Proxalutamide reduces SARS-CoV-2 infection and associated inflammatory response

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Over 3 y have passed since the first documented cases of COVID-19 arose from infection by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), yet many challenges remain worldwide in preventing and treating the disease (1). Robust vaccination campaigns led to rapid development, testing, and deployment of several vaccines effective against infection and serious illness from the initial SARS-CoV-2 genetic lineages (2–6). However, as the pandemic continued, waning vaccine protection and emergence of new variants have led to breakthrough infections, as well as many people now having been infected multiple times (5–9). Booster vaccines, including bivalent boosters effective against the highly transmissible omicron variant, have been developed in an effort to overcome these challenges (10). Oral antivirals such as molnupiravir and nirmatrelvir–ritonavir have been developed for high-risk individuals who contract COVID-19, but these are also met with obstacles like potential recurrent infections or contraindications with other commonly prescribed drugs (11–14). Together, these challenges highlight the ongoing critical need for new therapeutics to combat SARS-CoV-2.

As it is the initial step in the viral life cycle, the entry process has been intensely studied to understand how to potentially block SARS-CoV-2 infection (15). Early data during the pandemic showed that the spike (S) protein of SARS-CoV-2 binds to host angiotensin-converting enzyme 2 (ACE2), the SARS-CoV-2 receptor. However, proxalutamide inhibited SARS-CoV-2 infection with an IC50 value of 97 nM, compared to 281 nM for enzalutamide. Importantly, proxalutamide inhibited infection by multiple SARS-CoV-2 variants and synergized with remdesivir. Proxalutamide protected against cell death in response to tumor necrosis factor alpha and interferon gamma, and overall survival of mice was increased with proxalutamide treatment prior to cytokine exposure. Mechanistically, we found that proxalutamide increased levels of NRF2, an essential transcription factor that mediates antioxidant responses, and decreased lung inflammation. These data provide compelling evidence that proxalutamide can prevent SARS-CoV-2 infection and cytokine-induced lung damage, suggesting that promising clinical data may emerge from ongoing phase 3 trials.

Significance

Drugs that target androgen receptor (AR) signaling, including those that inhibit production of androgen ligands (degarelix) and those that bind to and directly block AR activity (enzalutamide), have been investigated in clinical trials for the treatment of COVID-19 but failed to produce positive results. Another AR antagonist, proxalutamide, is in ongoing phase 3 studies for COVID-19 after showing initial positive findings. Data from this study show that proxalutamide can inhibit infection of multiple variants of SARS-CoV-2 in vitro. These data suggest that proxalutamide should continue to be investigated in clinical studies as a potential therapy for COVID-19.


Competing interest statement: H.Y., Q.Z., and L.M. are part of Kintor Pharmaceutical Limited. The remaining authors declare no competing interests.

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Since TMPRSS2 is a well-known androgen receptor (AR)-regulated gene, early hypotheses suggested that inhibition of AR activity could be a potential treatment strategy for COVID-19 (26). As demographic data became available, many reports also observed that males had higher incidences of severe SARS-CoV-2 infections that required intensive care unit (ICU) admission or resulted in death (27–29). In further support of the initial hypothesis that AR activity may drive COVID-19 pathogenesis, a retrospective study during the first months of the pandemic observed a reduced incidence of SARS-CoV-2 infections in prostate cancer patients taking androgen deprivation therapy (ADT) compared to those not receiving ADT (30). Other small studies supported this observation and the premise that anti-androgens could be protective against severe COVID-19 (31, 32); however, these results quickly became debated as other studies found no association between ADT and SARS-CoV-2 infectivity (33–35).

These preliminary observations prompted a burst of basic science and clinical studies to attempt to elucidate the role of androgens in SARS-CoV-2 infection and determine whether AR inhibitors could be viable treatment options for COVID-19. Studies with AR antagonists prescribed for prostate cancer treatment (e.g., enzalutamide, apalutamide, and darolutamide) have since shown that SARS-CoV-2 infectivity can be decreased in vitro in certain contexts with these drugs (36–38). However, some randomized, controlled clinical trials of AR inhibition in COVID-19 patients have not produced encouraging results. For instance, in the Hormonal Intervention for the Treatment in Veterans with COVID-19 Requiring Hospitalization (HITCH) trial (NCT04397718) which showed degarelix, a gonadotropin-releasing hormone (GnRH) antagonist that rapidly suppresses testosterone levels, in male veterans hospitalized with COVID-19, no improvement in clinical outcome was observed compared to placebo (39). Similarly, the COVIDENZA trial (NCT04475601) found no improvement in outcome of COVID-19-positive male or female patients who were randomized to treatment with enzalutamide vs. standard of care (40).

In contrast, the AR antagonist proxalutamide was also tested as a possible treatment for COVID-19 in randomized, controlled trials and showed encouraging positive benefits (41–43), but these findings were met with caution from the scientific community after a retraction statement was issued for one of the publications, citing concerns over randomization (44). Proxalutamide is currently in additional phase 3 trials for COVID-19 in both outpatient (NCT04870606 and NCT04869228) and hospital (NCT05009732) settings in different countries, including the United States. Proxalutamide was originally developed as an AR antagonist for advanced prostate cancer and is in ongoing phase 3 clinical trials for COVID-19 pathogenesis, a retrospective study during the first months of the pandemic observed a reduced incidence of SARS-CoV-2 infections in prostate cancer patients taking androgen deprivation therapy (ADT) compared to those not receiving ADT (30). Other small studies supported this observation and the premise that anti-androgens could be protective against severe COVID-19 (31, 32); however, these results quickly became debated as other studies found no association between ADT and SARS-CoV-2 infectivity (33–35).

Since several variants of the SARS-CoV-2 virus have emerged throughout the pandemic, we examined the effect of proxalutamide on cell proliferation in vitro, but growth inhibition was greater with proxalutamide treatment compared to enzalutamide at the same concentrations (Fig. 1 C and D). Importantly, we found that proxalutamide not only suppressed AR signaling but also decreased AR protein levels, which were not altered by enzalutamide treatment (Fig. 1 E), indicating that proxalutamide possesses stronger inhibition of the AR signaling pathway than enzalutamide. Previously, we reported that enzalutamide can transcriptionally down-regulate SARS-CoV-2 entry factors TMPRSS2 and ACE2 (37). Here, we found that proxalutamide had the same ability to decrease TMPRSS2 and ACE2 (Fig. 1 F). Thus, we postulated that proxalutamide may block SARS-CoV-2 infection.

Employing a SARS-CoV-2 bioassay platform, we have established an in vitro system with which to examine the various strains of authentic SARS-CoV-2 viral infection (37, 52). In this system, cells were pretreated with the experimental compounds for 24 h prior to SARS-CoV-2 infection for an additional 72 h (Fig. 2 A). The results showed that proxalutamide decreased cellular infection by the WA1 strain of SARS-CoV-2 in a dose-dependent manner with an IC$_{50}$ value of 97 nM, whereas enzalutamide decreased infectivity with an IC$_{50}$ value of 281 nM (Fig. 2 B). Representative images of cellular infectivity by the WA1 strain of SARS-CoV-2 in control-, proxalutamide-, or enzalutamide-treated conditions confirmed that decreased infection could be achieved by the AR antagonists proxalutamide and enzalutamide in LNCaP cells (Fig. 2 C). Since several variants of the SARS-CoV-2 virus have emerged throughout the pandemic, we examined the effect of proxalutamide against infection of multiple strains. The results indicated that proxalutamide possessed robust inhibitory effects in blocking SARS-CoV-2 infection by the most common strains, including WA1, alpha, delta, and omicron, with IC$_{50}$ values of 69 nM, 48 nM, 98 nM, and 581 nM, respectively, in LNCaP cells (Fig. 2 D).

Furthermore, remdesivir is a Food and Drug Administration (FDA)-approved agent for treatment of SARS-CoV-2 infection (48, 49). The combinatorial effect of proxalutamide or enzalutamide against controls.

**Results**

Proxalutamide is an AR antagonist recently developed for castration-resistant prostate cancer (CRPC) (47), in comparison to enzalutamide which has been commonly prescribed for CRPC treatment for several years (51). To first compare the transcriptomic changes associated with proxalutamide and enzalutamide, RNA-sequencing (RNA-Seq) analysis was carried out in AR-positive prostate cancer Lymph Node Carcinoma of the Prostate (LNCaP) cells using either 20 μM proxalutamide or enzalutamide for 8 h of treatment. Gene set enrichment analysis was achieved by examining differentially expressed genes in either proxalutamide- or enzalutamide-treated cells compared to control. The normalized enrichment score results indicated that androgen responses were the top down-regulated hallmark in both proxalutamide- and enzalutamide-treated LNCaP cells (Fig. 1 A). Gene set enrichment analysis on androgen responses further confirmed that proxalutamide significantly down-regulated androgen-regulated genes that were suppressed by enzalutamide (Fig. 1 B), suggesting proxalutamide suppresses AR signaling. In addition, the effect of proxalutamide on cell proliferation was examined in LNCaP cells and a castration-resistant variant of LNCaP called C4-2B cells. In both LNCaP and C4-2B cells, proxalutamide and enzalutamide treatment resulted in dose-dependent inhibition of cell proliferation in vitro, but growth inhibition was greater with proxalutamide treatment compared to enzalutamide at the same concentrations (Fig. 1 C and D). Importantly, we found that proxalutamide not only suppressed AR signaling but also decreased AR protein levels, which were not altered by enzalutamide treatment (Fig. 1 E), indicating that proxalutamide possesses stronger inhibition of the AR signaling pathway than enzalutamide. Previously, we reported that enzalutamide can transcriptionally down-regulate SARS-CoV-2 entry factors TMPRSS2 and ACE2 (37). Here, we found that proxalutamide had the same ability to decrease TMPRSS2 and ACE2 (Fig. 1 F). Thus, we postulated that proxalutamide may block SARS-CoV-2 infection.

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Furthermore, remdesivir is a Food and Drug Administration (FDA)-approved agent for treatment of SARS-CoV-2 infection (48, 49). The combinatorial effect of proxalutamide or enzalutamide
and remdesivir in preventing infection by the SARS-CoV-2 alpha strain was examined in induced human alveolar cells (iAEC2) (Fig. 3A). The results indicated that proxalutamide had a strong synergistic effect with remdesivir in inhibition of alpha strain infection and achieved 100% protection against infection (Fig. 3B), with a synergy score of 14.516 (Fig. 3C). Similarly, the enzalutamide and remdesivir combination achieved synergy but with a slightly weaker synergistic effect than the proxalutamide and remdesivir
Both proxalutamide or enzalutamide and remdesivir combination treatments had no detrimental effects on the viability of iAEC2 cells (Fig. 3 D and G). These results suggest that proxalutamide may have clinical utility in combination with current SARS-CoV-2 treatments, such as remdesivir.

SARS-CoV-2-induced mortality is largely triggered by a cytokine storm that occurs in the pulmonary system and systemically (53). It has been reported that TNFα and INFγ can act synergistically to trigger inflammatory cell death in vitro and in vivo, which mimics the SARS-CoV-2-induced cytokine shock syndrome (CSS) that
occurs in COVID-19 patients (50). Specifically, TNFα and INFγ induce a type of inflammatory cell death called PANoptosis, which is regulated by the PANoptosome and involves molecular components of pyroptosis, apoptosis, and necroptosis (50, 54). In an AR-positive lung cell line, H1437, we demonstrated that the combination of TNFα and INFγ induced maximal cell death compared to either cytokine alone (Fig. 4A). Interestingly, the cell death induced by combination treatment with TNFα and INFγ was attenuated by proxalutamide and another AR antagonist darolutamide (SI Appendix, Fig. S1A). Additionally, the cell death triggered by TNFα and INFγ combination treatment was confirmed by elevated cleaved PARP (c-PARP) levels, which were dose-dependently blocked by proxalutamide and darolutamide (Fig. 4C) but not enzalutamide or apalutamide (SI Appendix, Fig. S1B). Similarly, AR protein levels were down-regulated by proxalutamide and darolutamide (Fig. 4D) but not enzalutamide or apalutamide (SI Appendix, Fig. S1C). This suggests that AR antagonists such as proxalutamide or darolutamide may provide additional benefits in terms of reducing CSS in vivo. In normal mouse prostate organoids, we confirmed that proxalutamide inhibited murine AR signaling by decreasing androgen (dihydrotestosterone, DHT)-stimulated induction of \( Fkbp5 \) and \( Psca \) target genes; additionally, proxalutamide decreased \( Ar \) mRNA levels (SI Appendix, Fig. S2A). These results prompted us to examine the in vivo efficacy of proxalutamide in preventing death in the TNFα and INFγ CSS model (50) in wild-type C57BL6 male mice. We tested two treatment regimens of proxalutamide prior to cytokine challenge with the TNFα and INFγ combination. The results showed that proxalutamide significantly reduced cell death and increased cell viability compared to controls (Fig. 3).
INFγ combination. The data showed that both proxalutamide treatment regimens reduced mortality induced by TNFα and INFγ (SI Appendix, Fig. S2 B and C). Histology evaluation of tissue damage triggered by TNFα and INFγ combination was examined in the small intestine and lung (SI Appendix, Fig. S2 D). Compared with the PBS treated group, atrophy of the villi and an increase in inflammatory cell infiltration in the lamina propria area of the intestine were observed post-TNFα and IFNγ treatment, which was largely alleviated with proxalutamide treatment. In addition, TNFα and IFNγ treatment induced interlobular septal thickening in the lungs of mice showing focal epithelial hyperplasia, and such effects were rescued by proxalutamide treatment. Thus, these results suggest that proxalutamide may reduce TNFα and IFNγ cytokine storm-induced cell death in vitro and in vivo.

The NRF2 pathway is an important part of cellular defense through the production of antioxidants, which occurs via binding of the NRF2 transcription factor to antioxidant response elements in target genes (55–57). The upregulation of NRF2 has been reported to control inflammation in several studies (56–60). Here, we found that proxalutamide increases NRF2 transcriptional activity by enhancing NRF2 DNA binding in RAW264.7 and THP-1 cells (Fig. 5 A). In RAW264.7 cells, proxalutamide also up-regulated NRF2 protein expression in lipopolysaccharide (LPS)-stimulated conditions (Fig. 5 B). In the in vitro CSS model triggered by TNFα and INFγ combination treatment, proxalutamide augmented NRF2 protein levels and decreased cell death in THP-1 cells (Fig. 5 C and D). Apoptotic cell death triggered by TNFα and INFγ combination treatment was attenuated by proxalutamide (Fig. 5 E). Next, we
examined proxalutamide in an acute lung injury animal model triggered by poly(I:C), and combination dexamethasone and roflumilast treatment was used as a positive control (Fig. 5F). In this model, proxalutamide significantly reduced the total mononuclear cells and neutrophils in alveolar lavage fluids from poly(I:C)-induced animals (Fig. 5G). Together, our data show that proxalutamide up-regulates NRF2 protein levels and decreases inflammation in the lungs induced by poly(I:C), suggesting a possible benefit of proxalutamide against SARS-CoV-2-associated inflammatory responses and mortality in COVID-19 patients.

**Discussion**

Proxalutamide was initially developed as an AR antagonist that could potentially have efficacy in CRPC patients, including those that had developed resistance to existing AR-targeted therapies.
that induces inflammatory responses in the lung similar to viral
H1437 and THP-1 cell lines (Figs. 4
G

GnRH antagonist that prevents release of follicle-stimulating hormone
diminishes the inflammatory response to SARS-CoV-2 infection in vitro,
and the compound is active against several strains of the virus (WA1, alpha, delta, and omicron). Synergy can be obtained when proxalutamide is combined with remdesivir. Interestingly, proxalutamide also increases levels of the Nrf2 transcription factor.

It is well established that COVID-19 can be associated with a cytokine storm, a hyperactivation of the immune system that can ultimately result in death (53). In this study, we employed two in vivo lines of experimentation to analyze the effect of proxalutamide on CSS and lung injury. Proxalutamide pretreatment in the TNFα/IFNγ model of CSS (50) results in a modest increase in overall survival (Supplementary Appendix, Fig. S2 B and C), mimicking the attenuation of mortality in vitro cell death observed with proxalutamide in the H1437 and THP-1 cell lines (Figs. 4B and 4D). Using poly(I:C) that induces inflammatory responses in the lung similar to viral infections (61), we observed that proxalutamide significantly decreases total cell and neutrophil levels in BALF (bronchoalveolar lavage fluid) (Fig. 5G). Altogether, results from these two in vivo models suggest that proxalutamide can decrease CSS responses and lung inflammation, but there are associated caveats to note. TNFα and IFNγ induce PANoptosis in mice that leads to CSS and death, which has been suggested to mimic severe COVID-19 in patients (50). However, TNFα/IFNγ-induced death in mice occurs within hours, whereas death from acute respiratory distress syndrome (ARDS) in COVID-19 patients happens over a much longer time (62).

Additionally, studies have implicated alternative cytokines (e.g., IL-6 and IL-1) rather than just TNFα and IFNγ as the primary inducers of ARDS in COVID-19 (63). In terms of the poly(I:C) model, it is prudent to also note that this is a model of lung injury, rather than lung epithelial cell death. Finally, these in vivo models are examples of the possible downstream effects of SARS-CoV-2 and did not directly involve animal infection with the virus. It is interesting to note, however, that proxalutamide increases the DNA binding activity and expression of Nrf2, and Nrf2 has been shown to be an essential factor for tempering the immune response and protecting against sepsis (64, 65). A recent study also shows that SARS-CoV-2 can inhibit Nrf2 signaling through one of its nonstructural proteins (66). In line with our findings, Nrf2 agonists consequently inhibited SARS-CoV-2 replication (66).

Combined, the data in this study support the notion that proxalutamide has antiviral activity against SARS-CoV-2 and suggest that it could show positive clinical benefit in cases of COVID-19, warranting further clinical exploration. In comparison, as mentioned above, clinical studies with degarelix (HITCH trial, NCT04397718) and enzalutamide (COVIDENZA trial, NCT04475601) did not find any improvements in clinical outcome with COVID-19 (39, 40). There are a multitude of explanations that could account for these disparate findings from different AR-targeting drugs. Degarelix is a GnRH antagonist that prevents release of follicle-stimulating hormone and luteinizing hormone, thereby leading to suppression of testicular testosterone release and a decrease in AR activity at the level of ligand availability (67). In contrast, proxalutamide, like enzalutamide, binds directly to the ligand-binding domain of AR to block receptor activation (47, 68). As shown in Fig. 1, proxalutamide and enzalutamide exert similar effects in LNCaP prostate cancer cells—decreasing or activating similar signaling pathways, decreasing androgen signaling, and decreasing cell proliferation. Relevant to SARS-CoV-2, both compounds decrease expression of host entry receptors ACE2 and TMPRSS2 (Fig. 1D). However, certain differences exist with these two compounds. For instance, a preclinical report on proxalutamide reported a 3.4-fold higher binding affinity for AR compared to enzalutamide (47). As shown here and previously (47), proxalutamide can also decrease AR protein expression, while enzalutamide does not lead to AR degradation (Fig. 1E). In the SARS-CoV-2 bioassays, proxalutamide exhibited increased potency in inhibiting infection compared to enzalutamide (IC50 of 97 nM for proxalutamide and 281 nM for enzalutamide, Fig. 2B) and a higher Bliss synergy score with remdesivir (14.516 and 11.685 for proxalutamide and enzalutamide, respectively, Fig. 3). Furthermore, in the cell line models of cytokine-mediated death with combined TNFα and IFNγ treatment, addition of proxalutamide prevented cell death (Fig. 4B), whereas enzalutamide was without effect, even at the high dose of 20 μM (Supplementary Appendix, Fig. S1A). These data show that although proxalutamide and enzalutamide are both AR antagonists, differences in their mechanisms of action exist. However, since both compounds decrease ACE2 and TMPRSS2 expression and ultimately prevent SARS-CoV-2 infectivity in vitro (albeit with different IC50 values), further research is needed to define the precise mechanisms that could account for disparate clinical outcomes in COVID-19 treatment.

Several phase 3 clinical trials of proxalutamide treatment for COVID-19, all sponsored by Kintor Pharmaceuticals, are ongoing in different countries, and these studies should provide more definitive answers as to its efficacy. One phase 3 randomized, placebo-controlled, multinational clinical trial of outpatients with mild or moderate COVID-19 (NCT04870606) primarily enrolled patients at centers across the United States (99%) (69). Efficacy data showed that proxalutamide reduced the risk of hospitalization or death compared to placebo, and proxalutamide continued to show a positive safety profile (69). An additional outpatient clinical trial of males with mild to moderate COVID-19 in Brazil is ongoing (NCT04869228), with the primary outcome being oxygen requirement at Day 28. Finally, NCT05009732 is an ongoing phase 3 trial of proxalutamide in hospitalized adults with COVID-19 that has participating locations across several countries, including the United States, China, Philippines, and South Africa. The primary end point for this study is time to clinical deterioration (need for ICU care, mechanical ventilation, or mortality). The data presented in our report suggest that proxalutamide can markedly decrease SARS-CoV-2 infectivity and associated inflammatory responses, which could result in positive clinical benefit, and results from the clinical studies above are eagerly awaited.

**Methods**

Cell Culture. LNCaP, RAW264.7, and THP-1 cells were purchased from the American Type Culture Collection (ATCC) and cultured in 5% CO2 at 37 °C in medium as suggested by ATCC. iAEC2 cells (iPSC (SPC2 IPSC line, clone SPC2-ST-B2, Boston University) derived alveolar epithelial type II cells) were maintained in different countries, and these studies should provide more definitive answers as to its efficacy. One phase 3 randomized, placebo-controlled, multinational clinical trial of outpatients with mild or moderate COVID-19 (NCT04870606) primarily enrolled patients at centers across the United States (99%) (69). Efficacy data showed that proxalutamide reduced the risk of hospitalization or death compared to placebo, and proxalutamide continued to show a positive safety profile (69). An additional outpatient clinical trial of males with mild to moderate COVID-19 in Brazil is ongoing (NCT04869228), with the primary outcome being oxygen requirement at Day 28. Finally, NCT05009732 is an ongoing phase 3 trial of proxalutamide in hospitalized adults with COVID-19 that has participating locations across several countries, including the United States, China, Philippines, and South Africa. The primary end point for this study is time to clinical deterioration (need for ICU care, mechanical ventilation, or mortality). The data presented in our report suggest that proxalutamide can markedly decrease SARS-CoV-2 infectivity and associated inflammatory responses, which could result in positive clinical benefit, and results from the clinical studies above are eagerly awaited.
SARS-CoV-2 Bioassay. SARS-CoV-2 isolates USA-WA1/2020, hCoV-19/USA/OR:OHsu-PHL00037/2021 (Lineage B.1.1.7; Alpha Variant), hCoV-19/USA/MD-HPS0285/2021 (Lineage B.1.617.2; Delta Variant), and hCoV-19/USA/GA-EHC-2811C/2021 (Lineage B.1.1.529; Omicron Variant) were obtained from BEI resources and propagated in VeroE6 cells (ATCC). Viral titers were established by TCID50 with the Reed and Muench method. LNCaP or iACE2 cells were plated in 384-well plates and treated with increasing concentrations of proxalutamide or enzalutamide for 24 h prior to SARS-CoV-2 virus infection in a Biosafety Level 3 facility. Cells were then incubated for 48 h post infection under culture conditions of 5% CO2 and 37°C. Assay plates were fixed, permeabilized, and labeled with anti-atrioless panSARS-CoV-2 primary antibody (Antibodies Online, Cat #: ABIN6952432) as previously described (52). The remaining of the assay proceeded as previously described (70).

Fluorescence Imaging and High-Content Analysis. A Thermo-Fisher CX5 high-content microscope with LED excitation (386/23 nm, 650/13 nm) at 10× magnification was used to image assay plates. Nine fields per well were imaged at a single z plane in these experiments. Imaging, processing, and normalization were performed as previously described (70, 71).

Gene Expression Analysis. RNA was extracted from LNCAP cells treated with DMSO, 20 μM proxalutamide, or enzalutamide for 8 h using a Qiagen RNA extraction kit. RNA quality was determined by using a Bioanalyzer RNA Nano Chip. Poly-A selection was performed with Sera-Mag Oligo(dT)-Coated Magnetic Particles (3815203101050; GE Healthcare Life Sciences), and libraries were generated using a KAPA RNA HyperPrep kit (KK8541; Roche Sequencing Solutions). RNA-seq was performed on an Illumina HiSeq 2500. Reads were aligned with the Spliced Transcripts Alignment to a Reference mapper to the human reference genome hg38. Gene differential expression analysis was carried out with edgeR70.

Mouse Prostate Organoid Culture. Whole mouse prostate was dissected from C57BL/6j wild-type mice, and organoid culture was generated according to previous publication (72). Mouse prostate organoids were treated with 5 μM or 10 μM proxalutamide or enzalutamide for 16 h prior to 10 nM DHT stimulation for previous publication (72). Mouse prostate organoids were treated with 5 μM or 10 μM proxalutamide, or enzalutamide for 8 h using a Qiagen RNA extraction kit. RNA quality was determined by using a Bioanalyzer RNA Nano Chip. Poly-A selection was performed with Sera-Mag Oligo(dT)-Coated Magnetic Particles (3815203101050; GE Healthcare Life Sciences), and libraries were generated using a KAPA RNA HyperPrep kit (KK8541; Roche Sequencing Solutions). RNA-seq was performed on an Illumina HiSeq 2500. Reads were aligned with the Spliced Transcripts Alignment to a Reference mapper to the human reference genome hg38. Gene differential expression analysis was carried out with edgeR70.

For western blotting analysis, cells were harvested and lysed in Pierce RIPA buffer (Thermo Fisher) with added phosphatase (Millipore) and protease (Roche) inhibitor cocktails. Protein quantification, sodium dodecyl-sulfate polyacrylamide gel electrophoresis, transfer, blocking, and antibody incubation proceeded as previously described (70). The remaining of the assay proceeded as previously described (70).

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Poly(I:C)-Induced Acute Lung Injury In Vivo Model. Six- to eight-week-old male BALB/c (Bagg Albino/c) mice were assigned to treatment groups by randomization in BioBook software to achieve similar group mean weight before treatment; 10 mice were allocated into each group. Group 1 was normal-vehicle; groups 2 to 5 were challenged with poly(I:C) with vehicle sodium carboxymethyl cellulose (CMC Na), 10 mg/kg dexamethasone and 20 mg/kg roflumilast combination, 20 mg/kg prozalutamide, or 40 mg/kg prozalutamide, respectively. Dexamethasone was dissolved in 0.5% CMC Na to make a suspension at a final concentration of 1 mg/mL. Roflumilast was dissolved in 0.5% CMC Na to make a suspension at a final concentration of 2 mg/mL. Mice were treated with vehicle, dexamethasone and roflumilast combination, or prozalutamide 16 h and 1 h prior to poly(I:C) injection and 6 h after poly(I:C) injection. Additional prozalutamide dose was given 18 h post poly(I:C) injection. Poly(I:C) solution was prepared to 0.06% solution in sterile PBS freshly prepared where 1.8 mg poly(I:C) was dissolved in 3 mL PBS to make a suspension at a final concentration of 0.6 mg/mL. Twenty-four hours post poly(I:C) injection, all mice were anesthetized with Zoletil (i.p., 25 to 50 mg/kg, containing 1 mg/mL Xylazine). Lungs were gently lavaged through the tracheal cannula with 0.5 mL PBS containing 1% fetal bovine serum (FBS), and the BALF was collected. Then, the lungs were gently lavaged with another 0.5 mL PBS containing 1% FBS. After lavage, the collected BALF was stored on ice. The total cell number in BALF was counted using a hemocytometer. After lavage by PBS, all mice were killed by exsanguination.

Liquid Mass Spectrometry Quantification after TFE (Transcription Factors Response Element) Enrichment. Mouse monocyte RAW264.7 cells (0, 2 h, 4 h, and 8 h) and human monocyte THP-1 (0, 0.5 h, 2 h, and 6 h) were treated with 10 μM proxalutamide, respectively. Cells were collected and cultured with TFE-binding beads, and the beads were rotated and combined for 1.5 h at 4°C. After the combined TFE beads were washed 3 times with NETN and 2 times with mass spectrometry (to remove the scale removing agent; if there were still bubbles, they were washed again with water). Then, 50 μL NH4HCO3, and 1.5 μg tyrosinase were added to the beads. The beads were hydrolyzed overnight, and the tube wall was lightly spritzed 1 to 2 times in the middle. Two hundred microliters of 50% acetonitrile + 0.1% formic acid was added to the suspension for 3 to 5 min, and then, the supernatant was transferred on a magnetic rack to a new Eppendorf tube; this was then repeated once. The supernatant was vacuum dried into peptide powder and stored at low temperature. Protein sequences were identified by liquid chromatography with tandem mass spectrometry.

Statistical Analysis. Statistical analyses were performed by the two-tailed, unpaired t-test, unless otherwise indicated in figure captions. Bars error bars indicate mean ± SEM. GraphPad Prism software (version 9) was used for statistical calculations. No data were excluded from the analyses.

Data, Materials, and Software Availability. All study data are included in the article and/or SI Appendix. Sequencing data are available through the National Center for Biotechnology Information Gene Expression Omnibus, accession number GSE234805 (74).

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