions of ER stress (23, 26, 49). Both the UPS and autophagy are activated in response to ER stress to facilitate degradation of misfolded proteins. To reduce levels of misfolded or ubiquitinated proteins during ER stress, the UPR inhibits protein translation and promotes ERAD by stimulating the UPS. When the proteasome is inhibited or when ER stress and unfolded protein burden overwhelms the degradation capacity of the UPS and ERAD, compensatory activation of autophagy can assist with misfolded protein clearance. This ER-activated autophagy (ERAA) is mediated by both UPR and UPR-independent mechanisms (25, 26).

In this study, we show that induction of Hsp27 after proteasome inhibition functions to facilitate UPS activity and clearance of ubiquitinated proteins under conditions of ER stress; moreover, Hsp27 knockdown can suppress UPS activity and induce ER stress, apoptosis, and ERAA, illustrating a key role for Hsp27 in ER stress–induced ERAD and highlighting activation of autophagy as a compensatory cytoprotective response to maintain proteostasis.

Under many cell stress conditions, including inhibition of UPS and nutrient deprivation, autophagy is rapidly upregulated for proteostasis or alternative energy source to promote cell survival (20, 50). However, in contrast, excessive or unquenched autophagy can lead to type II programmed cell death, which is morphologically distinct from apoptosis and usually caspase independent (51). Hsp27 and autophagy are activated by UPS inhibition and ER stress as adaptive responses to maintain proteostasis and block stress–induced cell death. It follows that cotargeting both Hsp27 and autophagy may increase cell death; however, it is important to recognize that the effect of manipulated autophagy can vary with intrinsic properties of the tumor and with the nature of combined therapy. Previous studies reported on combined targeting of proteasome and autophagy inhibitors in cancer (23, 24); for example, in vitro studies in prostate cancer cells by Zhu and colleagues (23) reported that combined inhibition of autophagy and the proteasome led to accumulation of toxic intracellular protein aggregates and apoptosis. Although this article shows that cotargeting proteostasis pathways can enhance anticancer activity, it is necessary to evaluate effects of autophagy activation or inhibition in response to specific treatments and under different contexts. This study identified autophagy as an adaptive cytoprotective response to Hsp27 silencing–induced UPS inhibition and tested whether cotargeting autophagy with Hsp27 inhibition amplified ER stress and unfolded protein burden in cancer cells to synergistically enhance treatment-induced apoptosis both in vitro and in vivo. We show that Hsp27 inhibition attenuates proteasome activity and induces the UPR similar to that seen with the classical proteasome inhibitor, MG132, and activated autophagy as a prosurvival mechanism. Cotargeted inhibition of both Hsp27 and autophagy in prostate cancer significantly enhanced antitumor effects in vitro and in vivo.

In summary, these results suggest that Hsp27 enhances the degradative capacity of ubiquitinated proteins by the UPS following ER stress. Furthermore, Hsp27 knockdown activated autophagic flux as a cytoprotective response, and that both inhibition of Hsp27 and autophagy enhanced cell death in prostate cancer cells in vitro and in vivo (Fig. 6). Cotargeting cytoprotective Hsp27 and autophagy represents a novel anticancer strategy to disrupt proteostasis and illustrates potential benefits of blocking activation of adaptive pathways to improve treatment outcomes in cancer.
Disclosure of Potential Conflicts of Interest

M.E. Gleave has ownership interest (including patents) in OGX-427 and is also a consultant and advisory board member of OncoGenex. The University of British Columbia has submitted patent applications on OGX-427, listing M.E. Gleave as inventor. This IP has been licensed to OncoGenex Technologies, a Vancouver-based biotechnology company that M.E. Gleave has founding shares in.

Authors’ Contributions

Conception and design: M. Kumano, J. Furukawa, A. Zoubeidi, M.E. Gleave

Development of methodology: M. Kumano, J. Furukawa, A. Zardan, F. Zhang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Furukawa, M. Shiota, R.M. Wiedmann, M.E. Gleave

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Furukawa, M. Shiota, A. Zardan, F. Zhang, L. Fazli, M.E. Gleave

Writing, review, and/or revision of the manuscript: M. Kumano, F. Zhang, L. Fazli, A. Zoubeidi, M.E. Gleave

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Furukawa, F. Zhang, E. Beraldi, L. Fazli

Study supervision: J. Furukawa

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References


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