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TITLE: Overcoming Autophagy to Induce Apoptosis in Castration-Resistant Prostate Cancer

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Survival mechanisms elicited by CRPC C4-2B cells when treated with Enza may be blocked by inhibiting autophagy with clomipramine and metformin. Combination of Enza with saracatinib and autophagy modulators significantly reduced cell proliferation in the LNCaP GRP CRPC model. Further in vivo studies will provide evidences to make autophagy targeting as a supplement to cancer therapeutic regimes.
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Introduction:

For our DOD award W81XWH-12-1-0529, we proposed to overcome autophagy as cell survival mechanism when treated with small molecule kinase inhibitor saracatinib or the arginine metabolism modulator ADI in prostate cancer therapy. We started our study with saracatinib but had to switch to enzalutamide due to the halted effort by the pharmaceutical companies for the Src kinase inhibitors (AstraZeneca for saracatinib and BMS for dasatinib). Enzalutamide (Enza) is the new generation anti-androgen that has been approved by FDA for the advanced prostate cancer treatment. We have identified that enzalutamide elicits autophagy in castration resistant prostate cancer cell lines LNCaP C4-2B and LNCaP GRP cells. This Enza-mediated autophagy is through activation of AMPK and repression of mTOR. Knocking down AMPK with siRNA reversed the survival mechanism and led cells to undergo apoptosis. Survival mechanisms elicited by CRPC C4-2B cells when treated with Enza may be blocked by inhibiting autophagy with clomipramine and metformin. Combination of Enza with saracatinib and autophagy modulators significantly reduced cell proliferation in the LNCaP GRP CRPC model.

Body:

I. To credential inhibition of autophagy via Src and androgen receptor signaling pathways as a new therapeutic strategy in vitro and short term in vivo xenograft models.

Autophagy modulators: It was reported that when cells treated with the Src inhibitor saracatinib, they would be growth inhibition mainly through growth arrest but not apoptosis due to the autophagy survival pathway [1]. Apoptosis was promoted when the autophagy inhibitor chloroquine (CQ) was added. We then tested more autophagy modulators such as clomipramine (CMI), metformin (Metf) and rapamycin (Rapa) on PC-3 cells. CQ did not show growth inhibition alone, whereas CMI, Metf and Rapa did. When combined with saracatinib, CMI and Rapa showed additive inhibition (figure 1). With a still broader experiment, PC-3, CWR22Rv1 and LNCaP GRP-Pro cells were treated with arginine deaminase inhibitor (ADI), docetaxol, CMI, Metf or Rapa alone or in combinations with saracatinib (2 or 5 μM). Each treatment alone displayed some inhibitory effect on cell proliferation and the degree varied from cell line to cell line. When combined with saracatinib, further cell killing was observed. Because of the lower toxicity and side effect of CMI (as an anti-depression drug) and Metf (as the diabetic drug) than CQ or Rapa, we decided to focus on the former two autophagy modulators in our studies.

LNCaP C42B-MDV resistant cells, second CRPC model
We used LNCaP and CWR22Rv1 cells stably transfected with LC3-eGFP construct to investigate autophagy as the potential cell survival mechanism with anti-androgen treatments. Both cell lines were treated with 10μM of Enzalutamide, a new generation of antiandrogen drug through MTA with Medivation. LC3-I in cells is localized in the cytosol; but upon induction of autophagy, it is lipitated into LC3-II and inserted into autophagosomes membrane and can readily be detected and visualized by the prominent change from diffuse cytoplasmic to bright, punctate fluorescence in the cytosol as shown in Figure 2A. Additional evidence of Enzalutamide-induced autophagy in LNCaP and CWR22r1 cells came from Western blot analysis as demonstrated by the significant increase in the LC3-I to LC3-II conversion (LC3-II/I ratio increased from 0.71 to 1.36) and the increased expression of ATG 5, both have been used as reliable marker of autophagy (Figure 2B) [2]. Flow cytometry was also used to measure and quantify increase of autophagosome formation upon Enzalutamide treatment as shown in Figure 2C. Because the AMPK activation has been implicated under androgen deprivation condition, we asked whether antiandrogen may activate this pathway, thereby inducing

Figure 2. A. Representative fluorescence microscopy of CWR-22Rv1-eGFP-LC3 (upper panel) and LNCaP-eGFP-LC3 (lower panel) cells treated with DMSO (vehicle control), 10 μM Enzalutamide for 48 h viewed by fluorescence microscopy, showing GFP-LC3 localization and puncta autophagosome formation represented by arrow. B. C4-2B and LNCaP cells were treated with 5 μM Enzalutamide, and cell lysates were harvested and subjected to Western blotting analysis using autophagy markers, LC3-I and LC3-II and ATG-5. PSA was used as internal control. β-actin was used as the loading control. C. Upon induction of autophagy, autophagosomes formed in eGFP-LC3 transfected cells may be gated and numbered to report the degree of autophagy using flow cytometry.

Figure 3. A. AMPK is activated with androgen receptor blockage. LNCaP and C4-2B cell lines were treated with DMSO (vehicle control), 5 μM CMI, 10 μM Enza, combination of CMI and Enzalutamide, 10μM bicalutamide (Bic), combination of Bic and autophagy inhibitor CMI for 48 h. Cell lysates were harvested and subjected to Western blotting analysis using autophagy markers, LC3-I and LC3-II and antibodies to phospho AMPK and total AMPK. B. Androgen deprivation and continuous androgen blockage by Enzalutamide induces autophagy and activates AMPK phosphorylation. Left panel showed representative Western analysis of C4-2B cells cultured under regular FBS (+Androgen) and charcoal stripped FBS (-Androgen). Right panel showed similar analysis using Enzalutamide resistance cells (C4-2B +R) and their counterpart parental line (C4-2B -WT).
autophagy.[3]. To determine the predominate mechanism involved in androgen receptor signaling inhibitor (ARSI) mediated autophagy, we subjected C4-2B and LNCaP cells to both bicalutamide and Enzalutamide treatment and analyzed for phosphorylated AMPK and AKT. As shown in Figure 3A, activation of AMPK significantly increased in cells treated with ARSI, while the level of phosphorylated AKT is minimally affected (data not shown). We next evaluated AMPK phosphorylation in cells that conferred resistant to ARSI; namely C4-2B and C4-2B+R (C4-2B cells selected after prolonged enzalutamide exposure) cells. Under androgen deprivation (Figure 3B left panel) or prolonged androgen receptor blockage with Enzalutamide (right panel), the induction of autophagy was coupled with the activation AMPK, again suggesting that AMPK plays a crucial role in the induction of autophagy. To prove the principle that activation of the AMPK pathway is responsible to the induction of autophagy mediated by ARSI, we used interference RNA to knock down the expression of AMPK in C4-2B cells and subsequently treated them with Enzalutamide. Autophagy was not observed in cells with diminished level of AMPK expression as evidenced by the lack of green fluorescence punctate, but can readily be detected and visualized by the prominent, bright punctate fluorescence in the cytosol of cells transfected with the scrambled control (Figure 4A). Previous studies demonstrated that the AMPK pathway directly interacts with TSC2/Raptor/mTOR complex to inhibit mTOR/S6K/4EBP signaling and the subsequent activation of autophagy [4-6].

Cells with knock down expression of AMPK were treated with vehicle and Enzalutamide, and then probed for phosphorylated Raptor, specifically detecting the phosphorylation of S792. As shown in Figure 4B, in the present of Enzalutamide and intact AMPK expression phosphorylated raptor level increased significantly, resulting in the consequential down regulation of pS6 and increased LC3-I to LC3-II conversion while pAKT remained unaffected.

When AMPK was effectively knocked down, Enzalutamide treatment did not affect the phosho-Raptor or phosho-S6 levels. These observations also correlated with reduced ATG-5 expression and reduced conversion of LC3-I to LC3-II. Hence, our data suggest the interaction between AMPK activation and suppression of mTOR via phosphorylation of Raptor at Serine 792 in the induction of ARSI mediated autophagy.

Figure 4 A. Knock down of AMPK in LNCaP-GFP cells blocks the induction of autophagy. LNCaP-eGFP-LC3 cells were transfected with negative control siRNA or siRNA targeting AMPK and treated with DMSO (vehicle control), 10 μM Enzalutamide for 48 h and were then analyzed by fluorescence microscopy. Shown are representative fluorescence microscopy of LNCaP-eGFP-LC3 showing GFP-LC3 localization and puncta autophagosome formation represented by arrow. B. Antiandrogen induced autophagy is mediated through activation of AMPK activation and inhibition of mTOR signaling via Raptor. C4-2B cells were transfected with negative control siRNA or siRNA targeting AMPK and treated with DMSO (vehicle control), 10 μM Enzalutamide for 72 h and cell lysates were analyzed by immunoblotting with antibodies as indicated. Controls were treated with vehicle alone, β-actin was detected as loading control. C. quantification of sub-G1 population in AMPK knock down cells 72 h after transfection.
Figure 4C showed enhanced cell killing when AMPK was knocked down in the Enzalutamide resistant cells. The data support the notion that once the upstream signals for autophagy induction is suppressed, the present of an autophagy inhibitor Clomipramine (CMI). Clomipramine is an FDA approved drug to treat depression and has been shown to be a potent inhibitor of autophagy with little toxic affects both in-vitro and in-vivo [7, 8]. Colony formation in cells treated with Enzalutamide or CMI alone was slightly reduced compared to control, but was markedly impaired in the combined treatment. Their proliferative potential was also markedly reduced, based on the size of the colonies as shown in Figure 5. We will address our hypothesis that targeting autophagy could overcome resistant to Enzalutamide therapy in CRPC in an in-vivo model next.

II. Inhibition of autophagy in a neuropeptide/Src mediated CRPC model

LNCaP GRP Pro cells had been characterized as CRPC cells. Growth of GRP Pro cells in androgen deprived environments was propelled by GRP mediated signaling pathway via Src kinase to activate androgen receptor (AR) in the absence of its cognate ligand [9]. Saracatinib was able to inhibit GRP Pro cell growth in vitro but not as significant in vivo. In addition to the ability of GRP Pro cells to synthesize intracrine androgen through up-regulating some crucial biosynthetic enzymes such as AKR1C3 and CYP11A, autophagy was considered the other contributing factor. We tested the two autophagy modulators CMI and Metf in combination with saracatinib on GRP Pro growth. Enzalutamide was also used with same dose of bicalutamide as a reference (Figure 6). Inhibiting Src kinase in GRP Pro cells rendered the CRPC cells more susceptible to anti-androgens such as bicalutamide and enzalutamide. Combining CMI and Metf with saracatinib also enhanced the cell killing. Metf also displayed some additive effect in inhibiting cell proliferation when combined with enzalutamide.

Figure 6. Cell proliferation inhibition by enzalutamide, bicalutamide, CMI, Metf alone or in combination with saracatinib, assayed by MTT or trypan blue cell counts.

Using all three inhibitors, saracatinib, enzalutamide and metformin reduced cell growth to the lowest. The trends were observed in two independent set of experiments using either MTT assay or trypan blue cell counts.
Since enzalutamide is known to block AR nuclear translocation, we extracted cells treated with various reagents into nuclear and cytosolic fractions and separated them by SDS-PAGE, probed with different antibodies (Figure 7). When grown in regular media, GRP Pro cells were sensitive to enzalutamide, showing reduction of AR protein in the nuclear fraction. Saracatinib showed no effect in this condition since AR was readily activated by its ligand. Metformin alone only decreased the AR translocation marginally. Yet, combination of enzalutamide and metformin almost totally blocked AR into nuclei. However, when in CS media, AR nuclear fraction was not affected by enzalutamide; whereas saracatinib reduced the nuclear translocation greatly which was in agreement with our previous observation [9]. Metformin treatment in CS conditions diminished nuclear AR almost to completion and also reduced cytosolic AR. The activation status of Akt decreased slightly in the regular serum condition but remained mostly unchanged except for enza+metf and enza+sara+metf. Activation of AMPKα and ribosomal S6 or transition from LC-3 I to II indicated autophagy.

**Key research accomplishments:**

1. Validated the additive effect of autophagy modulators on inhibition of cell proliferation to the kinase inhibitor and/or anti-androgen treatments.
2. Depicted the mechanism of anti-androgen mediated autophagy via the AMPK/mTOR signaling pathway in CRPC C4-2B cells.
3. Demonstrated the effects of autophagy modulators on the CRPC LNCaP-GRP model both in cell proliferation and the autophagy and AR signaling axes.

**Reportable outcomes:**

Poster presentation:

1. Enhanced in vivo inhibition of neuropeptide-mediated castration resistant prostate cancer progression by combination therapy with enzalutamide and saracatinib. JC Yang, HG Ngyuen, AC Gao, CP Evans. AACR 2013
2. Autophagy is a survival mechanism in mediating resistance to androgen receptor signaling inhibitors in castration resistant prostate cancer cells. HG Ngyuen, JC Yang, HJ Kung, AC Gao, CP Evans. AACR 2013

Manuscript in revision:

Conclusion:
Survival mechanisms elicited by CRPC C4-2B cells when treated with Enza may be blocked by inhibiting autophagy with clomipramine and metformin. Combination of Enza with saracatinib and autophagy modulators significantly reduced cell proliferation in the LNCaP GRP CRPC model. Further in vivo studies will provide evidences to make autophagy targeting as a supplement to cancer therapeutic regimes.

References: