Transcription Factor Stat5 Knockdown Enhances Androgen Receptor Degradation and Delays Castration-Resistant Prostate Cancer Progression In vivo

Christian Thomas1,2, Amina Zoubeidi1, Hidetoshi Kuruma1, Ladan Fazli1, Francois Lamoureux1, Eliana Beraldi1, Brett P. Monia3, A. Robert MacLeod3, Joachim W. Thüroff2, and Martin E. Gleave1

Abstract

Signal transducer and activator of transcription 5 (Stat5) plays an important role in the transition of prostate cancer (PCa) to its castrate-resistant state. Pharmacologic targeting of Stat5 is a rational approach to delay castrate-resistant progression, in part, because Stat5 cooperates with the androgen receptor (AR) to promote PCa progression. Immunostaining of tissue microarrays was used to correlate Stat5 expression with Gleason grade and to characterize changes in treatment-naive and androgen-deprived human PCa. Potency of a Stat5 antisense oligonucleotide (ASO) on Stat5 knockdown, cell growth, and apoptosis was assessed in LNCaP, C4-2, and DU145 cells. Effects of Stat5 knockdown on AR activity and stability was assessed using a PSA transactivation-luciferase assay and cyclohexamide plus MG132 treatment, respectively. LNCaP tumor-bearing mice were castrated and randomly assigned to treatment with Stat5-ASO or controls. Here, we show that the frequency of Stat5 expression is significantly increased in high Gleason grade as well as in hormone-treated PCa. Also, specific knockdown of Stat5 with ASO abrogates androgen-induced AR nuclear translocation and PSA transactivation despite R1881 stimulation. Moreover, Stat5 knockdown destabilizes AR, which leads to AR degradation via the proteasome. Shown for the first time as a preclinical proof-of-principle, Stat5 knockdown with Stat5-ASO significantly delays CRPC tumor progression in vivo. Thereby, we are able to recapitulate our in vitro results by reducing serum PSA and expression levels of target proteins in the xenograft tumors. Mol Cancer Ther; 10(2); 347–59. ©2011 AACR.

Introduction

Although highly effective and in most cases curative treatment options exist for clinically localized prostate cancer (PCa), treatment options for recurrent and metastatic PCa are limited and mostly palliative (1, 2). Androgen deprivation therapy (ADT) remains the most effective therapy for men with advanced disease, and although up to 80% show initial response, castration-resistant prostate cancer (CRPC) progression almost invariably occurs within 24 months. Androgen ablation induces apoptosis in subpopulations of PCa cells, but despite high initial response rates, remissions are temporary because surviving tumor cells usually recur with an AI phenotype (3–5). CRPC progression is a complex process by which cells acquire the ability to both survive and proliferate in the absence of androgens and involves variable combinations of clonal selection, adaptive upregulation of anti-apoptotic genes, and alternative growth factor pathways (6–18). Molecular targeting at this stage seems to be a promising approach (19).

The signal transducer and activator of transcription (Stat5) signaling cascade is known to play a pivotal role in the progression of PCa (20–23). Stat5 expression in human PCa tissue correlates with high histological grades of PCa and predicts early disease recurrence after initial treatment with radical prostatectomy (24, 25). Furthermore, ADT increases the frequency of Stat5 expression in human PCa (26). Stat5a is involved in the maintenance of prostate epithelial structures whereas Stat5b mediates cell growth (21, 27). In vitro, Stat5 promotes PCa proliferation by increasing the expression levels of cyclin D1 and Bcl-xL (23) and transcriptional activity of the androgen receptor (AR; ref. 26). This finding is of major interest as AR signaling pathway remains active in CRPC despite low levels of circulatory androgens after ADT (28, 29). The functional importance
of Stat5 in PCa in vivo has been shown in Stat5b-negative C2H cells that were unable to form tumors (21). Moreover, tumor growth in vivo was inhibited in CWR22Rv cells infected with adenovirus expressing dominant-negative Stat5a/b (23). The objectives of the current study were to evaluate changes in Stat5 after ADT and during development of CRPC in human and xenograft tissues and to test whether targeted knockdown of Stat5 can delay CRPC progression in xenograft PCa models. We show that the frequency of Stat5 expression in human PCa is significantly increased after ADT and in castrate-resistant tumors. Interestingly, specific knockdown of Stat5 abrogates nuclear translocation of AR, decreases AR protein stability, and induces its degradation via the proteasome. We also show that treatment with second generation antisense oligonucleotides (ASO) targeting Stat5 significantly delays CRPC tumor progression in vivo.

Materials and Methods

Prostate cancer cell lines and reagents

Stat5-positive human prostate cancer cell lines LNCaP, C4-2, and DU 145 were used. DU 145 cells were purchased from the American Type Culture Collection (ATCC) 1989, ATCC authentication by isoenzymes analysis. LNCaP and C4-2 cells were kindly provided by Dr. Leland W.K. Chung (1992, University of Texas MD Anderson Cancer Center, Houston, TX) and tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer IIX platform in July 2009. All 3 cell lines were passaged for less than 3 months after resuscitation. Western blotting and/or real time PCR was done for AR and PSA each time when LNCaP or C4-2 cells were resurrected. LNCaP and C4-2 cells were maintained in RPMI 1640 medium (Invitrogen) and DU 145 cells in DMEM medium (Invitrogen), each supplemented with 5% FBS. All cell lines were cultured without antibiotics at 37°C in 5% CO2 atmosphere.

Anti-Stat5 (sc-835), anti-Stat3 (sc-8019), anti-cyclin D1 (sc-718), anti-PSA (sc-7638), anti-AR (sc-816), and anti-Bcl-xL (sc-8392) were antibodies from Santa Cruz Biotechnology, PARP was assessed with an antibody (9542) from Cell Signaling Technology, vinculin with an antibody (sc-114) from Sigma-Aldrich, glyceraldehyde 3 phosphate dehydrogenase (GAPDH) with an antibody (NB-300-322) from Novus Biologicals, and actin with an antibody (MAB 1501R) from Millipore.

Tissue microarray construction and immunohistochemistry

A total of 163 PCa specimens (143 primary PCa per 20 CRPC) were obtained from the Vancouver Prostate Centre Tissue Bank after written informed consent and institutional study approval. The 143 patients with primary PCa underwent radical prostatectomy. Of those, 54 (37.8%) received preoperative ADT as short term (n = 16, average 1.91 months (range, 1–3 months); or long-term (n = 38, average 8.35 months (range, 5–12 months)). In 20 patients with CRPC, tissue was obtained by transurethral resection.

Tissue microarrays were created using a manual tissue microarrayer (Beecher Instruments). Each marked block was sampled twice with a core diameter of 1.0 mm arrayed in a rectangular pattern with 1.0 mm between the centers of each core, creating a duplicate tissue microarray layout. Immunohistochemical stains were done on formalin-fixed and paraffin-embedded 4-μm sections of the tissue microarray. Using polyclonal rabbit antibody against Stat5 (sc-835; Santa Cruz Biotechnology), immunohistochemical staining was conducted by Ventana autostainer model Discover XT (Ventana Medical System) with an enzyme-labeled biotin streptavidin system and solvent-resistant 3,3’-diaminobenzidine Map kit. The samples were scored by a pathologist (L.F.) for the level of immunoeexpression of Stat5 on a scale of 0 to 1, wherein 0 was undetectable and 1 represented positive staining. All comparisons of staining intensities were made at 200× magnifications.

Treatment of prostate cancer cells with oligonucleotides

A 2'-methoxyethyl-modified phosphorotiate ASO for the coding region of Stat5 and Stat5b (ISIS 424751) was synthesized by ISIS Pharmaceuticals. The sequence of the Stat5-ASO is 5’-TCCACGGCGGTACTGCTGCAG-3’. The control oligonucleotide (ScrB) used is designed not to match any mRNA in human or mouse transcriptomes and has the sequence 5’-CCCTCCCTGAGGTCTCC-3’. C4-2 and DU145 cells were plated at the density of 1.25 × 10⁴ per cm² (LNCaP 2.5 × 10⁴ per cm²) and treated a day later at a confluence of 50% to 60% for 2 consecutive days with ISIS 424751 or ScrB. Transfection was done in serum-free OPTI-MEM (Invitrogen Life Technologies, Inc.) for 4 hours, allowed to recover in standard culture media for 20 hours. Oligofectamine (Invitrogen), a cationic lipid, was used as a transfection reagent.

Cell growth assays

Cell growth was assessed using crystal violet assay, as described previously (30). Cells were plated in 12-well plates and treated with ASO as described previously. Crystal violet staining was carried out for dose-dependent treatment 3 days posttransfection and for timecourse 1, 2, and 3 days posttransfection. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware) at 562 nm. Cell survival after ASO treatment was calculated as the percentage of the absorbance in vehicle-treated cells.

Apoptosis assays

Detection and quantitation of apoptotic cells were done by flow cytometric analysis. Cells were plated in 10-cm² dishes and transfected as described previously. Two days later, cells were trypsinized and fixed in 70% ethanol.
overnight at 4°C and then incubated with 1 μg/mL RNase (Sigma) for 30 minutes at 37°C before being stained with 5 μg/mL propidium iodide (PI; Sigma) for 30 minutes at room temperature. The stained cells were analyzed for relative DNA content on a dual laser flow cytometer (Beckman Coulter Epics Elite; Beckman, Inc.). Each assay was done in triplicate.

Caspase-3 activity was assessed 2 days after treatment using the kit CaspACE Assay System, Fluorometric (Promega). Aliquots of 50 μg of total cell lysate were incubated with caspase-3 substrate AC-DEVD-AMC at 37°C before being stained with 5/14 nmol/L R1881 for 15 minutes and thereafter with 30 nmol/L Stat5 ASO as described previously. Two days after second transfection, cells were treated with 30 nmol/L 2 days after second transfection using TRIzol reagent (Invitrogen Life Technologies, Inc.). After DNase I (Invitrogen) treatment, total RNA (2 μg) was reversed transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). For detection of Stat5b complementary DNA (cDNA), we used DNA primers 5'-AAGTCGAGAGCAACCGGGGC-3' (forward) and 5'-TGTGGTGGGATCCACTG-3' (reverse) for AR 5'-TACCACTCACAAGTCTCTC-3' (forward) and 5'-GCTTCACTGGGTGGAAAT-3' (reverse), and for 80 ng/mL to 20 ng/mL nonfat milk before AR antibody addition overnight (1:250). Antigens were visualized using anti-rabbit antibodies coupled to FITC or rhodamine (1:500; 30 minutes). Photomicrographs were taken at 40X magnification using Zeiss Axioplan II fluorescence microscope, followed by analysis with imaging software (Northern Eclipse; Empix Imaging, Inc.).

Reverse transcriptase-PCR
Total RNA was extracted from cultured cells 2 days after transfection using TRIZol reagent (Invitrogen Life Technologies, Inc.). After DNase I (Invitrogen) treatment, total RNA (2 μg) was reversed transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). For detection of Stat5b complementary DNA (cDNA), we used DNA primers 5'-AAGTCGAGAGCAACCGGGGC-3' (forward) and 5'-TGTGGTGGGATCCACTG-3' (reverse) for AR 5'-TACCACTCACAAGTCTCTC-3' (forward) and 5'-GCTTCACTGGGTGGAAAT-3' (reverse), and for 80 ng/mL to 20 ng/mL nonfat milk before AR antibody addition overnight (1:250). Antigens were visualized using anti-rabbit antibodies coupled to FITC or rhodamine (1:500; 30 minutes). Photomicrographs were taken at 40X magnification using Zeiss Axioplan II fluorescence microscope, followed by analysis with imaging software (Northern Eclipse; Empix Imaging, Inc.).

Western blot analysis
Cell population was harvested 3 days posttransfection in RIPA buffer (50 mmol/L Tris, pH 7.2; 1% NP-40; 0.1% deoxycholate; 0.1% SDS; 10 mmol/L NaCl; and Roche complete protease inhibitor cocktail) incubated on ice for 60 minutes and centrifuged at 13,000 rpm for 20 minutes. Forty micrograms of whole cell lysate was subjected to SDS-PAGE transferred to nitrocellulose filters and immunoblotted with the appropriate antibodies. After washing 3 times with washing buffer, membranes were incubated with Alexa Fluor 680 or 800 secondary antibodies (Invitrogen) for 1 hour. Detection of specific bands their densiometric quantification was done using the ODYSSEY IR imaging system (Li-COR Biosciences).

Luciferase assay
LNCaP and C4-2 cells were plated on 12-well plates and transfected with Stat5-ASO or ScrB together with PSA-Luciferase-Plasmid (6.1) using lipofectin (1.5 μL per well; Invitrogen Life Technologies, Inc.). The total amount of PSA-plasmid DNA used was normalized to 0.5 μg per well by the addition of a control plasmid. Cells were transfected at different concentrations for 2 consecutive days. Media were replaced by charcoal-stripped serum (CSS; Thermo scientific)/C6/C14/C2/C0/C before being stained with 5/14 nmol/L R1881 for 15 minutes and thereafter with 30 nmol/L Stat5 ASO as described previously. Two days after second transfection, cells were treated with 30 nmol/L 2 days after second transfection using TRIzol reagent (Invitrogen Life Technologies, Inc.). After DNase I (Invitrogen) treatment, total RNA (2 μg) was reversed transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). For detection of Stat5b complementary DNA (cDNA), we used DNA primers 5'-AAGTCGAGAGCAACCGGGGC-3' (forward) and 5'-TGTGGTGGGATCCACTG-3' (reverse) for AR 5'-TACCACTCACAAGTCTCTC-3' (forward) and 5'-GCTTCACTGGGTGGAAAT-3' (reverse), and for 80 ng/mL to 20 ng/mL nonfat milk before AR antibody addition overnight (1:250). Antigens were visualized using anti-rabbit antibodies coupled to FITC or rhodamine (1:500; 30 minutes). Photomicrographs were taken at 40X magnification using Zeiss Axioplan II fluorescence microscope, followed by analysis with imaging software (Northern Eclipse; Empix Imaging, Inc.).

Protein stability and degradation
To assess the effect of Stat5-ASO on protein stability, cyclohexamide (CHX; Calbiochem; chemical structure see Supplemental Fig. 1A) was added at 10 μg/mL to the culture media of LNCaP and C4-2 cells for 4, 8, and 16 hours following transfection. Thereafter, Western blot was done using AR, Stat5, and GAPDH antibodies. Degradation was tested in LNCaP cells by a 6-hour incubation with RPMI ± 5% FBS media containing MG132 (Calbiochem; chemical structure see Supplemental Fig. 1B) at 10 μmol/L 2 days after second transfection. Western blot was done for AR, Stat5, GAPDH, and vinculin.

Animal treatment
Male athymic nude mice (Harlan Sprague-Dawley, Inc.) were injected s.c. with 2 × 10⁶ LNCaP cells suspended in 0.1 mL Matrigel; BD Biosciences) and castrated when PSA values exceeded 50 ng/mL. Mice were randomly selected for treatment with 12.5 mg/kg ISIS 424751 or ScrB when PSA relapsed to precastration level and

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Mol Cancer Ther; 10(2) February 2011 349

Published OnlineFirst January 7, 2011; DOI: 10.1158/1535-7163.MCT-10-0850

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then injected i.p. once daily for 7 days and 3 times per week thereafter (castrate-resistant model). Each experimental group consisted of 7 mice. Serum PSA measurements were done once weekly by enzymatic immunoassay (Abbott IMX). Data points were expressed as average tumor volume ± SE or average PSA concentration ± SE. Animals were sacrificed after 8 weeks of treatment or earlier when tumor volume reached 10% or more of body weight. After sacrifice, tumors were harvested and 3 samples per group evaluated for protein expression of total Stat5, AR, and cyclin D1. All animal procedures were done according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

**Statistical analysis**

All results were expressed as the average ± SE, and the significance of differences were measured by Student’s t test or Fisher’s exact test (Medcalc software). *, $P \leq 0.05$; **, $P \leq 0.01$; and ***, $P \leq 0.001$ were considered significant.

**Results**

**Stat5 expression is associated with high Gleason grade, ADT, and castration-resistant disease**

To characterize levels of Stat5 expression in primary PCa with progression to CRPC, we analyzed staining patterns in human prostate cancer tissues before and after ADT. Figure 1A illustrates that Stat5 expression is low in untreated tumors but increases after androgen ablation. Stat5 expression is predominantly nuclear with weaker cytoplasmic expression in the PCa cells. Stat5 expression is significantly more frequent (*, $P < 0.05$) in high Gleason grade compared with low Gleason grade (Fig. 1B, left). Furthermore, Stat5 expression increased in short-term (87.5%, *, $P < 0.05$) and long-term (94.7%, **, $P < 0.001$) ADT tissue as well as CRPC tissue (90.0%, *, $P < 0.05$) compared with untreated primary PCa (60.7%). The frequency of Stat5 expression in different human PCa states is illustrated in Figure 1B (right).

**Expression of Stat5 protein and mRNA levels in prostate cancer cell lines**

We next assessed expression of Stat5 at the protein and mRNA levels. Using an antibody that detect both isoforms a and b, Western blot analysis showed that Stat5 is expressed in LNCaP, C4-2, and DU145 cells. Stat5 protein expression was higher in the AR-positive A1 cell line C4-2 and the AR-negative A1 cell line DU 145 compared with the AR-positive, androgen-sensitive cell line LNCaP (Fig. 1C).

Using qRT-PCR (quantitative RT-PCR), we observed that the mRNA expression level of Stat5b was significantly higher compared with Stat5a (**, $P < 0.001$) in LNCaP, C4-2, and DU 145 cells (Fig. 1D). The ratio between both isoforms was on average 3.0, 5.4, and 4.8 for LNCaP, C4-2, and DU 145 cells, respectively.

**Sequence-specific, dose-dependent knockdown of Stat5 by ASO in PCa cell lines in vitro**

ISIS 424751 potently suppressed Stat5 expression in a dose-dependent and sequence-specific fashion at both protein and mRNA levels. In LNCaP, C4-2, and DU 145 cells, Stat5 protein was potently suppressed at 30 nmol/L. ISIS 424751 had no effect on the expression of Stat3, confirming its specificity to Stat5 (Fig. 2A). Stat5b mRNA levels were significantly reduced in LNCaP, C4-2, and DU145 cells in a dose-dependent manner up to 75%, 85%, and 93%, respectively (***, $P < 0.001$). For Stat5a, maximum mRNA knockdown of 59%, 47%, and 59% was reached at 30 nmol/L in LNCaP, C4-2 (*, $P < 0.05$), and DU145 cells (**, $P < 0.01$), respectively (Fig. 2B).

**Stat5 knockdown inhibits PCa cell growth in vitro**

LNCaP, C4-2, and DU 145 cells were treated for 2 days with 1 to 50 nmol/L ISIS 424751 or ScrB and growth rates were examined. Figure 2C shows a significant reduction of cell growth in all 3 cell lines 3 days after treatment with ISIS 424751 alone compared with ScrB in a dose-dependent manner (***, $P < 0.001$). IC50 was reached at 30, 20, and 30 nmol/L for LNCaP, C4-2, and DU145 cells. In PC3 cells used as a Stat5-negative control, ISIS 424751 decreased cell growth by 8.0% at 30 nmol/L (data not shown). ISIS 424751 significantly decreased cell growth rates at 30 nmol/L in LNCaP cells compared with control. In C4-2 and DU 145 cells, time-dependent cell growth was already significantly affected at 10 nmol/L (Fig. 2D).

**Stat5 ASO treatment induces apoptosis PCa cells in vitro**

To assess effects of Stat5 knockdown using ISIS 424751 on cell apoptosis, we evaluated its effect on cell-cycle population using flow cytometry, caspase-3 activity, and cleaved PARP. As shown in Figure 3A, the fraction of cells undergoing apoptosis (sub-G0/G1) was significantly increased in a dose-dependent manner for Stat5-ASO-treated LNCaP, C4-2, and DU 145 cells compared with ScrB (9.5% vs. 4.5% at 30 nmol/L for LNCaP, *, $P < 0.05$; 43.7% vs. 26.4% at 50 nmol/L for C4-2, **, $P < 0.01$; 4.7% vs. 2.7% at 30 nmol/L for DU145, **, $P < 0.01$).

Caspase-3 activity was significantly increased in LNCaP, C4-2, and DU 145 cells treated with ISIS 424751 compared with ScrB starting at 30 nmol/L (*, $P < 0.05$), 50 nmol/L (**, $P < 0.01$), and 30 nmol/L (**, $P < 0.01$), respectively (Fig. 3B). Furthermore, cells treated with ISIS 424751 expressed higher amounts of cleaved PARP compared with ScrB. Moreover, we found that ISIS 424751 induces a decrease of cyclin D1 and Bcl-xL expression in a dose-dependent manner in all 3 cell lines (Fig. 3C).

**Effect of Stat5 on AR transcriptional activity and nuclear translocation**

To determine effects of Stat5 on androgen-induced, AR-mediated gene activation, LNCaP and C4-2 cells were treated with ISIS 424751 or ScrB and evaluated for
Figure 1. Expression of Stat5 in human prostate tissues and prostate cancer cell lines. A, Stat5 immunostaining in human prostate tissue microarrays, ×200 magnification. From left to right, untreated primary PCa, primary PCa after short term ADT (1–3 months), primary PCa after long-term ADT (4–12 months), and CRPC. Untreated PCa shows low nuclear and cytoplasmatic immunoreactivity in contrast to hormonal treated PCa tissue. Post ADT and CRPC tissue illustrates stronger nuclear staining and generally more highly intense and uniform immunostaining. B, left and right, data from 163 samples were calculated. Columns, percentage; bars, SE. Samples are scored for the level of immunostaining of Stat5 (0 is undetectable and 1 represents positive staining). Left, Stat5 expression is significantly more frequent (*, \(P < 0.05\)) in high Gleason grade compared with low Gleason grade by Fisher’s exact test. Right, frequency of Stat5 expression is significantly higher after short- and long-term ADT (*, \(P < 0.05\) and ***, \(P < 0.001\)) as well as in CRPC (*, \(P < 0.05\)) compared to untreated primary PCa by Fisher’s exact test. C, expression of Stat5 protein in human prostate cancer cell lines. Stat5 expression is increased in C4-2 and Du 145 cells compared with LNCaP cells. D, mRNA expression levels of Stat5 isoforms in human prostate cancer cell lines. Stat5a and Stat5b mRNA levels were normalized to levels of GAPDH mRNA and Stat5b set as 100%. Columns, mean; bars, SE. Stat5b expression is significantly higher (***, \(P < 0.001\)) compared with Stat5a in all 3 cell lines.
Figure 2. Sequence-specific and dose-dependent suppression of Stat5 expression and inhibition of cell growth by ISIS 424751 in LNCaP, C4-2, and DU 145 cells. A, LNCaP, C4-2, and DU 145 cells were treated with 10 to 70 nmol/L ISIS 424751 or ScrB for 2 days. Three days after treatment, protein was extracted and Stat5, Stat3, and vinculin protein levels were analyzed by Western blotting. B, quantitative analysis of Stat5a and Stat5b mRNA levels by quantitative RT-PCR 2 days after treatment; Stat5a and Stat5b mRNA levels were normalized to levels of GAPDH mRNA and expressed as mean $\pm$ SE. CTR indicates cells treated with oligofectamine only. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ differ from CTR by Student's $t$ test. C, dose-dependent inhibition of cell growth in all 3 cell lines treated with ISIS 424751 or ScrB. Three days after treatment, cell viability was determined by crystal violet assay. Data points are means of 2 independent triplicate analysis $\pm$ SE. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ differ from ScrB by Student's $t$ test. D, timecourse for cell growth in all 3 cell lines. Cell growth rates were compared with control (after first transfection) using crystal violet assay at day 1, 2, and 3 after second transfection. *, $P < 0.05$ and **, $P < 0.01$ differ from control (oligofectamine only) by Student's $t$ test.
Figure 3. Effect of ISIS 424751 on LNCaP, C4-2, and DU 145 cell apoptosis. A, ISIS 424751 induces cell-cycle arrest. Cells were treated with ISIS 424751 or ScrB. Two days after treatment, cells were fixed and permeabilized and then stained with PI and cell-cycle population was analyzed by flow cytometry. Histograms represent the means of 2 independent triplicate analysis ± SE. *, P < 0.05 and **, P < 0.01 differ from ScrB by Student’s t test. B, treatment with ISIS 424751 increases caspase-3 activity. All 3 cell lines were treated from 10 to 50 nmol/L with ISIS 424751 or ScrB for 2 days, and total cell lysate was extracted 2 days later. Results are expressed in arbitrary units and corrected for protein content. Columns show means of triplicate analysis ± SE. *, P < 0.05 and **, P < 0.01 differ from ScrB by Student’s t test. C, treatment with ISIS 424751 induces PARP cleavage and decreases expression of Stat5 dependent genes. Western blot analysis of selected genes altered by Stat5 knockdown in all 3 cell lines. Cells were treated from 10 to 70 nmol/L with ISIS 424751 or ScrB for 2 days, and protein was extracted 3 days later for detection of cyclin D1, PARP, and Bcl-xL. vinculin was used as an internal control.
changes in R1881-stimulated PSA transactivation (Fig. 4A). Stat5 knockdown significantly abrogated the transactivation of the androgen-regulated PSA reporter in a dose-dependent manner in LNCaP (*, $P < 0.05$ at 10 nmol/L; **, $P < 0.05$ at 30 nmol/L; ***, $P < 0.001$ at 50 nmol/L) and C4-2 cells (*, $P < 0.05$ at 10 Nmol/L; *, $P < 0.05$ at 30 nmol/L; ***, $P < 0.001$ at 50 nmol/L). AR localization using immunofluorescence after ISIS 424751 ± R1881 in LNCaP and C4-2 cells showed that Stat5 knockdown abrogates R1881-induced AR nuclear translocation compared with ScrB-treated cells. Interestingly, AR staining was lower after Stat5 knockdown (Fig. 4B).

**Stat5 knockdown decreases AR stability and induces its degradation via the proteasome**

To further explore the role of Stat5 knockdown on AR expression, we evaluated changes in AR proteins levels after treatment with ISIS 424751 in AR-positive LNCaP and C4-2 cells. ISIS 424751 decreased AR and PSA protein levels in a dose-dependent manner (Fig. 5A). In LNCaP cells, AR as well as PSA was potently suppressed at 30 nmol/L, whereas in C4-2 cells, knockdown for both proteins was reached at 50 nmol/L. AR-mRNA expression levels were not affected by Stat5-ASO treatment (Fig. 5B). The effect of Stat5 knockdown on AR protein
Figure 5. Effect of Stat5 downregulation on AR protein expression and stability. A, LNCaP and C4-2 cells were treated in a dose-dependent manner with ISIS 424751 or ScrB, and AR protein levels were determined 3 days later by Western blotting. GAPDH was used as a loading control. B, Stat5 knockdown does not affect AR-expression on mRNA level. LNCaP and C4-2 cells were treated with ScrB or Stat5-ASO at 30 nmol/L and AR and Stat5b mRNA levels determined 2 days later by RT-PCR. C, Stat5 knockdown alters AR stability. AR protein levels were determined after incubation of cell lines with CHX for the indicated time period. D, Stat5 knockdown accelerates proteasomal degradation of AR. LNCaP cells were treated with ISIS 424751 or ScrB and 10 µmol/L MG-132 for 6 hours. AR protein level was measured by Western blot analysis.
stability was next evaluated using CHX, which inhibits protein synthesis by blocking ribosomes (Figure 5C). AR protein levels decreased with rapid degradation after Stat5 knockdown by ISIS 424751 compared with ScrB treatment in LNCaP and C4-2 cells. AR degradation after Stat5-knockdown occurs via the proteasome pathway because treatment with the proteasome inhibitor MG-132 suppressed Stat5 knockdown-induced AR protein degradation in LNCaP cells (Fig. 5D). In summary, these findings indicate that Stat5 knockdown induces AR protein degradation via the proteasome pathway.

**ISIS 424751 treatment inhibits castrate-resistant LNCaP tumor growth and PSA progression**

We next evaluated the effect of ISIS 424751 treatment on the growth of castrate-resistant LNCaP tumors in vivo. Castrated male nude mice bearing LNCaP xenograft tumors were randomly selected after PSA relapse to precastration level and treated with 12.5 mg/kg ISIS 424751 or ScrB 3 times per week by i.p. injection (first-week daily treatment). Mean LNCaP tumor volume and serum PSA levels at baseline were similar in both groups. Figure 6A shows that ISIS 424751 treatment significantly delayed tumor progression by more than 71% within the first 35 days (*, P < 0.05). In the control group 3 animals required sacrifice by 4 weeks and 1 animal after 6 weeks because tumor volume reached 10% or more of body weight. In the ISIS 424751–treated group, no animal was sacrificed within 6 weeks of treatment.

Serum PSA levels were reduced in the ISIS 424751–treated group compared with control. To quantify treatment effects on target protein reduction, protein expression levels for Stat5, AR, and cyclin D1 in tumor tissue (3 animals per group) were evaluated using Western blot analysis (Fig. 6C). Total Stat5 and AR protein expression was significantly downregulated in ISIS 424751–treated mice by 67.6% and 90.1%, respectively, compared with control mice (*, P < 0.05), whereas cyclin D1 showed no significant difference between both groups.

**Discussion**

Stat5 has been previously linked to PCa progression (31,32). Li and colleagues reported a strong positive correlation with Stat5 expression and high Gleason score in 114 human PCa (24). In the current study, we confirmed that the frequency of Stat5-positive staining significantly increases in high Gleason grade (>3) compared with low Gleason grade (≤3) cancers. Interestingly, and in accordance to the study of Tan and colleagues (26), we found that the frequency of Stat5 expression is significantly increased after short-term and long-term ADT as well as in CRPC.

This result supports the notion that androgen deprivation leads to an upregulation of the JAK2-Stat5 signaling cascade, which allows cancer cells to survive in low-androgen environments. Following androgen ablation, prolactin and other mitogens may function as promoters of the JAK2-Stat5 signaling cascade (22, 33–35). On the basis of these findings, we selected castrate-recurrent LNCaP xenografts to model the human situation of CRPC (36).

The individual roles of the Stat5a and Stat5b isoforms remain unclear. Several studies suggest that both isoforms are expressed at different levels in different human PCa cell lines (21,24). Data on the ratio of isoform expression levels in human PCa have not been previously reported. In the current study, we show that mRNA expression of Stat5b was 3.0- to 5.4-fold higher than isoform Stat5a in several PCa cell lines. These findings support the assumption of several studies that Stat5b plays a greater role in cancer cell growth and cancer progression than does Stat5a (21, 37).

Although targeting Stat5 in vitro using ASO or siRNA has been reported previously (20, 23), no study so far has reported on pharmacologic Stat5 inhibition in vivo. In this study, we used a second generation ASO incorporating a 2′-O-methoxymethyl gapmer modified backbone, which enables favorable physicochemical, biochemical, and pharmacokinetic properties in vivo (38). The improved resistance against nuclease-mediated metabolism results in a significantly improved tissue half-life in vivo, resulting in a longer duration of action and a more intermittent dosing schedule.

Our data show that ISIS 424751 suppresses Stat5 expression at protein and mRNA levels in LNCaP, C4-2, and DU145 cells (Fig. 2A and B). Expression of other members of Stat-family such as Stat3 was not affected. The downregulation achieved with ISIS 424751 was observed on Stat5 dependent genes such as cyclin D1 and Bcl-xL, as shown by Western blot (Fig. 3C). Interestingly, Stat5 knockdown was associated with an increase of apoptosis-markers such as sub-G0/G1, cleaved PARP and caspase-3 activity. These results reflect the observations of Dagvadorj and colleagues (23), where Stat5 inhibition by ASO induced morphologic changes in LNCaP cells consistent with apoptotic cell death. In addition, ISIS 424751 decreased cell growth with IC50 at concentrations as low as 30 nmol/L. Taken together, these in vitro data support pharmacologic targeting of Stat5 in by ASO ISIS 424751.

To gain further insight into the mechanisms of apoptosis induction by targeting Stat5 in PCa cells, we analyzed the effects of Stat5 knockdown on AR axis. The reactivation of AR signaling despite low androgen levels is a characteristic of CRPC (28) and results from persisting low levels of intracrine testosterone production (29). Supported by the finding that the frequency of Stat5 expression is increased in different stages of hormonal treated PCa (Fig. 1A and B), Stat5 may act as a coactivator of AR in androgen deprived PCa.

We found that Stat5 knockdown leads to a decrease of AR protein levels and stability without affecting AR mRNA levels (Fig. 5A–C). Furthermore, treatment with
the proteasome inhibitor MG-132 suppressed Stat5 knockdown–induced AR degradation (Fig. 5D). Taken together, these findings indicate that Stat5 knockdown induces AR degradation via a proteasome-mediated pathway.

We also investigated the effects of Stat5 knockdown on AR-dependent genes such as PSA. Targeting Stat5 with ISIS 424751 abrogated R1881-induced AR nuclear translocation and transcription activity (Fig. 3). These results are supported by Tan and colleagues (26), suggesting that Stat5 synergizes with AR by inducing increase of AR transcriptional activity and nuclear localization. In turn, ligand-bound AR increased the transcriptional activity of Stat5.

This study provides preclinical proof-of-principle that targeting Stat5 with antisense ISIS 424751 significantly affects tumor growth and delays CRPC progression in vivo. Our results are in accordance with the findings of several studies in which subcutaneous tumor growth was reduced in CWR22Rv and DU145 PCa cells overexpres-
sing dominant-negative Stat5α/b (23, 37). In addition, we recapitulated our in vitro results in vivo by affecting serum PSA levels as well as protein expression of our target genes Stat5, AR, and cyclin D1.

In conclusion, the current study shows for the first time that progression of CRPC in vivo can be delayed by pharmacologic targeting and knockdown of Stat5, thereby affecting regulators of apoptosis and cell cycle as well as enhancing degradation of the AR.

Potential conflict of interest

B.P. Monia and R. MacLeod are employees of Isis Pharmaceuticals. No other potential conflicts of interest were disclosed.

References


Molecular Cancer Therapeutics

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Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-10-0850

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