Cancer Therapy: Preclinical

Clinical Cancer Research

Elevation of c-FLIP in Castrate-Resistant Prostate Cancer Antagonizes Therapeutic Response to Androgen Receptor–Targeted Therapy

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Abstract

Purpose: To characterize the importance of cellular Fas-associated death domain (FADD)–like interleukin 1β-converting enzyme (FLICE) inhibitory protein (c-FLIP), a key regulator of caspase-8 (FLICE)–promoted apoptosis, in modulating the response of prostate cancer cells to androgen receptor (AR)–targeted therapy.

Experimental Design: c-FLIP expression was characterized by immunohistochemical analysis of prostatectomy tissue. The functional importance of c-FLIP to survival and modulating response to bicalutamide was studied by molecular and pharmacologic interventions.

Results: c-FLIP expression was increased in high-grade prostatic intraepithelial neoplasia and prostate cancer tissue relative to normal prostate epithelium ($P < 0.001$). Maximal c-FLIP expression was detected in castrate-resistant prostate cancer (CRPC; $P < 0.001$). *In vitro*, silencing of c-FLIP induced spontaneous apoptosis and increased 22Rv1 and LNCaP cell sensitivity to bicalutamide, determined by flow cytometry, PARP cleavage, and caspase activity assays. The histone deacetylase inhibitors (HDACi), droxinostat and SAHA, also downregulated c-FLIP expression, induced caspase-8- and caspase-3/7–mediated apoptosis, and increased apoptosis in bicalutamide-treated cells. Conversely, the elevated expression of c-FLIP detected in the CRPC cell line VCaP underpinned their insensitivity to bicalutamide and SAHA *in vitro*. However, knockdown of c-FLIP induced spontaneous apoptosis in VCaP cells, indicating its relevance to cell survival and therapeutic resistance.

Conclusion: c-FLIP reduces the efficacy of AR-targeted therapy and maintains the viability of prostate cancer cells. A combination of HDACi with androgen deprivation therapy may be effective in early-stage disease, using c-FLIP expression as a predictive biomarker of sensitivity. Direct targeting of c-FLIP, however, may be relevant to enhance the response of existing and novel therapeutics in CRPC. *Clin Cancer Res; 18(14); 3822–33. ©2012 AACR.*

Introduction

Androgen-deprivation therapy (ADT) is initially effective in the treatment of localized prostate cancer (1) and was developed on the basis that prostate tumor outgrowth is stimulated by androgens acting via the androgen receptor (AR; ref. 2). Bicalutamide (Casodex) is currently one of the principal antiandrogenic drugs used in prostate cancer therapy, interrupting androgen signaling by antagonizing ligand-induced activation of the AR (3). The onset of resistance to AR-targeted therapies is a major clinical problem resulting in patient relapse and the emergence of an incurable castrate-resistant condition. Bicalutamide does not provide effective biochemical control in all patients, and the effectiveness of the drug is often transient. Knowledge of the mechanisms underpinning bicalutamide resistance and the transition to castrate-resistant prostate cancer (CRPC) may identify more effective combinatorial treatments to increase the proportion of patients that respond, and the duration of response to bicalutamide or alternate ADT strategies.

Elevated expression of antiapoptotic proteins has been proposed to represent one mechanism of resistance to anticancer drug strategies (4), including ADT. Antiapoptotic c-FLIP [cellular Fas-associated death domain (FADD)–like interleukin 1β-converting enzyme (FLICE) inhibitory protein], is an endogenous inhibitor of caspase-8 (FLICE)

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Translational Relevance

Androgen receptor (AR) signaling is the principal pathway driving prostate cancer growth. Inhibition of androgen-driven signaling is the predominant therapeutic strategy for prostate cancer, reinforced by the recent development of 2 clinically effective agents, abiraterone acetate and MDV3100. Here we show that c-FLIP, an AR-regulated antiapoptotic protein, constitutes a novel mode of resistance to AR-targeted therapeutics. Downregulation of c-FLIP in response to siRNA-targeting or histone deacetylase inhibitors (HDACi) induced apoptosis coincident with downregulation of c-FLIP expression and potentiated the response to the AR-targeted drug bicalutamide in noncastrate-resistant cells. In contrast, the elevated c-FLIP expression detected in castrate-resistant cells antagonized HDACi and bicalutamide response. Accordingly, HDACi may be useful in prolonging the therapeutic response to AR-targeted drugs in noncastrate conditions. Moreover, the increase in c-FLIP expression determined in castrate-resistant disease may assist in predicting patient response to existing and emerging androgen deprivation strategies and, alternatively, may itself be a rationale therapeutic target to improve treatment of CRPC.

that inhibits the activation of the extrinsic apoptosis pathway mediated by death receptors such as Fas, DR4 (TRAIL-R1), and DR5 (TRAIL-R2; refs. 5–8). Overexpression of c-FLIP has been characterized in the cancer cells of prostate biopsy tissue and has been shown to promote the androgen-independent growth of tumors in nude mice (9). We and others have also determined that transcription of the gene encoding c-FLIP is regulated in part by the AR (9–11), although NF-κB activity can also potentiate the transcription and expression of the gene in LNCaP and PC3 prostate cancer cell lines (11). Thus, increased expression of c-FLIP, induced as a result of failure to repress androgen signaling or alternatively promoted by compensatory cytokine/growth factor–induced signaling, may represent an important feature during the transition to CRPC.

The objective of this study was to characterize the expression of c-FLIP in prostatectomy tissue and determine whether expression of c-FLIP modulates the sensitivity of androgen-dependent prostate cancer cells to bicalutamide. In addition to direct targeting of c-FLIP using siRNA, we also show that 2 histone deacetylase inhibitors (HDACi), droxinostat and SAHA (Vorinostat), can repress c-FLIP expression in androgen-dependent cells and that these agents can potentiate the apoptosis induced by bicalutamide in noncastrate models. Conversely, we show that SAHA is ineffective in cell-based models of castrate-resistant disease, suggesting that combinations of HDACi with bicalutamide are applicable during salvage therapies but may be ineffective in rescuing castrate-resistant disease. Moreover, we show that c-FLIP plays an important role in the viability of castrate-resistant prostate cancer cells.

Materials and Methods

Chemicals and reagents

All chemicals were supplied by Sigma unless otherwise stated. 4-(4-chloro-2-methylphenoxy)-*N*-hydroxybutanamide (droxinostat) was purchased from Chembridge. Bicalutamide was provided under a MTA agreement with AstraZeneca. Suberoylanilide hydroxamic acid (SAHA; Vorinostat) was purchased from Selleck. All drugs were reconstituted in 100% dimethyl sulfoxide (DMSO) vehicle before further dilution for use in experiments; appropriate controls to account for effects of DMSO in biologic assays were conducted in parallel.

Cell culture

Authenticated LNCaP and 22Rv1 cells were obtained from American Type Culture Collection and cultured for a maximum of 4 months, as previously described (12). Human VCaP cells (European Collection of Cell Cultures) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% v/v fetal calf serum (FCS). All cells were maintained in a humidified chamber at 37°C in 5% CO_2. LN-Abl cells were provided by Prof. Bill Watson (University College Dublin, Ireland).

Western blotting

Protein lysates were prepared as previously described (11, 12), subjected to SDS-PAGE and transferred onto nitrocellulose membranes for 2 hours. Membranes were blocked for 1 hour at 25ºC in 5% (w/v) Marvel/PBS/3% (v/v) Tween-20 (PBS-T), then incubated overnight at 4°C with either monoclonal antibodies to c-FLIP (NF6; 1:1,000 dilution; Enzo Life Sciences), PARP (1:2,000; eBiosciences), AR (1:400; Millipore), or BCL-2 (1:1,000; Cell Signaling) reconstituted in 5% (w/v) Marvel/PBS-T. Membranes were washed for 10 minutes 3 times in PBS-T, then probed with the respective horseradish peroxidise–conjugated secondary antibodies (Amersham Life Sciences) for 1 hour at 25ºC. Following 3×10 minutes washes in PBS-T, bands were detected using enhanced chemiluminescence (ECL+ reagents; Amersham). Equal protein loading was assessed by reprobing membranes with an antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:3,000; AbD Serotec).

siRNA transfections

Cells (density of 1×10^6 per P90 in FCS-supplemented growth media) were grown to 50% confluency, replenished in serum-free Optimem 1, then transfected for 4 hours with a siRNA oligonucleotide pool targeting both c-FLIP splice variants (FT; Dharmacon) or oligonucleotides specifically targeting the c-$FLIP_L$ (FL) or c-$FLIP_S$ (FS) isoforms (13), using oligofectamine according to manufacturer's instructions. Cells were replenished in 30% FCS-enriched medium, harvested after 24 hours or treated with bicalutamide for a further 48 hours. A nontargeting oligonucleotide pool

(NT; Dharmacon) was used at the same concentration as a transfection control.

Flow cytometry

Flow cytometry was carried out on siRNA- or drug-treated cells using previously described protocols (11, 14). Following treatment, detached and attached cells were collected, centrifuged 2,000 × *g* for 5 minutes, washed twice in PBS containing 1% FCS, and fixed in 100% ethanol. Cell pellets were resuspended in 500-μL propidium iodide (PI)/RNaseA solution, incubated at 37°C for 30 minutes and analyzed on a FACSCalibur machine (Becton Dickinson). Ten thousand events were captured using forward and sidescatter detectors. Gating of the sub-G_0/G_1 fraction of the cell cycle calculated the percentage of cells staining positive for PI and undergoing cell death. In some experiments, apoptosis was calculated as the sum of the fluorescein isothiocyanate (FITC)–Annexin V positive/PI negative (early apoptosis) and FITC–Annexin V positive/PI positive (late apoptosis) cell populations (BD Biosciences). To assess mitochondrial membrane potential, tetramethyl-rhodamine ethyl ester perchlorate (TMRE) was added to drug-treated cells at a final concentration of 25 nmol/L and incubated for 15 minutes at 37°C. Cells were collected, pelleted by centrifugation at 2,000 r.p.m at 4°C for 5 minutes and resuspended in 300 μL PBS. TMRE fluorescence was analyzed immediately by flow cytometry using the FL2 filter. Geometric means were calculated from the FL2 fluorescence signal, normalized against background fluorescence in non-TMRE–treated cells, and expressed as percentage TMRE staining compared with vehicle control.

Quantitative real-time PCR analysis

Samples were prepared as previously described (11, 12). The primer sequences used were as follows: 18S: Forward, 5′-CATTCGTATTGCGCCGCTA-3′; Reverse, 5′-CGACGGTATCTGATCGTC-3′; AR: Forward, 5′-CGGAAGCTGAAGAAACTTGG-3′; Reverse, 5′-CGTGTCCAGCACACACTACA-3′; c-$FLIP_L$: Forward, 5′-CCTAGGAATCTGCCTGATAATCGA-3′; Reverse, 5′-TGCGATATACCATGCATACTGAGA-3′; c-$FLIP_S$: Forward, 5′-GCAGCAATCCAAAAGAGTCTCA-3′; Reverse, 5′-ATTTCCAAGAATTTTCAGATCAGG-3′; PSA: Forward, 5′-TGAGCCTCCTGAAGAATCGA-3′; Reverse, 5′-TTGCGCACACACGTCATT-3′; BCL-2: Forward, 5′-GGATGCCTTTGTGGAACTG-3′; Reverse, 5′-AGAGACAGCCAGGAGAAATCA-3′. Real-time PCR was carried out in a 96-well plate using a LC480 light cycler instrument (Roche Diagnostics). The threshold cycle (C_P) was calculated for each reaction and expression levels determined using the relative standard curve method, normalized against 18S expression.

Transfection and luciferase assay

Luciferase reporter assays to assess AR and NF-κB transcriptional activity were carried out as previously described (11, 12). Transfected cells were incubated for 24 hours before drug treatment; SAHA was administered for 24 hours before the addition of bicalutamide for a further 48 hours. Samples were analyzed using a Dual-Luciferase Reporter assay kit (Promega) according to manufacturer's instructions. Transcriptional activity was calculated by adjusting for transfection efficiency using *Renilla*, followed by normalization to sample-matched pGL3 readouts.

Immunohistochemistry

Expression of c-FLIP was analyzed in 50 prostate specimens obtained from the Pathology Services of the Polytechnic University of the Marche Region–United Hospitals, Ancona, Italy. The clinical characteristics associated with these specimens are defined in Supplementary Table S1. Forty radical prostatectomies were included, taken from men with clinically detected prostate cancer. Twenty of these prostatectomies were obtained from patients who had not received any hormone treatment and high-grade prostatic intraepithelial neoplasia (HGPIN) was present in all samples. A further 20 prostatectomies were studied in which patients had been under total androgen ablation for 3 months before surgery; HGPIN was detected in 18 of these specimens. Tissue from 10 transurethral resections of the prostate (TURP) was also included in which patients had locally recurrent hormone-independent prostate cancer, defined as a minimum of 2 consecutive rises in PSA, obtained at least 2 weeks apart, in the presence of castrate levels of testosterone. All tissue samples were obtained from the peripheral zone of the prostate to avoid the influence of zonal distribution upon the determination of c-FLIP expression. In addition, the cancers were all stage pT2a. In the group of 20 specimens with untreated prostate cancer, we had a balance of Gleason score $3 + 3 = 6$ (10 cases) and $4+4 = 8$ (10 cases). In the group of 20 specimens with treated prostate cancer, the Gleason score, as determined in the initial prostate biopsy, was $3 + 3 = 6$. For each case, the microscope slide with the greatest amount of cancer, HGPIN, and normal tissue of the peripheral zone was selected. Five-micron thick sections were cut from archival, formalin-fixed, paraffin-embedded specimens and mounted on silane-coated slides.

c-FLIP expression was carried out by standard indirect biotin–avidin immunohistochemical analysis. Antigen retrieval was carried out using EDTA buffer in a pressure steamer at 100°C for 90 minutes. Slides were stained on an automated immunostainer (DakoCytomation) using a polyclonal anti–c-FLIP antibody (Enzo Life Sciences; 1:50 dilution). Bound antibodies were detected with the Dako Envision System. As a negative control, primary antibody was omitted and replaced with PBS. In addition, nonspecific rabbit antibody was used, resulting in clean negative results in all cases tested (not shown). Slides were counterstained with a light hematoxylin.

At least 1,000 cells were counted in contiguous 400× microscopic fields, counting separately for normal epithelium, HGPIN, and prostate cancer. Each slide was assessed independently by 2 pathologists (FB and RMa). Discrepancies were resolved by a concurrent reexamination by both investigators using a double-headed microscope. c-FLIP

expression was evaluated in a semiquantitative manner, whereby the levels of expression are represented as the percentage of positive cells and the intensity of the staining, as follows: [H-score = 1 × (% weak) + 2 × (% moderate) + 3 × (% intense) generating a ranking between 0 and 300; ref. 15].

Statistical analysis

Experimental results were compared using a 2-tailed Student *t* test analysis, comparing the statistical significance between means (GraphPad Prism). For immunohistochemistry studies, statistical analysis was done using SPSS software (SPSS Inc.) using the Mann–Whitney test function. Results were considered significant at $P < 0.05$ for all analyses.

Results

c-FLIP expression in diseased prostate tissue correlates with sensitivity or resistance to androgen-deprivation therapy

Expression of c-FLIP was evaluated by a semiquantitative immunohistochemical analysis of radical prostatectomy and TURP tissue (Supplementary Table S1). c-FLIP expression ranged from a minimal level in histologically normal prostate epithelium (NPE; Fig. 1A) to a maximal level in CRPC tissue (Fig. 1B; Supplementary Table S2).

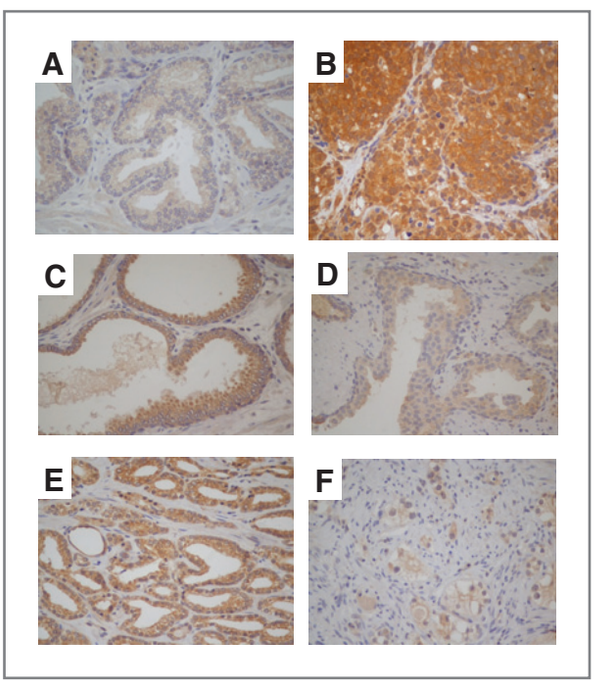

Figure 1. Characterization of c-FLIP expression in prostate tissue. Expression of c-FLIP was determined by immunohistochemistry. Images shown depict (A) normal prostate epithelium [×10], (B) castrate-resistant tissue [×20], (C) prostatic-intraepithelial neoplasia [×10], (D) hormonally-treated prostatic intraepithelial neoplasia [×10], (E) untreated Gleason pattern 4 cancer [×10], and (F) hormonally treated Gleason pattern 4 cancer [×20]. Magnification of the image is shown in brackets.

Expression of c-FLIP in hormone-naive normal prostate epithelium, HGPIN, and prostate cancer tissue. c-FLIP was detected at low levels in NPE (Supplementary Table S2; Fig. 1A). There was no statistically significant difference in the expression of c-FLIP in the adjacent normal tissue between Gleason pattern 3+3 or Gleason pattern 4+4 cancer. Upon the transition to HGPIN, we observed a statistically significant increase in mean H-score for c-FLIP relative to expression in NPE in untreated Gleason score 3+3 = 6 group ($P = 0.008$) and normal untreated Gleason score 4+4 = 8 group ($P = 0.008$; Supplementary Table S2; Fig. 1C). However, as previously observed in normal tissue, the Gleason score of the adjacent cancer did not affect the level of c-FLIP expression detected within HGPIN. There was no observed increase in the expression of c-FLIP in cancer relative to HGPIN; the mean H-score value was 193 ± 4 in the untreated Gleason score 3 + 3 = 6 cancers and 202 ± 15 in the untreated Gleason score 4 + 4 = 8 cancers (Supplementary Table S2; Fig. 1E).

Effect of hormone treatment upon c-FLIP expression. Hormone treatment had no effect on c-FLIP expression in NPE. However, there was a statistically significant decrease in c-FLIP expression in HGPIN foci within hormonally treated prostate tissue relative to untreated HGPIN tissue ($P < 0.001$; Fig. 1D). Similarly, c-FLIP expression was significantly lower in cancers treated for 3 months with hormonal therapy (Fig. 1F); this decrease was also statistically significant when compared with c-FLIP expression in the untreated Gleason score 3 + 3 = 6 and hormone-treated groups ($P = 0.001$) and between the untreated Gleason score 4 + 4 = 8 and hormone-treated groups ($P = 0.001$). Of major significance, the expression of c-FLIP was elevated in locally recurrent castrate-resistant tissue, with a mean H-score of 282.9 ± 12.13. This increase in the level of expression in castrate-resistant tissue was statistically significant when compared with expression in untreated Gleason score 3 + 3 = 6 cancers ($P = 0.001$), untreated Gleason score 4+4 = 8 cancers, and that detected in hormonally treated cancers ($P < 0.001$; Fig. 1B).

Silencing of c-FLIP induces spontaneous apoptosis in prostate cancer cells

The significance of c-FLIP expression to prostate cancer cell viability was studied *in vitro* using a previously validated c-FLIP–targeted siRNA strategy (14). We used splice form–selective oligonucleotides (FL and FS, respectively) to target the 2 predominant splice variants expressed in human cells, c-FLIP$_L$ and c-FLIP$_S$, and a nonselective oligonucleotide (FT) that targets both c-FLIP splice forms. Transfection of 22Rv1 (left) and LNCaP cells (right) with increasing concentrations of the nonselective FT-siRNA resulted in a dose-dependent increase in the apoptotic cell population (Fig. 2A), compared with the effects of a nontargeting-siRNA (NT-siRNA) control. Immunoblotting confirmed the selectivity of the respective siRNAs used and, secondly, confirmed enhanced PARP cleavage, consistent with apoptosis, in cells transfected with the dual c-FLIP$_{L/S}$–targeting FT siRNA (Fig. 2B, left and right panels; Supplementary Fig.

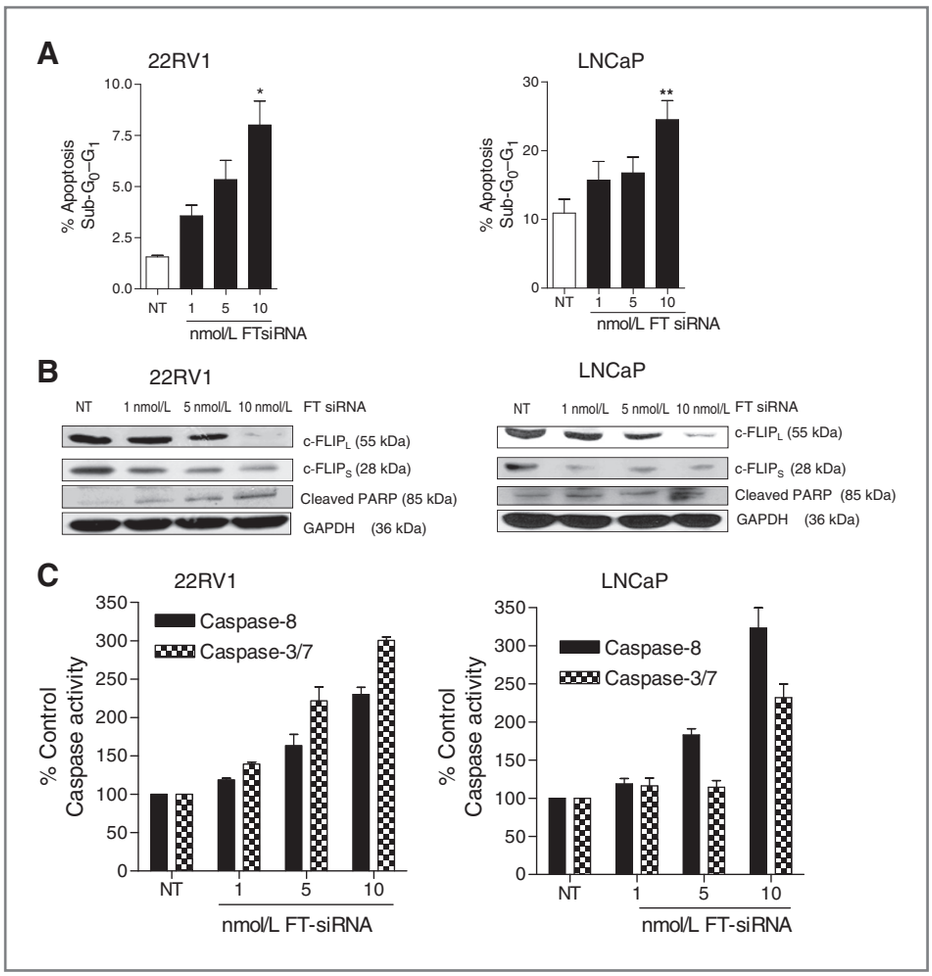

Figure 2. Silencing of c-FLIP induces spontaneous apoptosis in prostate cancer cells. A, histograms showing a dose-dependent induction of apoptosis following FT-siRNA targeted silencing of c-FLIP for 24 hours in 22Rv1 (left) and LNCaP cells (right, respectively). B, immunoblots illustrating the specificity of the siRNA pools in decreasing c-FLIP expression and the resultant cleavage of PARP in 22Rv1 (left) and LNCaP cells (right). Membranes were reprobed with anti-GAPDH to confirm equal loading of protein in all wells. C, bar graphs presenting the levels of caspase-8 and caspase-3/7 activity detected in 22Rv1 (left) and LNCaP cells (right) following transfection with increasing concentrations of the FT-oligonucleotide. All data points represent mean ± SEM, determined from 4 independent experiments. Statistically significant differences were obtained using a Student 2-tailed *t* test; *, $P < 0.05$; **, $P < 0.01$.

S1). We also characterized a dose-dependent increase in caspase-8 and caspase-3/7 activity in 22Rv1 and LNCaP cells (Fig. 2C, left and right panels, respectively). In contrast, 22Rv1 and LNCaP cells displayed a minimal induction of apoptosis upon transfection with either FL-siRNA (c-FLIP$_L$–targeted siRNA) or FS-siRNA (c-FLIP$_S$–targeted siRNA; Supplementary Fig. S1), suggesting that expression of either c-FLIP splice form can maintain the viability of these prostate cancer cell lines.

Silencing of c-FLIP potentiates the level of apoptosis in bicalutamide-treated prostate cancer cells

We next investigated whether knockdown of c-FLIP modulated cellular sensitivity to the AR antagonist bicalutamide. Administration of 10 μmol/L bicalutamide decreased c-FLIP expression in 22Rv1 cells but not to a level sufficient to significantly increase apoptosis (Fig. 3A and B). However, transfection with FT-siRNA significantly increased apoptosis levels in bicalutamide-treated 22Rv1 cells ($P < 0.05$, Fig. 3A and B). In LNCaP cells, bicalutamide failed to induce apoptosis (Fig. 3A, right panel) and had no effect on c-FLIP expression (Fig. 3B, right panel). Bicalutamide-induced apoptosis was significantly increased in LNCaP cells following transfection with FT-siRNA (Fig. 3B). This potentiation of apoptosis was confirmed by measurement of caspase-8 and caspase-3/7 activity. In both 22Rv1 and LNCaP cells (Fig. 3C), the induction of caspase activation was maximal in bicalutamide-treated cells in the presence of the FT-siRNA.

HDAC inhibitors downregulate c-FLIP expression in androgen-dependent prostate cancer cells and potentiate bicalutamide-induced apoptosis

Droxinostat was initially identified by its capacity to potentiate apoptosis in a Fas-resistant prostate cancer cell line because of its ability to repress c-FLIP expression (16). Droxinostat was adopted as an initial pharmacologic approach to target c-FLIP expression in androgen-dependent prostate cancer cells. Administration of droxinostat repressed c-FLIP expression and induced PARP cleavage in 22Rv1 and LNCaP cells at concentrations of 30 and 60 μmol/L, respectively (Supplementary Fig. S2A). Flow cytometry confirmed statistically significant increases in apoptosis in response to droxinostat in 22Rv1 ($P < 0.05$) and LNCaP cells ($P < 0.01$) at these concentrations (Supplementary Fig. S2B). Although bicalutamide was ineffective as

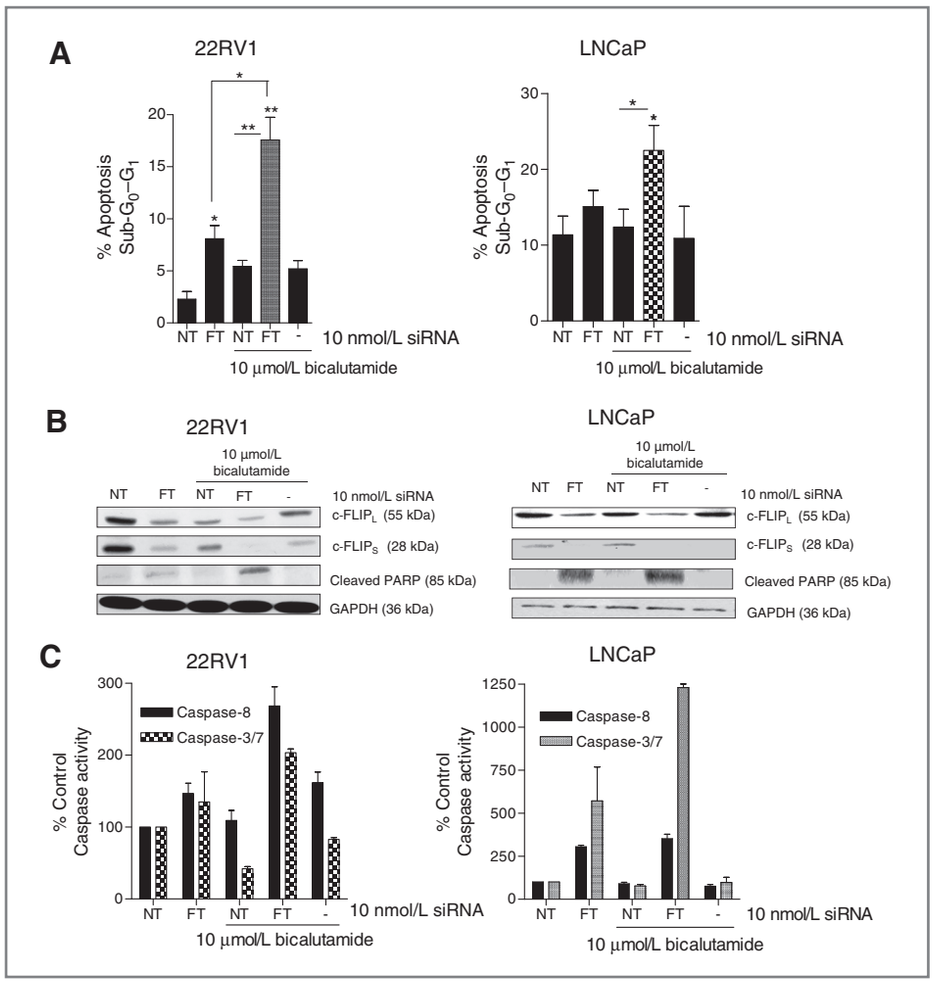

Figure 3. Silencing of c-FLIP potentiates the level of apoptosis in bicalutamide-treated androgen-dependent prostate cancer cells. A, histograms presenting the extent of apoptosis detected in 22Rv1 (left) and LNCaP cells (right) transfected with FT-siRNA and bicalutamide. B, representative immunoblots confirming that c-FLIP expression is reduced in bicalutamide-treated 22Rv1 (left) and LNCaP cells (right) following transfection with the FT-siRNA oligonucleotides and is coupled to enhanced cleavage of PARP protein. Membranes were reprobed with anti-GAPDH to confirm equal protein loading. C, the increased apoptotic index in siRNA-transfected cells treated with bicalutamide is dependent on the activation of caspase-8 and caspase-3/7 in (left) 22Rv1 and (right) LNCaP cells. All data points presented represent the mean ± SEM values, calculated from 4 independent experiments. Statistically significant differences were determined using a Student 2-tailed *t* test; *, $P < 0.05$; **, $P < 0.01$.

a single agent, combination of bicalutamide with droxinostat further increased the level of apoptosis in 22Rv1 cells ($P < 0.001$) and LNCaP cells ($P < 0.05$). Maximal repression of c-FLIP was detected in both cells by combined treatment with droxinostat and bicalutamide (Supplementary Fig. S2C, left and right panels).

In further experiments, we used a more clinically relevant HDACi, SAHA. SAHA also promoted a concentration-dependent decrease in c-FLIP expression that correlated with apoptosis induction, determined by PARP cleavage (Supplementary Fig. S3A). Moreover, SAHA repressed c-FLIP mRNA expression consistent with inhibition of gene transcription (Supplementary Fig. S3B). 22Rv1 cells were especially sensitive to SAHA-induced apoptosis (Supplementary Fig. S3C). Cell viability curves determined the IC_{50} of SAHA as 2.2 μmol/L in 22Rv1 cells and 3.9 μmol/L in LNCaP cells, respectively (Supplementary Fig. S3D).

We next examined the effect of SAHA on the sensitivity of 22Rv1 and LNCaP cells to bicalutamide. In 22Rv1 cells, the apoptosis induced by 0.5 or 1 μmol/L SAHA was significantly increased in cells cotreated with 10 μmol/L bicalutamide; this was paralleled by demonstrable knockdown of c-$FLIP_L$ and c-$FLIP_S$ expression in these cells and by an enhanced level of cleaved PARP protein (Fig. 4A and B). Likewise, in LNCaP cells, SAHA promoted a significant increase in apoptosis, either in the absence or presence of bicalutamide, compared with bicalutamide alone ($P < 0.001$; Fig. 4A). Addition of 2 μmol/L SAHA to bicalutamide (10 μmol/L)-treated cells increased apoptosis levels from 4.0 ± 0.4% to 13.0 ± 0.8% ($P < 0.001$). Combination of a lower concentration of SAHA (1 μmol/L) with bicalutamide also increased apoptosis levels compared with bicalutamide alone ($P < 0.001$). However, apoptosis levels observed in SAHA/bicalutamide-treated cells was not different from that in SAHA-treated cell populations, as determined by PI- or Annexin V–based detection (Supplementary Fig. S4). Immunoblotting experiments showed that the combination of SAHA with bicalutamide decreased c-FLIP isoform expression (Fig. 4B).

The relevance of caspase activation in drug-induced apoptosis was addressed. In 22Rv1 cells, SAHA alone or in combination with bicalutamide (10 μmol/L) increased caspase-8 and caspase-3/7 activity relative to vehicle control or treatment with bicalutamide alone. At the highest concentration of SAHA, cotreatment induced a rise of caspase-8

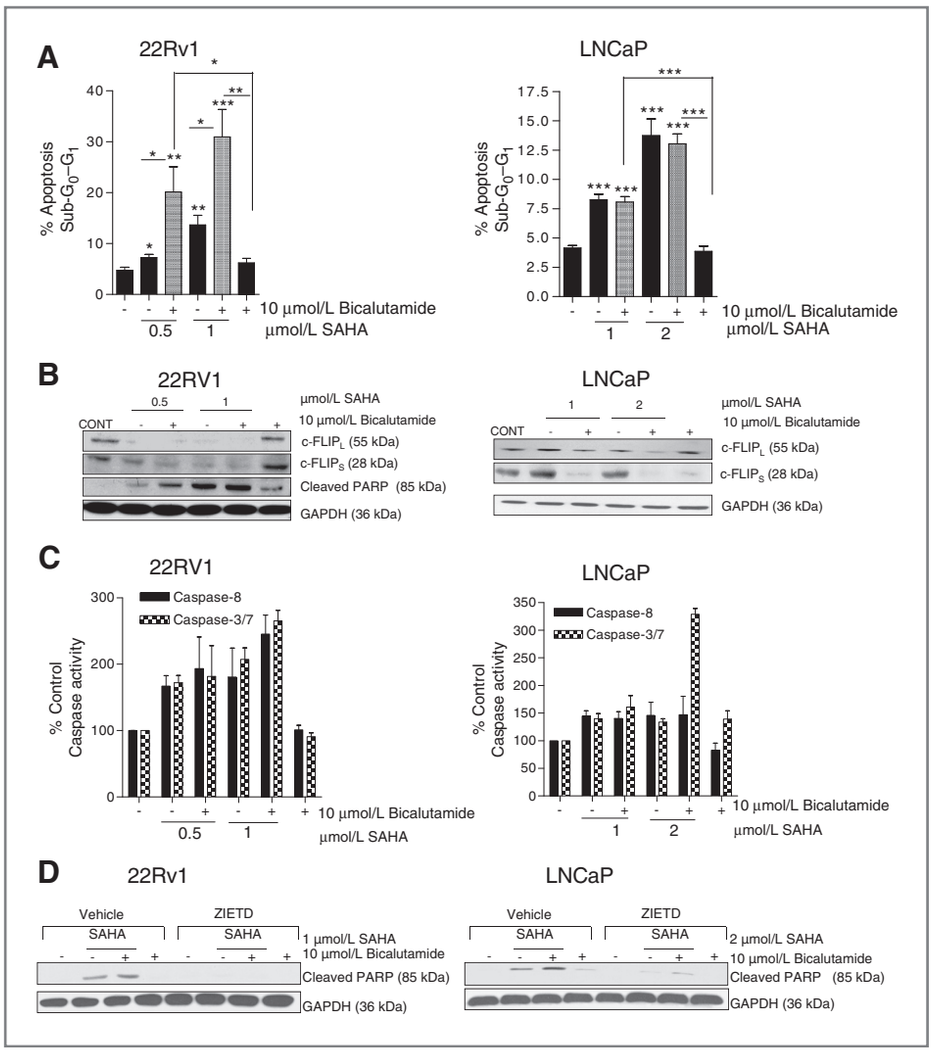

Figure 4. SAHA downregulates c-FLIP expression and increases the sensitivity of prostate cancer cells to undergo apoptosis in the presence of bicalutamide. A, bar graph illustrating the sub-G_0/G_1 cell population in 22Rv1 (left) and LNCaP (right) cells in the absence and presence of 10 μmol/L bicalutamide, administered as a single agent or in combination with increasing concentrations of the HDAC inhibitor, SAHA. B, immunoblots showing the single-agent activity of bicalutamide or SAHA or effect upon their combined administration on the expression of c-FLIP and/or the processing of PARP. Equal protein loading was confirmed by GAPDH. C, bar graph illustrating the effect of bicalutamide and SAHA upon the induction of caspase-8 or caspase-3/7 activity in 22Rv1 cells (left) and LNCaP cells (right). D, immunoblots characterizing the impact of SAHA and/or bicalutamide upon the induction of PARP cleavage, in the absence or presence of the pan-caspase inhibitor, ZIETD. ZIETD was incubated with the cells for 12 hours before the experiment. All data points shown in A and C represent the mean ± SEM. value, calculated from 4 independent experiments. Statistically significant differences were obtained using a Student 2-tailed *t* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and caspase-3/7 activity to 245.3 ± 28.9% and 265.5 ± 15.6%, respectively (Fig. 4C, left panel). Similarly, in LNCaP cells, caspase activity was augmented in response to SAHA and SAHA/bicalutamide, in which there was a more than 3-fold increase in caspase-3/7 activity with 2 μmol/L SAHA in combination with bicalutamide (Fig. 4C, right panel). To confirm the importance of caspase-8 in initiating drug-induced apoptosis, caspase-8 activity was inhibited by the addition of Z-IETD-fmk before the drug treatments (Fig. 4D). In the absence of Z-IETD-FMK, SAHA, and SAHA/bicalutamide induced PARP cleavage in 22Rv1 and LNCaP cells, however, SAHA- or SAHA/bicalutamide-induced cleavage of PARP was completely abrogated in 22Rv1 cells and attenuated in LNCaP cells following pre-treatment with Z-IETD-FMK (Fig. 4D).

SAHA modulates AR and NF-κB pathways in prostate cancer cells

We next examined how SAHA impacted upon AR and NF-κB activity, given their importance in modulating c-*FLIP* gene transcription. Concentrating on the AR pathway initially, we confirmed a higher level of AR expression and activity in 22Rv1 cells relative to LNCaP cells (Supplementary Fig. S5A and B). SAHA decreased AR transcript levels and AR protein expression in both cell lines. In combination experiments, AR mRNA (Fig. 5A) and protein levels (Fig. 5B) were further downregulated in cells cotreated with SAHA and bicalutamide. Furthermore, in 22Rv1 cells, addition of 1 μmol/L SAHA was as effective as the AR antagonist bicalutamide (10 μmol/L) in decreasing ARE-driven luciferase activity (to 77% of control). Combination of these 2 inhibitors had an additive effect in repressing AR activity to 54.8% of control ($P < 0.05$ relative to individual treatments; Fig. 5C, left panel). In contrast, whereas bicalutamide decreased AR activity in LNCaP cells, SAHA increased ARE-driven luciferase activity to 144.7 ± 10.1% of control. Cotreatment with SAHA and bicalutamide resulted in no overall change in AR activity relative to untreated control LNCaP cells (Fig. 5C, right panel).

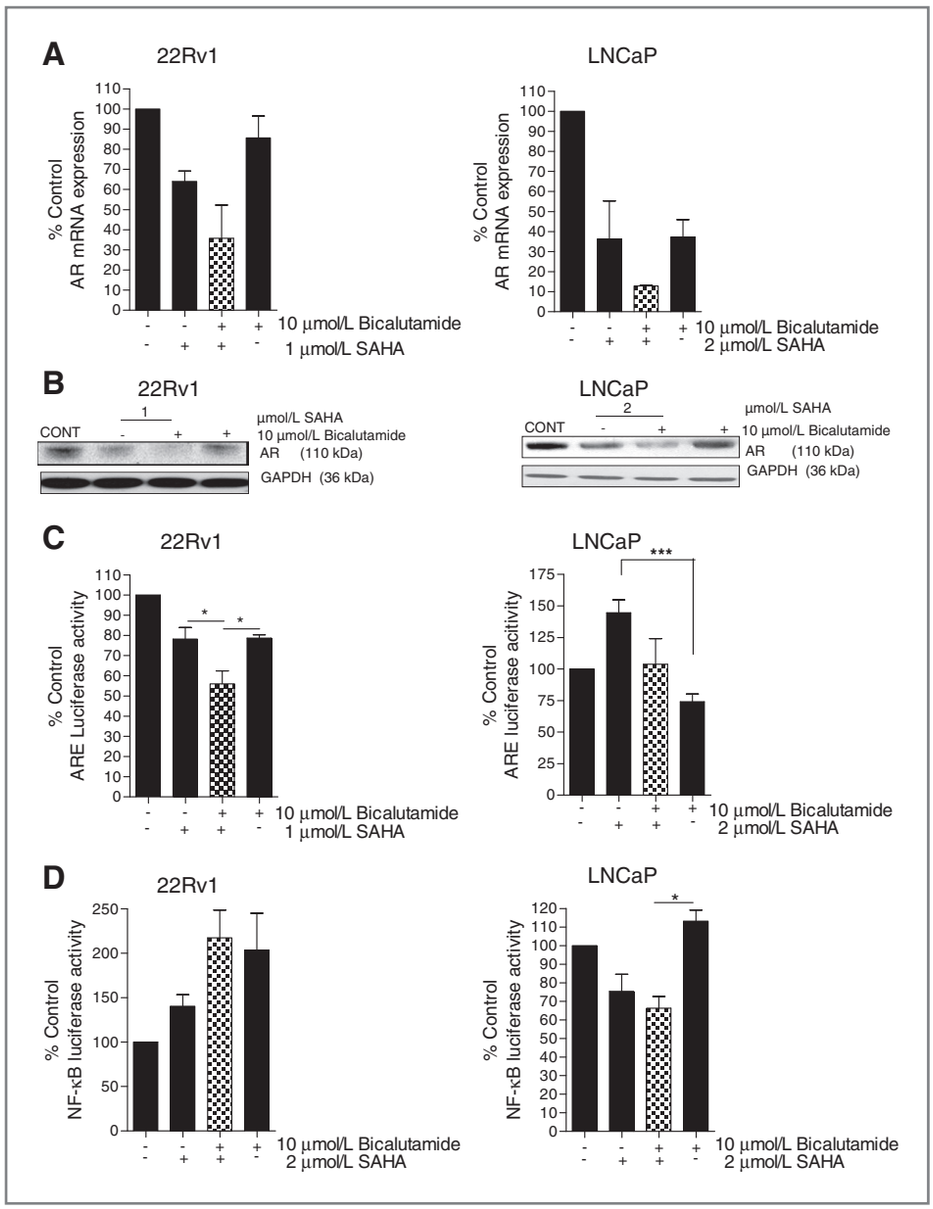

Figure 5. SAHA modulates the AR and pathway in androgen-dependent prostate cancer cells. A, bar graphs illustrating the effect of SAHA, bicalutamide, or their combination upon the transcript levels encoding the *AR* gene in 22Rv1 cells (left) or LNCaP cells (right). B, immunoblots illustrating the effect of SAHA, bicalutamide, or their combined administration upon the expression of the AR in 22Rv1 (left) or LNCaP cells (right). Membranes were reprobed with anti-GAPDH to ensure equal loading of protein in the wells. C, bar graphs showing the effect of SAHA, bicalutamide, or a combined SAHA/bicalutamide administration upon AR activity in 22Rv1 (left) and LNCaP cells (right). D, bar graphs illustrating the effect of SAHA, bicalutamide, or their combined administration upon NF-κB transcriptional activity in 22Rv1 and LNCaP cells (left and right, respectively). All data points shown in bar graphs represent the mean ± SEM value, calculated from a minimum of 3 to 6 independent experiments. Immunoblots are representative of at least 3 experiments. Statistically significant differences between values were determined using a Student 2-tailed *t* test; *, $P < 0.05$; ***, $P < 0.001$.

Increased NF-κB transcriptional activity is implicated in the development of castrate resistance (17, 18) and underpins transcription of the *AR* (19), *c-FLIP* (20, 21), and *PSA* (22) genes. Intrinsic levels of NF-κB activity were approximately 2-fold higher in LNCaP cells relative to 22Rv1 cells (Supplementary Fig. S5C). Administration of 1 μmol/L SAHA or 10 μmol/L bicalutamide, alone or in combination, increased NF-κB activity in 22Rv1 cells (Fig. 5D, left panel). In contrast, the combination of SAHA and bicalutamide reduced NF-κB activity to 65% of control levels in LNCaP cells (Fig. 5D, right panel).

The cell-specific effects of SAHA and bicalutamide on AR and NF-κB activity was investigated by reverse transcriptase PCR (RT-PCR) analysis of downstream genes. Consistent with the SAHA/bicalutamide combination reducing NF-κB activity in LNCaP cells, we observed a reduction in Bcl-2 mRNA transcript levels for Bcl-2 in LNCaP cells but not 22Rv1 cells (Supplementary Fig. S5D). In further analysis, SAHA induced expression of the AR- and NF-κB–regulated gene *PSA* in either cell line, consistent with SAHA-induced AR activity in LNCaP cells and NF-κB activity in 22Rv1 cells. The combination of SAHA with bicalutamide reduced *PSA* gene expression back to levels detected in untreated 22Rv1 cells or to below basal levels in LNCaP cells (Supplementary Fig. S5E). Finally, RT-PCR analysis confirmed that the SAHA/bicalutamide combination reduced *CXCL8* gene expression in LNCaP cells consistent with the reduction of NF-κB activity. In contrast, due to drug-induced elevation of NF-κB activity in 22Rv1 cells, *CXCL8* gene expression increased in this model (Supplementary Fig. S5F).

Increased expression of c-FLIP correlates with cellular insensitivity to bicalutamide and SAHA

VCaP is a CRPC cell model (23). VCaP cells exhibited a significantly higher level of AR activity (~4-fold) compared with 22Rv1 cells (Supplementary Fig. S6A). Immunoblots also confirmed a higher level of c-FLIP$_S$ and BCL-2 expression in VCaP cells relative to 22Rv1 and LNCaP cells (Supplementary Fig. S6B). VCaP cells were more tolerant to SAHA treatment; cell viability assays determined an IC_{50} value of 9.2 μmol/L (data not shown). SAHA was ineffective in inducing apoptosis (Supplementary Fig. S6C), in downregulating c-FLIP expression at concentrations less than 10 μmol/L (Supplementary Fig. S6D) and consistently reduced caspase-8 and caspase-3/7 activity across the concentration range (Supplementary Fig. S6E). At higher concentrations, SAHA (10 μmol/L) alone or in combination with bicalutamide was shown to decrease mitochondrial TMRE retention, indicative of mitochondrial outer membrane permeabilization (MOMP). Bicalutamide did not affect MOMP (Supplementary Fig. S6F). This suggested that at higher concentrations, SAHA alone or in combination with bicalutamide, induces a mitochondrial-dependent, caspase-independent mode of cell death.

SAHA was unable to restore sensitivity to AR-directed therapy in VCaP cells; use of SAHA at a clinical achievable concentration of 1 μmol/L with bicalutamide did not induce apoptosis (Fig. 6A) or downregulate the expression of antiapoptotic c-FLIP protein (Fig. 6B) and was incapable of potentiating caspase-8 or caspase-3/7 activity in this cell line (Fig. 6C). Furthermore, the combination of SAHA with bicalutamide was unable to reduce AR or NF-κB activity, measured by *PSA* and *Bcl-2* gene readouts (Supplementary Fig. 7A and B). To investigate the role of c-FLIP overexpression in modulating the survival and therapeutic resistance of VCaP cells, cells were transfected with the FT-siRNA before exposure to bicalutamide. Loss of c-FLIP alone significantly increased apoptosis in these CRPC cells (Fig. 6D). In addition, we detected an equivalent level of apoptosis in c-FLIP-depleted VCaP cells treated with bicalutamide. This experiment was repeated using the androgen-ablated derivative of the LNCaP cells, the LN-Abl model (24). Consistent with prior reports, LN-Abl cells had increased AR activity relative to LNCaP cells, but did not attain the AR activity observed in VCaP cells (Supplementary Fig. S7C). Correspondingly, we observed an intermediate level of c-FLIP expression in this model (Supplementary Fig. S7D). Although bicalutamide reduced the level of death in these cells, the knockdown of c-FLIP was shown to once again potentiate apoptosis (Supplementary Fig. S7E).

Discussion

c-FLIP is an antiapoptotic protein which antagonizes caspase-8–mediated apoptosis. Transcription of the c-*FLIP*

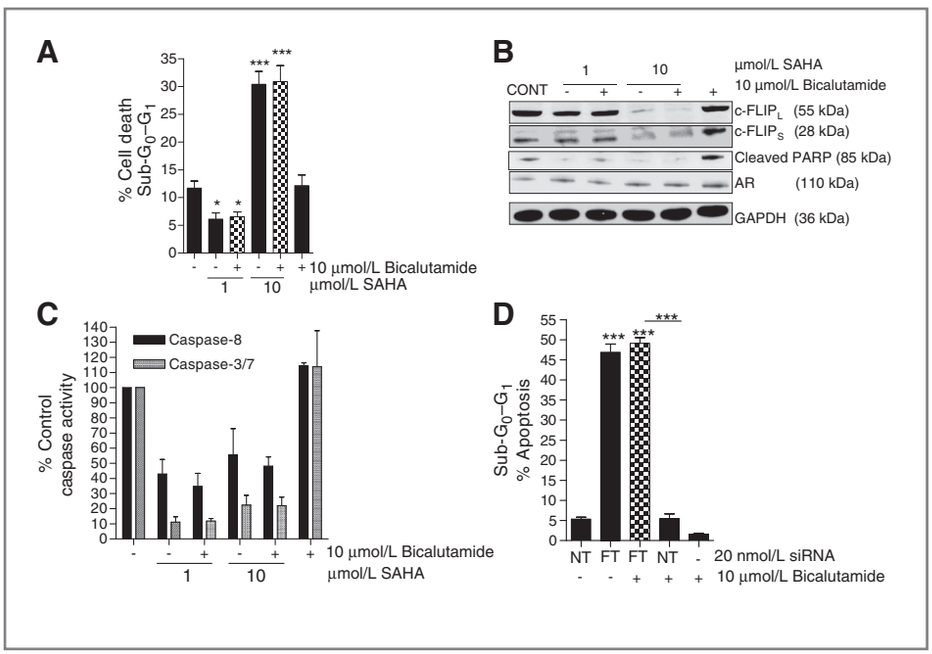

Figure 6. Castrate-resistant VCaP cells are insensitive to bicalutamide and SAHA. A, bar graph showing the percentage apoptosis detected in VCaP cells treated with 1 or 10 μmol/L SAHA, in the absence or presence of 10 μmol/L bicalutamide. B, immunoblot characterizing the level of c-FLIP or AR expression or the promotion of PARP cleavage in VCaP cells following treatment with SAHA in the absence or presence of bicalutamide at the indicated concentrations. Membranes were reprobed with anti-GAPDH to ensure equal loading. C, bar graph illustrating the level of caspase-8 and caspase-3/7 activity in SAHA, bicalutamide, or SAHA/bicalutamide-treated VCaP cells. D, bar graph showing the effect of a FT-siRNA to modulate the sensitivity of VCaP cells to bicalutamide. All data points shown in the figure represent the mean ± SEM value, calculated from a minimum of 3 to 5 independent experiments. Statistically significant differences were determined using a Student 2-tailed *t* test; *, $P < 0.05$; ***, $P < 0.001$.

gene (*cFLAR*) in prostate cancer cells has been shown by us and others to be in part under AR regulation (9–11), suggesting that this antiapoptotic protein may constitute an important component of androgen-driven, AR-mediated survival. *c-FLIP* gene expression is also regulated by NF-κB (20, 21), a transcription factor whose activation is elevated in more aggressive prostate cancers and has been associated with the transition to castrate resistance (17, 18, 25, 26). Therefore, we proposed that c-FLIP may be relevant to all stages of disease progression and may modulate the onset of resistance to AR-targeted therapeutics enabling the outgrowth of CRPC cells. Although we have previously shown that c-FLIP confers resistance of metastatic prostate cancer cells to docetaxel, TRAIL, and oxaliplatin (27), its relevance to AR-targeted therapeutics has not been previously investigated.

Our data shows that c-FLIP expression is increased in premalignant PIN foci and is retained in untreated Gleason pattern 3 and 4 cancers. Silencing c-FLIP using siRNA oligonucleotides resulted in a spontaneous apoptosis in 2 prostate cancer cell lines, 22Rv1 and LNCaP, supporting an important role of this protein in maintaining cell survival. Splice form-selective siRNA oligonucleotides further indicated that both of the predominant human isoforms of this protein have equal importance in regulating prostate cancer cell survival. Therefore, the increased c-FLIP expression may enable cancer cells to remain viable in the face of the accumulating genetic instability present within HGPIN and locally confined disease.

We have further defined the significance of c-FLIP as a key regulator of ADT response. Analysis of prostatectomy tissue confirmed that hormonal treatment can reduce c-FLIP expression, consistent with AR-regulated expression of this gene. *In vitro*, bicalutamide only partially reduced c-FLIP expression in prostate cancer cells and therefore had a limited effect in inducing apoptosis, even when administered at concentrations up to 10 μmol/L. However, siRNA-mediated silencing of c-FLIP resulted in a marked potentiation of apoptosis in bicalutamide-treated 22Rv1 cells while underpinning the high levels of spontaneous apoptosis induced in bicalutamide-treated LNCaP cells. These results confirm that c-FLIP expression can dampen the sensitivity of prostate cancer cells to undergo ADT-induced apoptosis.

Of major clinical relevance, we also report a very significant increase in the expression of c-FLIP in the tumors of castrate-resistant patients. This increased level of expression in tissue may relate to increased activity of its transcriptional regulators in CRPC cells, and equally, may reflect the selection of therapy-resistant cells as a consequence of high c-FLIP expression. I*n vitro* experiments conducted on 2 cell-based models of CRPC clearly show that the overexpression of c-FLIP is fundamental to the viability of these cells, given the prominent induction of spontaneous apoptosis in these cells following siRNA targeting of c-FLIP. Thus, repressing the expression of c-FLIP may be an appropriate strategy to target what may be an Achilles heel of CRPC.

HDACis have been shown to repress c-FLIP expression in cancer cells (15, 28–31). We used droxinostat and SAHA (Vorinostat) as representative HDACi to modulate c-FLIP expression and determine the impact on bicalutamide sensitivity in AR-expressing prostate cancer cells. Droxinostat and SAHA both induced spontaneous apoptosis in 22Rv1 and LNCaP cells, coinciding with a potent downregulation of both c-FLIP isoforms and moreover, potentiated or retained the maximal level of apoptosis observed in bicalutamide-treated 22Rv1 and LNCaP cells. The mode-of-action of SAHA was observed to be complex and cell specific. SAHA-promoted downregulation of c-FLIP expression paralleled the downregulation of c-FLIP transcript levels in prostate cancer cells, consistent with a predominantly transcription-mediated regulation of this gene. SAHA was subsequently shown to decrease the transcriptional activity of AR or NF-κB signaling in a cell line–specific manner, consistent with promoting decreased transcription of c-FLIP in the prostate cancer cells. In the 22Rv1 cell line, the synergy in apoptosis induction resulting from the combination of bicalutamide with HDACi reflects the capacity of SAHA to attenuate, but not abrogate, AR transcriptional activity, with the further reduction in AR transcription and survival mediated by the presence of bicalutamide. In contrast, SAHA had greater effect in attenuating NF-κB as opposed to AR activity in LNCaP cells. Therefore, in LNCaP cells, it is apparent that SAHA-induced apoptosis results predominantly from the reduction in NF-κB signaling and consequently, the addition of bicalutamide provides no further enhancement of apoptotic induction in this model. Therefore the combination of SAHA and bicalutamide is effective in attenuating the activity of 2 major transcription factors implicated in the progression to CRPC and sensitizes cells to undergo an increased level of apoptosis irrespective of the principal progression-driving pathway in the tumor.

HDACis have been trialled in CRPC but with limited success (32). Using VCaP as a model of CRPC, we found that HDACis were unable to downregulate c-FLIP expression, suggesting that this class of agent may be unable to surmount the high level of AR and NF-κB signaling present in CRPC. Targeted knockdown of c-FLIP using the FT-siRNA, however, did induce a spontaneous apoptosis in CRPC cells, indicating that the elevated c-FLIP expression and an output of AR and NF-κB signaling in this castrate-resistant cell line may render these cells resistant to apoptosis following treatment with HDACi. Previous studies have shown that elevated BCL-2 expression in VCaP cells diminishes the sensitivity of PC3 cells to HDACi *in vitro* (33). However, our studies show that c-FLIP overexpression is equally important in underpinning the resistance of VCaP cells and CRPC to AR- and molecularly targeted therapeutics. Targeting the marked overexpression of antiapoptotic proteins in CRPC must therefore be a prime consideration for the development of more effective treatments in this currently incurable condition.

In summary, we have shown that c-FLIP expression is increased in premalignant and malignant prostate cancer, with further increased expression detected within castrate-

resistant tumors. Prostate cancer cells exploit the increased expression of c-FLIP to maintain their viability and to diminish the sensitivity of the cells to androgen ablation therapy. Although HDACi can downregulate c-FLIP expression through downregulation of AR and NF-κB activity and increase the sensitivity of androgen-dependent prostate cancer cells to undergo caspase-8- and caspase-3/7–promoted apoptosis in the presence of the AR antagonist bicalutamide, our data indicates that there are levels to which this strategy is effective. The elevated expression of c-FLIP in the castrate-resistant VCaP cells and CRPC is consistent with these cells being insensitive to HDACi *in vitro* and the suboptimal performance of HDACi in trials conducted on metastatic prostate cancer patients, respectively. Consistent with a prior publication (34), our data suggests that an earlier introduction of HDACi may increase the effectiveness of bicalutamide in treating progressive prostate cancer, in which increasing response rates or the duration of response to androgen-targeted therapies would significantly impact on patient outcomes, retarding the rate and incidence of progression to castrate-resistant disease. Moreover, the expression of c-FLIP in diagnostic tissue may assist in predicting response to androgen-deprivation therapies, in which elevated expression of this protein is indicative of resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. McCourt, R. Mazzucchelli, R. Montironi, D.B. Longley, D.J.J. Waugh

Writing, review, and/or revision of the manuscript: C. McCourt, P. Maxwell, M. Scarpelli, M.S. Tellez, J.M. O'Sullivan, D.B. Longley, D.J.J. Waugh

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Study supervision: P. Maxwell, D.B. Longley, D.J.J. Waugh

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