Transcription factor Stat 5 knockdown enhances androgen receptor degradation and delays castration-resistant prostate cancer progression in vivo

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ABSTRACT

Purpose: Signal Transducer and Activator of Transcription 5 (Stat5) plays an important role in the transition of prostate cancer (PCa) to its castrate-resistant state. Pharmacological targeting of Stat5 is a rational approach to delay castrate resistant progression, in part because Stat5 cooperates with androgen receptor (AR) to promote PCa progression.

Experimental Design: 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.

Results and Conclusions: Here, we demonstrate that the frequency of Stat5 expression is significantly increased in high Gleason grade as well as in hormonal 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.
INTRODUCTION

While 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 while up to 80% show initial response, castrate 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 (Stat)-5 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 while Stat5b mediates cell growth (21, 27). In vitro, Stat5 promotes PCa progression by increasing the expression levels of Cyclin-D1 and Bcl-xL (23) and transcriptional activity of AR (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 demonstrated in Stat5b-negative C2H cells which 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 test whether targeted knockdown of Stat5 can delay CRPC progression in xenograft PCa models. We demonstrate 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 (1989, ATCC-authentication by isoenzymes analysis). LNCaP and C4-2 cells were kindly provided by Dr. Leland W.K. Chung (1992, MDACC, Houston Tx) and tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer IIx platform in July 2009. All three cell lines were passaged for less than 3 months after resurrection. Western blotting and/or real time PCR was performed 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% fetal bovine serum. All cell lines were cultured without antibiotics at 37.0°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 (Danvers, MA) Vinculin with an antibody (V 9131) from Sigma-Aldrich (St. Louis, MO), GAPDH with an antibody (NB-300-322) from Novus Biologicals (Littleton, CO) and Actin with an antibody (MAB 1501R) from Millipore (Billerica, MA).

TMA construction and immunohistochemistry
A total of 163 PCa specimens (143 primary PCa / 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 1-3months]) or long-term
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 1.0mm arrayed in a rectangular pattern with 1.0mm between the centers of each core, creating a duplicate tissue microarray layout. Immunohistochemical stains were performed on formalin-fixed and paraffin-embedded 4 μm sections of the TMA. 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 enzyme labeled biotin streptavidin system and solvent resistant 3,3’-diaminobenyidine Map kit. The samples were scored by a pathologist (L.F.) for the level of immunoexpression of Stat5 on a scale from 0 to 1, wherein 0 was undetectable and 1 represented positive staining. All comparisons of staining intensities were made at 200x magnifications.

Treatment of prostate cancer cells with oligonucleotides

A 2’-methoxyethyl-modified phosphorotiate ASO for the coding region of Stat5a and Stat5b (ISIS 424751) was synthesized by ISIS Pharmaceuticals (Carlsbad, CA). The sequence of the Stat5-ASO is 5’ TCCACGCGGTACTGCTGCAG-3’. The control oligonucleotide (ScrB) used is designed not to match any mRNA in human or mouse transcriptomes and has the sequence 5’-CCTTCCCTGAAGGTTCCTCC-3’. C4-2 and DU145 cells were plated at the density of 1.25x10⁴/cm² (LNCaP 2.5x10⁴/cm²) and treated a day later at a confluence of 50-60% for two consecutive days with ISIS 424751 or ScrB. Transfection was performed in serum-free OPTI-MEM (Invitrogen Life Technologies, Inc.) for 4hrs, allowed to recover in standard culture media for 20hrs. 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 above. Crystal violet staining was carried out for dose-dependent treatment 3 days post transfection and for time-course 1, 2 and 3 days post transfection. 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 quantification of apoptotic cells were done by flow cytometric analysis. Cells were plated in 10 cm² dishes and transfected as described above. 2 days later cells were trypsinized and fixed in 70% ethanol over night at 4 C, then incubated with 1 µg/mL RNase (Sigma) for 30 min at 37 C before stained with 5µg/mL propidium iodide (Sigma) for 30 min 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 two days after treatment using the kit CaspACE Assay System, Fluorometric (Promega, Madison, WI, USA). Aliquots of 50µg of total cell lysate were incubated with caspase-3 substrate AC-DEVD-AMC at room temperature for 16 h. The caspase activity was quantified in a fluorometer with excitation at 360 nm and emission at 460 nm.

**Western blot analysis**

Cell population was harvested three days post transfection in RIPA buffer (50nM Tris, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 10nM NaCl, Roche complete protease inhibitor cocktail) incubated on ice for 60 min and centrifuged at 13,000 rpm for 20 min. 40 µg of whole cell lysate was subjected to SDS-PAGE transferred to nitrocellulose filters and immunoblotted with the appropriate antibodies. After washing thrice with washing buffer, membranes were incubated with Alexa Fluor 680 or 800 secondary antibodies (Invitrogen) for 1 h. 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 uL 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 two consecutive days. Media were replaced by charcoal stripped serum (CSS; Thermo scientific) + 0.1nm R1881 (PerkinElmer) 24h after last transfection for another 24h. Luciferase activities measured using the Dual-Luciferase Reporter Assay System (Promega) with the aid of a microplate luminometer (EG&G Berthold). All experiments were carried out in triplicate wells and repeated two times.

Immunofluorescence
LNCaP and C4-2 cells were grown on coverslips in 12-well plates. For Stat5 knockdown, cells were transfected with 30nM Stat5 ASO as described above. 2 days after second transfection, cells were treated +R1881 for 15min and thereafter fixed in ice-cold methanol completed with 3% acetone for 10min at -20C. Cell were then washed thrice with PBS and incubated with 0.2% Triton/PBS for 10 min, followed by washing and 30 min blocking in 3% nonfat milk before AR antibody addition overnight (1:250). Antigens were visualized using anti-rabbit antibodies coupled to FITC or rhodamine (1:500; 30 min). Photomicrographs were taken at 40x magnification using Zeiss Axioplan II fluorescence microscope, followed by analysis with imaging software (Northern Eclipse, Empix Imaging, Inc.).
Reverse transcription-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 (2µg) 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’-AAGTCAAGGAGCAACCGGGGC-3’ (forward) and 5’-TGTGCGTGCGGGATCCACTG-3’ (reverse), for AR 5’-TACCAGCTCAACAGCTCTCT-3’ (forward) and 5’-GCTTCACCGTGGGTGGAAAT-3’ (reverse) and for β-Actin 5’-AACTCGGACCACACCCTTC-3’ (forward) and 5’-AGCAGCTGTGTTGGCGACAG-3’ (reverse). Real time monitoring of PCR amplification of cDNA was performed using DNA primers 5’-TGCAGCTCTCCCGGCTGGGAT-3’ (forward) and 5’-CAGGCGTACGGTGGCTGCA-3’ (reverse) targeting Stat5a, 5’-AAGTGCAGAGCAACCGGGGC-3’ (forward) and 5’-TGTGCGTGCGGGATCCACTG-3’ (reverse) targeting Stat5b and 5’-CAATGACCCTTCTTGACC-3’ (forward) and 5’-GACAAGCTTTCCGTCTCAG-3’ (reverse) targeting human GAPDH on a ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) with SYBR PCR Master Mix (Applied Biosystems). Target gene expression was normalized to GAPDH levels in respective samples as an internal standard, and the comparative cycle threshold (Ct) method was used to calculated relative quantification of target mRNAs. Each assay was performed in triplicate.

Protein stability and degradation

To assess the effect of Stat5-ASO on protein stability, cyclohexamide (CHX; Calbiochem / chemical structure see supplemental Figure 1A) was added at 10μg/ml to the culture media of LNCaP and C4-2 cells for 4, 8 and 16 h following transfection. Thereafter western blot was done using AR, Stat5 and GAPDH antibodies. Degradation was tested in LNCaP cells by 6h incubation with RPMI ± 5% FBS media containing MG132 (Calbiochem / chemical structure see supplemental Figure 1B) at 10µM 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 2x10^6 LNCaP cells (suspended in 0.1ml Matrigel; BD Biosciences) and castrated when PSA values exceeded 50ng/ml. Mice were randomly selected for treatment with 12.5 mg/kg ISIS 424751 or ScrB when PSA relapsed to pre-castration level and then injected i.p. once daily for 7 days and three times per week thereafter (castrate-resistant model). Each experimental group consisted of 7 mice. Serum PSA measurements were performed once weekly by enzymatic immunoassay (Abbott IMX, Montreal, Quebec, Canada). 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% 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 performed 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 ≤ 0.05 (*), p ≤ 0.01 (**) and p ≤ 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 to low Gleason grade (Fig. 1B left). Furthermore, Stat5 expression increased in short- (87.5%, *p<0.05) and long- (94.7%, ***p<0.001)) term ADT tissue as well as CRPC tissue (90.0%, *p<0.05) compared to 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 detecting 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 AI cell line C4-2 and the AR-negative AI cell line DU 145 compared to the AR-positive, androgen sensitive cell line LNCaP (Fig. 1C).

Using qRT-PCR, we observed that the mRNA expression level of Stat5b was significantly higher compared to 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 30nM. 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 30nM in LNCaP, C4-2 (*, p<0.05) and DU145 cells (**, p<0.01) (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 50nM ISIS 424751 or ScrB and growth rates were examined. Figure 2C shows a significant reduction of cell growth in all three 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 30nM, 20nM and 30nM 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 30nM (data not shown). ISIS 424751 significantly decreased cell growth rates at 30nM in LNCaP cells compared to control. In C4-2 and DU 145 cells, time-dependent cell growth was already significantly affected at 10nM (Figure 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 poly (ADP-ribose) polymerase (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 to ScrB (9.5% vs. 4.5% at 30nM for LNCaP / *, p<0.05; 43.7% vs. 2.6% at 50nM for C4-2 / **, p<0.01; 4.7% vs. 2.7% at 30nM for DU145 / **, p...
Caspase-3 activity was significantly increased in LNCaP, C4-2 and DU 145 cells treated with ISIS 424751 compared to ScrB starting at 30nM (*, p<0.05), 50nM (**, p<0.01) and 30nM (**, 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 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 10nM; *, p<0.05 at 30nM; ***, p<0.001 at 50nM) and C4-2 cells (*, p<0.05 at 10nM; *, p<0.05 at 30nM; ***, p<0.001 at 50nM). AR localization using immunofluorescence after ISIS 424751 +/- R1881 in LNCaP and C4-2 cells demonstrated that Stat5 knockdown abrogates R1881-induced AR nuclear translocation compared to 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 30nM, while in C4-2 cells knockdown for both proteins was reached at 50nM. AR- mRNA expression levels were not affected by Stat5-ASO treatment (Fig. 5B). The effect of Stat5 knockdown on AR protein 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 to 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 pre-castration 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 >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% of body weight. In the ISIS 424751 treated group no animal required sacrifice within 6 weeks of treatment.

Serum PSA levels were reduced in the ISIS 424751 treated group compared to 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 analysis (Fig. 6C). Total Stat5 and AR protein expression were significantly downregulated in ISIS 424751 treated mice by 67.6% and 90.1%, respectively, compared to control mice (*, p<0.05), while Cyclin D1 showed no significant difference between both groups.
DISCUSSION

Stat5 has been previously linked to PCa progression (31,32). Li et al. 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 to low Gleason grade (<3) cancers. Interestingly and in accordance to the study of Tan et al (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). Based on 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 demonstrate 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).

While 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-methoxyethyl 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-live in vivo, resulting in a longer duration of action and a more intermittent dosing schedule.

Our data shows that ISIS 424751 suppresses Stat5 expression at protein and mRNA levels in LNCaP, C4-2 and DU145 cells (Fig.2A,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 et al. (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 nM. Taken together, these in vitro data support pharmacological 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, B), Stat5 may act as a co-activator 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 et al (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 where subcutaneous tumor growth was reduced in CWR22Rv and DU145 PCa cells overexpressing dominant-negative Stat5a/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 pharmacological targeting and knockdown of Stat5, thereby affecting regulators of apoptosis and cell cycle as well as enhancing degradation of the AR.
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REFERENCES


LEGENDS

Figure 1.
Expression of Stat5 in human prostate tissues and prostate cancer cell lines. A, Stat5 immunostaining in human prostate tissue microarray, 200x 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 to 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 to 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 to Stat5a in all three cell lines.

Figure 2.
Sequence-specific and dose-dependent suppression of Stat5 expression and inhibition of cell growth by ISIS 424751 in LNCaP, C 4-2 and DU 145 cells. A, LNCaP, C4-2 and DU 145 cells
were treated with 10 to 70nM ISIS 424751 or ScrB for 2d. 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 two days after treatment; Stat5a and Stat5b mRNA levels were normalized to levels of GAPDH mRNA and expressed as mean ± SE. CTR indicates cells treated with oligofectamine only.*, p<0.05 and **, p<0.01 and ***, p<0.001 differ from CTR by Student's t-test. C, dose-dependent inhibition of cell growth in all three 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 two independent triplicate analysis ± SE. *, p<0.05 and **, p<0.01 and ***, p<0.001 differ from ScrB by Student's t-test. D, time course for cell growth in all three 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, then stained with PI and cell cycle population was analyzed by flow cytometry. Histograms represent the means of two 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 three cell lines were treated from 10 to 50 nM with ISIS 424751 or ScrB for 2d, and total cell lysate was extracted two 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 nM with ISIS 424751 or ScrB for 2d, and protein was extracted three days later for
detection of Cyclin D1, PARP and Bcl-xL. Vinculin was used as an internal control.

Figure 4.
Effect of Stat5 on genomic activity and nuclear translocation of AR. A, ISIS 424751 abrogates R1881 induced AR transcription activity. LNCaP and C4-2 were treated with ISIS 424751 and co-transfected with PSA-luciferase. 24 hours after transfection, media was replaced by CSS + 0.1 nM R1881 for additional 24h. Luciferase activity was measured using a luminometer. Columns show means of triplicate analysis ± SE. *, p<0.05 and **, p<0.01 differ from ScrB by Student’s t-test. B, ISIS 424751 inhibits androgen-stimulated nuclear translocation of AR: Two days after transfection with 30nM of ISIS 424751 or ScrB, LNCaP and C4-2 cells were treated + 0.1 nM R1881 for 15min and fixed in methanol/acetone for immunofluorescence staining with anti-AR antibodies. Nucleus is stained with DAPI. In both cell lines treated with ScrB, R1881 induces nuclear translocation of AR (columns 1 and 2). After ISIS 424751 treatment, AR remains in the cytoplasm despite R1881 stimulation.

Figure 5.
Effect of Stat5 down-regulation on AR protein expression and stability. A, LNCaP and C4-2 cells were treated in a dose-depentent manner with ISIS 424751 or ScrB, and AR protein levels 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 30nM 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 10µM MG-132 for 6h. AR protein level was measured by western blot analysis.
Figure 6.

Effect of ISIS 424751 treatment on castrate-resistant LNCaP-xenograft growth in vivo. A, ISIS 424751 treatment was started when serum PSA values relapsed to pre-castration levels. After 1wk of daily i.p. injection with 12.5 mg/kg/mouse ISIS 424751 or ScrB, treatments were administered 3 times per week. Each data point represents the mean tumor volume in each group containing 7 mice + SE. *, p<0.05 differs from ScrB by Student’s t-test. ††† indicating that 3 mice after 4 weeks and † indicating that 1 mouse after 6 weeks required sacrifice in the ScrB group because tumor volume exceeded > 10% of bodyweight. B, serum samples were obtained from the tail vein of the mice once weekly to measure serum PSA by ELISA. † and ††† see above. C, total proteins were extracted from xenografts treated with ISIS 424751 or ScrB at 8 wk or when tumor volume exceeded ≥ 10%. Target genes were normalized to Actin. *, p<0.05 differs from ScrB by Student’s t-test.

Supplemental Figure 1.

A, chemical structure of cyclohexamide. B, chemical structure of MG132.
Figure 1

A

untreated 1-3 m ADT 4-12m ADT CRPC

B

Stat 5 negative Stat 5 positive

C

LNCaP C 4-2 DU 145

Stat 5 Vinculin

D

mRNA expression (% CTR)

LNCaP C4-2 DU 145

Stat 5b Stat 5a
Figure 2

A

Stat 5
LNCaP
C 4-2
DU 145

Stat 3

Vinculin

ScrB (nM)
Ctr 10 30 50 70
Stat5-ASO (nM)
10 30 50 70

B

LNCaP

C 4-2

DU 145

mRNA expression

ScrB (nM)
Ctr 10 30 50
Stat5-ASO (nM)
10 30 50

C

LNCaP

C 4-2

DU 145

% of viable cells

ScrB (nM)
0 10 20 30 40 50
Stat5-ASO (nM)

D

LNCaP

C 4-2

DU 145

% of viable cells

Control ScrB 30nM Stat5-ASO 10nM Stat5-ASO 30nM

day
Figure 3

A

LNCaP

C4-2

DU 145

% cells in sub G0/G1 arrest

sub G 10nM sub G 30nM sub G 50nM

0 10 20 30 40 50 60 70

ScvB Stat5-ASO

B

LNCaP

C4-2

DU 145

Substrate cleavage (AU)

10nM 30nM 50nM

0 5 10 15 20 25 30 35 40

ScvB Stat5-ASO

C

LNCaP

C4-2

DU 145

PARP

Cyclin D1

Bcl-XL

Vinculin

ScvB (nM) Stat5-ASO (nM)

ScvB (nM) Stat5-ASO (nM)

ScvB (nM) Stat5-ASO (nM)
Figure 4

A

LNCaP + R1881

C 4-2 + R1881

B

LNCaP

ScrB mono
ScrB + R1881
Stat5-ASO mono
Stat5-ASO + R1881

AR

DAPI

MERGE

C 4-2

ScrB mono
ScrB + R1881
Stat5-ASO mono
Stat5-ASO + R1881

AR

DAPI

MERGE
Figure 5

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Figure 6

A

![Graph showing tumor volume (mm³) over weeks after treatment for ScrB and Stat5-ASO groups.](image)

B

![Graph showing serum PSA (ng/ml) over weeks after treatment for ScrB and Stat5-ASO groups.](image)

C

![Image with Western blots showing expression of Stat5, AR, Cyclin D1, and Actin for ScrB and Stat5-ASO groups.](image)
Transcription factor Stat 5 knockdown enhances androgen receptor degradation and delays castration-resistant prostate cancer progression in vivo


Mol Cancer Ther Published OnlineFirst January 7, 2011.

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

Supplementary Material
Access the most recent supplemental material at:
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