SYK is a candidate kinase target for the treatment of advanced prostate cancer

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Precis: Striking preclinical findings offer a mechanistic rationale to immediately reposition SYK kinase inhibitors currently in early clinical trials for evaluation in patients with metastatic prostate cancer.
ABSTRACT

Improved targeted therapies are needed to combat metastatic prostate cancer. Here we report the identification of the spleen kinase SYK as a mediator of metastatic dissemination in zebrafish and mouse xenograft models of human prostate cancer. While SYK has not been implicated previously in this disease, we found that its expression is upregulated in human prostate cancers and associated with malignant progression. RNAi-mediated silencing prevented invasive outgrowth in vitro and bone colonization in vivo, effects that were reversed by wild-type but not kinase-dead SYK expression. In the absence of SYK expression, cell surface levels of the progression-associated adhesion receptors integrin α2β1 and CD44 were diminished. RNAi-mediated silencing of α2β1 phenocopied SYK depletion in vitro and in vivo, suggesting an effector role for α2β1 in this setting. Notably, pharmacological inhibitors of SYK kinase currently in Phase I-II trials for other indications interfered similarly with the invasive growth and dissemination of prostate cancer cells. Our findings offer a mechanistic rationale to reposition SYK kinase inhibitors for evaluation in patients with metastatic prostate cancer.
INTRODUCTION

Prostate cancer is the most common cancer in males and the second leading cause of cancer deaths among men in the Western world (1). Non-detectable micro-metastatic disease may be present in up to 40% of patients (2) while 8–14% may have visible or symptomatic bone metastases at diagnosis (3). Although the majority of prostate cancers are diagnosed as organ-confined disease, which is curable by prostatectomy or radiation therapy, 20-25% of patients will experience relapse within 5 years of treatment (4). Androgen deprivation therapy is used when prostate cancer reappears but in most cases resistance develops within 1-3 years. Chemotherapy, particularly docetaxel is able to prolong overall survival in these cases but it also causes significant toxicity and not all patients receive this therapy. In order to more successfully combat prostate cancer, screening programs for early diagnosis and treatment of localized disease are important. In addition, some alternatives for docetaxel as first line treatment for metastatic disease and options for those cases where docetaxel failed have become available (5). Nevertheless, once the disease has spread beyond the prostate, no curative treatments are currently available (6). Hence, there is an urgent need for novel targeted therapies to improve treatment of metastatic prostate cancer.

SYK is a non-receptor tyrosine kinase containing two adjacent Src homology 2 (SH2) domains, a kinase domain, but no SH3 domain. SYK is expressed in hematopoietic cells where it binds phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) to mediate immune receptor signaling (7). For malignant hematopoietic cells that rely on immune receptor-mediated survival signals, SYK might represent an attractive drug target. Indeed, pharmacological inhibition of SYK has shown promising results in the context of non-Hodgkin
lymphoma and leukemias (8). SYK is also widely expressed in a variety of cell types outside the hematopoietic system and it is required for proper development of blood and lymph vessels during embryonic development (9, 10). The role of SYK in epithelial cancers appears diverse. SYK abundance negatively correlates with breast cancer progression and SYK suppresses tumor growth and metastasis in breast cancer xenografts (11, 12). Conversely, SYK levels in head and neck squamous cell carcinomas and lymph node metastases are high compared to corresponding normal tissue and SYK promotes migration of squamous carcinoma cells (13). SYK has not been implicated in prostate cancer.

In the current study, we find that SYK adenoviral shRNAs interfere with PC3 human prostate cancer dissemination using a semi-automated whole animal bioimaging platform (14). Further investigation of SYK in patient cohorts, three-dimensional (3D) in vitro cultures, and zebrafish and mouse xenografts indicate that SYK may represent a novel candidate drug target for further study in prostate cancer.
MATERIALS AND METHODS

Cell lines, antibodies, and pharmacological inhibitors. LNCaP, PC3, DU-145, and HEK293T cells were obtained from ATCC and cultured for fewer than 6 months after receipt or resuscitation according to the provided protocol. ATCC characterized the cell lines using STR profiling. PC3-derived PC3-M-Pro4luc cells (15) and LNCaP-derived cell lines C4-2 and C4-2 B were grown in DMEM and T-Medium, respectively. For FACS, primary antibodies included AIIB2 anti-human integrin α1, 4A10 anti human integrin α2, and sc-18849 anti-human CD44 (Santa-Cruz). Goat-anti-mouse APC and donkey-anti-rat PE (Jackson laboratories) were used as secondary antibodies. For immunohistochemistry in patient tumor samples and Western blot rabbit anti-human SYK monoclonal antibody (clone EP573Y, ab40781; Abcam) was used. For Western blot, anti-human AKT (#4691; Cell Signaling), anti-phospho-Ser473 AKT (#9271; Cell Signaling), anti-human CD44 MAb (kindly provided by Dr. Marcel Spaargaren, Academic Medical Center, Amsterdam the Netherlands), and α-tubulin (B-5-1-2; Sigma) were used. R-406 and BAY-61-3606 were obtained from Selleckchem and Sigma, respectively.

Zebrafish xenotransplantation experiments. For quantification of tumor cell spreading, tumor cells were labeled with CM-Dil (Invitrogen), mixed with 2% PVP, and injected into the yolk sac of enzymatically dechorionated, two-day old Casper fli-EGFP transgenic zebrafish embryos using an air driven microinjector (20 psi, PV820 Pneumatic PicoPump; World precision Inc). Embryos were maintained in egg water at 34°C for 6 days and subsequently fixed with 4% paraformaldehyde. Imaging was done in 96 well plates containing a single embryo per well using a Nikon Eclipse Ti confocal laser-scanning microscope. Z stacks (12 x 30 μm) were obtained using a Plan Apo 4X Nikon dry objective with 0.2 NA and 20 WD. Images were converted into a single Z
projection in Image-Pro Plus (Version 6.2; Media Cybernetics). Automated quantification of tumor cell spreading per embryo, cumulative distance of cells per embryo, and mean cumulative distance for all embryos (MCD) was determined using an in-house built Image-Pro Plus plugin as previously described (14).

**Transient adenoviral shRNA transduction.** PC3 cells were transduced one day after seeding with adenoviral shRNA constructs from Galapagos BV (Leiden, the Netherlands) using an MOI of 15 for 24 h. After 3 days, medium was replaced and after an additional two days, the transduced PC3 cells were detached with trypsin and single cell suspensions were used for zebrafish xenotransplantation.

**Stable shRNA and cDNA expression.** PC3 or PC3-M-Pro4luc cells were transduced using Sigma’s MISSION library lentiviral shRNAs (shSYK#1: TRCN0000003167, shSYK#2:TRCN0000199566; shITGB1#1:TRCN00000029645, shITGB1#2:TRCN00000029646; shITGA2#1: TRCN00000057730, shITGA2#2: TRCN000000057731). For lentivirus production, HEK293T cells were transfected with the short hairpin constructs together with the packaging plasmids REV, GAG and VSV in a 1:1:1:1 ratio using PE (Sigma) as transfection reagent. Lentiviral supernatant was collected 48 h after transfection and used for transduction or target cells in the presence of 8 μg Polybrene (Sigma). Transduced cells were bulk selected in medium containing 2 μg/ml puromycin. Lentiviral shRNA vector targeting TurboGFP was used as a negative control. Retroviral cDNAs for wild type and kinase dead SYK were a gift from Drs. Wei Zou and Steven Teitelbaum, Washington University, St Louis MO (16). Retrovirus was produced in Plat-E packaging cells and used for transduction of PC3-M-Pro4-luc cells stably expressing shRNA targeting SYK 3’UTR, followed by bulk blasticidin selection.
mRNA expression analysis. For qPCR, total RNA was extracted using RNA easy Plus Mini Kit (Qiagen). cDNA was randomly primed from 50 ng total RNA using iScript cDNA synthesis kit (BioRad) and real-time qPCR was subsequently performed in triplicate using SYBR green PCR (Applied Biosystems) on a 7900HT fast real-time PCR system (Applied Biosystems). The following qPCR primer sets were used:

GAPDH: forward AGCCACATCGCTCAGACACC, reverse ACCCGTGGACTCCGACCTT;
SYK forward GATGCTGGTTATGGAGATG, reverse TCTATGATGTTCCTACTTGAC;
CD44 forward TGGCACCCTGCTATGTCCAG, reverse GTAGCAGGGATTCTGTCTG;
ITGB1 forward ATTGACCTCTACTACCTT, reverse GTGTTGTGCTAATGTAAG;
ITGA2 forward AACTCTTTGGATTTGCGTGTG, reverse TGGCAGTCAGAATAGGCTTC.

Data were collected and analyzed using SDS2.3 software (Applied Biosystems). Relative mRNA levels after correction for GAPDH control mRNA, were expressed using $2^{-\Delta\Delta Ct}$ method.

For mRNA expression analysis of human prostate cancer patient material either directly or following xenografting in mice, existing datasets were queried as described (17).

Colony formation assay. Cells were seeded into a 96-well plate containing ~1 cell per well. After 1 to 3 weeks, percentage of wells showing colonies and colony size was determined by microscopy (Zeiss Axiovert 200M).

3D invasion assays. Cell suspensions in PBS containing 2% polyvinylpyrrolidone (PVP; Sigma-Aldrich) were microinjected (~1x10^6 cells/droplet) using an air driven microinjector (20 psi, PV820 Pneumatic PicoPump; World precision Inc) into solidified 3D collagen gels in 8 well μslides (IBIDI) as previously described (18). Collagen gels were prepared from 2.5 mg/ml acid-
extracted rat tail collagen type I. Collagen was diluted to working concentration of 1 mg/ml in complete medium containing 44 mM NaHCO3 (stock 440 mM, Merck) and 0.1 M Hepes (stock 1M, BioSolve). Tumor cell spheroids were monitored for ~1 week using Nikon eclipse TS100. For immunostaining gels were incubated for 1 hour with 5 μg/ml collagenase (Clostridium histolyticum, Boehringer Mannheim) at room temperature, fixed with 4% paraformaldehyde, and permeabilized in 0.2% Triton X-100. After fixation, collagen gels were stained using a cocktail containing 4% paraformaldehyde, 0.2% Triton X-100 (Sigma) and 0.1 μM rhodamine Phalloidin (Sigma) for 3 hrs. Thereafter, wells were washed with PBS. Preparations were then mounted in Aqua-Poly/Mount solution (Polysciences, Inc) and imaged using a Plan Apo 4X Nikon dry objective with 0.2 NA and 20 WD. A total of 15 Z planes at an interval of 30 μm were captured. Image stacks were converted into two dimensional maximum intensity projections using ImagePro 7.0. Cell spheroids were analyzed using an automated Image pro 7-based plugin to calculate surface area of spheroid, number of cells migrating out of the cell spheroid and cumulative distance travelled by these cells.

**Immunohistochemistry.** Immunohistochemistry was performed on a series 30 formalin-fixed, paraffin-embedded radical prostatectomies and prostate lymph node metastases. 5 micron sections were dewaxed and rehydrated using xylene and ethanol. Endogenous peroxidase was blocked in 0.3% H2O2 and antigen retrieval was performed under pressure (0.9 bar) in TRIS-EDTA buffer (pH=9, Klinipath). SYK antibody (ab40781, Abcam) was diluted 1:300 in normal antibody diluent (Scytek) and incubated overnight at 4°C. Envision (DAKO) was used to visualize the antibody, counterstaining was performed with hematoxylin. The percentage and intensity (negative 0, weak 1+, moderate 2+, strong 3+) of positive SYK staining was estimated in benign luminal epithelial cells and prostate adenocarcinoma. Lymphocytes served as internal positive
control in all prostate and lymph node samples. Mann-Whitney U testing was performed to compare median expression levels.

**Experimental bone metastasis assay.** Male nude (BALB/c nu/nu) mice were anesthetized and injected with a single-cell suspension of $10^5$ cells/100 μl in PBS into the left cardiac ventricle. Outgrowth of spread PC3-M-Pro4luc cells was monitored weekly by whole body bioluminescent imaging (BLI) using an intensified charge-coupled device (I-CCD) video camera of the in vivo Imaging System (IVIS100; Xenogen, Alameda, CA, USA) as described previously (15). Values are expressed as RLUs in photons/sec. Bone metastases in a subset of mice were also examined by Goldner staining after mice were sacrificed using decalcified bone.

**Flow cytometry and Western blot.** For flow cytometry, surface expression levels were determined using primary antibodies, followed by fluorescence-conjugated secondary antibodies, and analysis on a FACSCanto or sorting on a FACSCalibur (Becton Dickinson). For Western blot, cells were lysed with modified RIPA buffer [150 mM NaCl, 1.0% triton-X 100, 0.5% Na deoxycholate, 0.1% 50mM Tris pH 8, and protease cocktail inhibitor (Sigma-Aldrich)]. Samples were separated by sodium dodecyl sulfate Polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore), incubated with primary antibodies then HRP-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.), and developed with enhanced chemiluminescence substrate mixture (ECL plus, Amersham, GE Healthcare). Blots were scanned on a Typhoon 9400 (GE Healthcare).

**Statistical analysis.** Data are presented as mean ± SEM of at least 3 independent biological replicates unless otherwise stated. Student's t test (two-tailed) was used to compare groups.
except for immunohistochemistry on human prostatectomies and lymph node metastasis where Mann-Whitney U testing was used to compare the median expression levels between groups.
RESULTS

Identification and validation of SYK in prostate cancer zebrafish xenografts

A panel of human prostate cancer cell lines was xenografted in the yolk of zebrafish embryos and dissemination was analyzed using a whole animal automated bioimaging platform as described (14). Prostate cancer cell lines reported to be androgen-independent and/or metastatic in mice (LnCaP-derived C4-2 and C4-2B; DU145 and PC3) showed enhanced dissemination in comparison with androgen-dependent non-metastatic LnCAP cells (19-22) (Fig S1a-d).

As a first step towards an adenovirus-based RNAi screening platform for regulators of prostate cancer dissemination we used adenoviruses targeting two genes previously implicated in prostate cancer (Fig 1a). These were the CD44 cell surface hyaluronan receptor and the SRC tyrosine kinase (23-26). Additionally, the SYK tyrosine kinase was included since it plays apparently opposite roles in different epidermal malignancies and has not been analyzed in prostate cancer (11-13). In agreement with their reported link to growth and progression of prostate cancer, targeting CD44 or SRC, each by two independent shRNAs and in two independent experiments using ~25 embryos per condition, led to a significant reduction in PC3 spreading throughout the embryos (Fig 1b,c). Interestingly, these criteria were also fulfilled for SYK (Fig 1b,c; Fig S2).

Stable expression of either of two independent lentiviral SYK shRNAs further confirmed the effect of SYK gene silencing in the zebrafish xenograft model (Fig 1d-f). Reduced SYK protein expression in the presence of lentiviral shSYK was confirmed by Western blot and by
immunohistochemistry on agar embedded cells, using the same antibody as used for immunohistochemistry on human tissue sections (Fig S3a,b). Reduced SYK abundance also effectively blocked migration in a model where cell spheroids are embedded in 3D extracellular matrix (ECM) scaffolds (18) (Fig 1g,h; Fig S4a). In addition, SYK gene silencing attenuated spheroid expansion and in vitro and tumor outgrowth at the primary injection site in zebrafish xenografts (Fig 1g,h; Fig S2,S4a). No signs of increased nuclear fragmentation in SYK-depleted spheroids were observed, pointing to decreased proliferation rather than cell death as the underlying mechanism (Fig S4b). We also analyzed the effect of silencing SYK on the ability of PC3 cells to form colonies when plated as single cells in vitro (Fig S5a). Reduced SYK levels led to a significant decrease in colony number and size. This was not associated with an apparent decrease in PI3K/AKT signaling since shSYK did not affect AKT phosphorylation on Ser473 reporting AKT activity (Fig S5b). These findings point to a role for SYK in growth and migration of PC3 prostate cancer cells.

Expression of SYK in human prostate cancer

Based on these findings, we next analyzed SYK expression levels in human prostate cancer. Breast cancer cells with reported low and high levels of SYK mRNA expression were used as controls (27,28). Compared to LnCaP, mRNA expression was increased in the androgen-independent LNCaP-derived C4-2 and C4-2B sublines and in DU145 and PC3 androgen-independent, metastatic prostate cancer cell lines (Fig 2a). Likewise, in a series of human prostate cancer xenografts (29), SYK mRNA expression was higher in androgen-independent tumors (Fig 2b). Moreover, SYK RNA expression in prostate cancer metastasis resection specimens was significantly increased compared to primary prostate cancer in two different datasets (Fig 2c). The EMC dataset used had GEO number: GSE41410 (30); The Taylor dataset
had GEO number GSE21032 (31). The expression of SYK was also compared between normal adjacent prostate (NAP) and primary prostate cancer (PCa) and in both datasets there was no significant difference in expression between NAP and PCa (PCa/NAP ratios: 0.97 (EMC), 0.94 (Taylor)). This was further confirmed in the Brase dataset (GEO number GSE29079; ref. 32) where the PCa/NAP ratio was 1.01. These findings indicated that SYK is unlikely to play a role in the early prostate cancer development but rather may have a role in progression of the disease.

Since the stromal compartment may affect mRNA analysis in clinical samples, SYK protein expression was analyzed subsequently in a set of radical prostatectomies for human prostate adenocarcinoma and lymph node metastases. SYK expression was variable in prostate adenocarcinoma ranging from complete absence of staining to moderate (2+) staining in the majority of tumor cells. Expression of SYK in normal luminal glandular epithelium and low-grade prostate cancer (Gleason score <7) did not differ significantly (Fig 2d). Notably, in pre-existent epithelial glands, SYK was expressed in basal cells while luminal cells were only rarely weakly positive (Fig 2e). Intermediate (Gleason score 7) and high-grade (Gleason score 8-10) prostate cancer demonstrated significantly higher expression of SYK than normal luminal epithelium or low-grade (Gleason score <7) prostate cancer (Fig 2d,e). SYK expression in prostate cancer lymph node metastasis ranged from undetectable to 100% moderate (2+) staining (Fig 2e). Median SYK expression in all metastases was significantly higher than that in all intermediate and high-grade primary prostate cancers taken together (Fig 2d). These results further supported the notion that SYK expression is associated with progression, rather than early development of prostate cancer.

**SYK supports cell surface expression of CD44 and integrin α2β1**
In acute lymphoid leukemia (AML), inhibition of SYK promotes differentiation (33). We analyzed a set of transcripts previously associated with undifferentiated characteristics of prostate cancer cells (34) but observed no gross changes in the expression of these genes upon depletion of SYK. However, while mRNA levels of the prostate cancer progression-associated markers CD44 and integrin α2β1 (23, 24, 35-37) were unaffected; their cell surface expression, but not total CD44 or integrin β1 protein levels, was suppressed following SYK silencing (Fig 3a-c; Fig S5c and data not shown). SYK has been previously reported to regulate surface expression of transmembrane receptors (38, 39) and two adenoviral CD44 shRNAs decreased PC3 dissemination in zebrafish (Fig 1b). Moreover, lentiviral silencing of α2 or β1 integrin subunits, each by two independent shRNAs, suppressed invasive outgrowth in 3D ECM as well as dissemination in the zebrafish xenograft model (Fig 3d-h). Together, these results identify regulation of surface expression of adhesion receptors as a potential underlying mechanism for the support of prostate cancer dissemination by SYK.

**SYK kinase activity supports formation of bone metastases**

We next addressed the role of SYK in a preclinical mouse xenograft model for prostate cancer bone metastasis. This preclinical in vivo model has been extensively characterized and the PC-3M-Pro4Luc cells were selected, by multiple in vivo passaging, for extremely high bone tropism (which reflects CRPC with bone metastasis in advanced prostate cancer patients) and virtually exclusively colonize bone (marrow) (15). Depletion of SYK led to a strong reduction in metastatic bone tumor burden following intracardiac inoculation of PC3M-Pro4Luc cells (Fig 4a,b). Similar to its effect *in vitro* and in zebrafish xenografts; shRNA targeting the integrin α2-subunit mRNA, phenocopied shSYK in this model (Fig 4a). To interrogate the specific role for SYK kinase activity in colony formation and prostate cancer bone colonization, wild type or kinase dead SYK was
expressed in PC3M-Pro4luc cells expressing an shRNA targeting the SYK 3’UTR. The reduced capacity to form colonies as well as colony growth in PC3M-Pro4luc-shSYK cells was restored to control levels by wild type but not kinase dead SYK (Fig 4c). Moreover, effective bone colonization of shSYK cells was restored by wild type SYK while expression of kinase dead SYK even further suppressed the process with very few detectable metastases (Fig 4d-f).

**Pharmacological inhibition of SYK prevents in vitro invasion and in vivo dissemination**

Based on the dependency on SYK kinase activity determined in the mouse model, we performed initial experiments to evaluate whether pharmacological inhibition of SYK could interfere with *in vitro* invasive outgrowth and *in vivo* dissemination using zebrafish xenografts. Small molecule inhibitors of SYK are in clinical development for autoimmune diseases and lymphoid malignancies (8, 40). Two of these compounds, R-406 and BAY-61-3606 show efficacy in preclinical leukemia and retinoblastoma studies (33, 41-44). When used at 1-10\(\mu\)M, the concentration widely used *in vitro* (33, 41-44) these compounds reduced spheroid outgrowth and ECM invasion of PC3 as well as C4-2B cells (Fig 5a-c). Moreover, R-406 significantly inhibited dissemination of PC3 cells (Fig 5d) without significant signs of toxicity at 10 \(\mu\)M (e.g. no effects were observed when yolk sac edema, cardiac edema, bending of the tail, hepatic necrosis, and impaired cardiovascular function were compared for R-406 and vehicle control treated animals). Thus, pharmacological inactivation of SYK recapitulated the effect of silencing the SYK gene *in vitro* and in zebrafish xenografts.
DISCUSSION

There is an urgent need for further insights into aspects of prostate cancer progression that provide new avenues for targeted therapy. Our study demonstrates that a semi-automated whole animal bioimaging assay based on zebrafish xenotransplantation (14) can be productive in RNAi-based preclinical prostate cancer drug target discovery. Efficacy of human prostate cancer cell spreading throughout the embryo correlates with androgen-independence, a major hallmark of prostate cancer progression, and with behavior in rodent models. Two signaling proteins, previously associated with prostate cancer progression, Src and CD44 are effectively identified using adenoviral shRNAs (23-26). Although the pipeline is currently only partly automated, screening of small adenoviral RNAi sub-libraries (~100 genes) is feasible. Integration of the established automated imaging and quantitative image analysis with recently described methods for automated injection and sorting of zebrafish embryos can widen applicability to larger scale screening (45).

Our findings indicate that the protein tyrosine kinase, SYK supports growth and migration of prostate cancer cells. The evidence comes from two transiently expressed adenoviral and two stably expressed and bulk-sorted lentiviral shRNA vectors. In addition, expression of wild type SYK rescues the attenuated *in vitro* clonogenic outgrowth and *in vivo* formation of bone metastases of shSYK cells, further arguing against off-target effects. This suggests that the role of SYK in prostate cancer is opposite to its proposed “progression suppressor” role in breast cancer (11, 12). In further support of that, we show that expression of SYK is somewhat increased in more aggressive prostate cancer cell lines and in metastases as compared to primary prostate cancer lesions. A wider analysis of SYK protein expression and activity in a large
A cohort of prostate cancer patients will be needed to firmly establish if SYK is positively correlated to progression of prostate cancer, as it appears to be for head and neck squamous cell carcinoma (13).

In hematopoietic cells, immune receptors provide the ITAM for recruitment and activation of SYK. In the epithelial cell types where SYK is expressed it has not been established if and how SYK may be activated. Src family kinases are responsible for the ITAM phosphorylation that is required to recruit SYK (7, 46). Src is has been associated with prostate cancer progression and RNAi targeting Src also interfered with PC3 dissemination in the zebrafish model. Src may act on a large number of substrates in prostate cancer cells. One potential target in the context of SYK activation that we evaluated was “migration and invasion enhancer 1” (Mien1; also termed C35/C17orf37). Expression of Mien1 is correlated with progression of breast, ovarian, and colon cancer, it contains an ITAM, and it has been reported to require SYK for its breast cancer promoting activity (47-51). However, stable silencing of MIEN1 in PC3 cells did not affect outgrowth or invasion in 3D cultures, indicating that this is unlikely to be involved in prostate cancer growth or invasion (not shown). Further studies will address additional possible mechanisms in the context of prostate cancer.

It is not known how SYK contributes to - or, in the case of breast cancer, interferes with tumor progression but modulation of NKκB activity may be one aspect involved (52). In immune cells, SYK mediates the activation of MAP kinase signaling, calcium fluxes, and cytoskeletal remodeling when immune receptors are engaged (7, 46). In addition, SYK has been previously reported to support the surface expression of integrins (38, 39). Our findings indicate that stimulation of α2β1 and CD44 cell surface expression may play a role in the stimulation by SYK of invasive
growth in 3D ECM in vitro and dissemination in the zebrafish model. A role for β1 integrins in intravascular locomotion of MDA-MB-435 breast cancer cells in zebrafish has been previously reported (53). In our study, silencing either α2 or β1 subunits prohibits effective migration in the zebrafish. Moreover, silencing α2β1 phenocopies the effect of silencing SYK in the experimental mouse bone metastasis model. Thus, SYK-mediated stimulation of the cell surface expression of adhesion receptors may contribute to aspects of prostate cancer progression.

Our experiments using a kinase-dead mutant show that stimulation of clonogenic growth in vitro and experimental bone metastasis in the mouse, depend on SYK kinase activity. Moreover, R-406 and BAY-61-3606 SYK kinase inhibitors that were effective in preclinical leukemia and retinoblastoma studies (33, 41-44) interfere with invasive growth in 3D ECM in vitro and dissemination in the zebrafish model. So, genetic or pharmacological inactivation of SYK kinase activity inhibits invasive growth and dissemination of prostate cancer. We verify that SYK mRNA and protein is detected in human prostate cancer tissues and SYK inhibitors have already been tested in phase I-II clinical trials for other diseases. Altogether, this establishes SYK as a potential new drug target in prostate cancer for which existing pharmacological inhibitors with known toxicological profiles can be tested for clinical efficacy.

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AUTHOR CONTRIBUTIONS

REFERENCES


FIGURE LEGENDS

Figure 1. SYK supports growth, invasion, and dissemination of human prostate cancer cells. (a) Schematic overview of the in-vivo screening procedure. (b) Mean cumulative distance (MCD) of tumor foci relative to site of injection for PC3 cells transiently transduced with indicated adenoviral shRNAs calculated from >40 xenografts obtained from two independent experiments. (c) Representative scatter plots showing tumor foci detected by automated confocal imaging and automated image analysis as described (14) in zebrafish injected with PC3 cells expressing indicated adenoviral shRNAs. Each color shows foci detected in one embryo. Arrows indicate foci in tail region. (d) qPCR verification of SYK silencing in PC3 cells expressing two independent lentiviral shSYK vectors. (e,f) MCD (e) and representative images (f) for dissemination in zebrafish of PC3 cells stably expressing indicated lentiviral SYK shRNAs (combined data from 2 independent experiments using >32 embryos per condition are shown; arrowheads indicate tumor foci in tail region). (g,h) Representative images (g) and quantification of expansion (mean spheroid area) and ECM invasion (MCD) (h) for control and shSYK PC3 cell spheroids at 6 days post-injection in collagen gels (blue, Hoechst; red, Phalloidin). Scale bars, 100 μm. ns, non significant; *p<0.05; ** p<0.01; ***p<0.005.

Fig 2. SYK is expressed in human prostate cancer. (a) SYK RNA expression determined by qPCR in indicated cell lines. MDA-MB-435S (hypermethylated SYK gene promoter (27, 28)) and HCC-1954 breast cancer cells (high SYK expression (28)) were used as negative and positive controls, respectively. (b) SYK RNA expression in human prostate cancer resection specimens xenografted in mice. (c) Ratio between SYK RNA expression in prostate cancer metastases (PCa Met) and primary prostate cancers (PCa) and significance (TTEST) in two different datasets. (d) Semi
quantitative analysis (mean and SEM) of SYK expression in normal luminal epithelium, primary prostate cancer, and prostate cancer lymph node metastases. **(e)** Immunohistochemical detection of SYK in human benign glandular prostate epithelium (1; note staining of basal but rarely of luminal cells), prostate adenocarcinoma (2; weak, and 3; moderate SYK expression), and prostate lymph node metastasis (4; negative, and 5; strong SYK expression). Arrows point to prostate epithelial / cancer cells. Original magnifications 200x (1,4,5) and 100x (2, 3). Strong staining in lymphocytes served as internal positive control in all cases (arrowheads). ns, non significant; *p<0.01; ** p<0.005; ***p<0.001 (Mann-Whitney U test).

**Figure 3.** SYK regulation of adhesion receptor surface expression in human prostate cancer cells modulates invasive outgrowth in vitro and dissemination in zebrafish and metastatic colonization in mice. **(a)** FACS analysis of surface expression of CD44 and integrin subunits α2 (ITGA2) and β1 (ITGB1) in PC3 cells expressing control or SYK shRNAs. MFI, mean fluorescence intensity. **(b,c)** qPCR analysis of ITGA2 and ITGB1 mRNA expression in PC3 cells expressing control or SYK shRNAs. **(d)** FACS verification of ITGA2 and ITGB1 silencing in PC3 cells expressing two independent lentiviral vectors targeting ITGA2 or ITGB1, respectively. **(e,f)** Quantification of expansion (mean spheroid area) and ECM invasion (MCD) **(e)** and representative images **(f)** for control, shITGA2, and shITGB1 PC3 cell spheroids at 6 days post-injection in collagen gels (blue, Hoechst; red, Phalloidin). **(g,h)** Representative images **(g)** and quantification of MCD **(h)** for dissemination in zebrafish of PC3 cells expressing indicated lentiviral shRNAs (combined data from 2 independent experiments using > 40 embryo’s per condition are shown; arrowheads indicate tumor foci in tail region). Scale bars, 100 μm. ** p<0.01; ***p<0.005.
Figure 4. SYK kinase activity supports metastatic bone colonization in mice. (a) Total metastatic tumor burden determined by BLI monitoring at indicated time points following intra-cardiac inoculation in immune-compromised mice for PC3M-Pro4luc variants expressing indicated lentiviral shRNAs (data obtained from at least 9 mice per experimental group). (b) Bones of mice collected 31 days after intracardiac inoculation with PC3M-Pro4-luc shCTR or shSYK cells and stained with Goldner staining. *, tumor lesion. (c) Quantification of colony formation assay for PC3M-Pro4-luc cells expressing control or SYK shRNAs in combination with wild type (SYKwt) or kinase dead SYK (SYKkd) expression vectors. Grey, small; white, medium; black, large colonies. (d) BLI images of PC3M-Pro4luc variants expressing control or SYK shRNAs in combination with wild type (SYKwt) or kinase dead (SYKkd) expression vectors taken 31 days following intra-cardiac inoculation. (e,f) Quantification of the experiment shown in (d) where total metastatic tumor burden was determined by BLI monitoring at indicated time points (e) and the number of metastatic colonies was determined by counting of BLI foci at 31 days following intra-cardiac inoculation (data obtained from at least 10 mice per experimental group) (f). ns, non significant; *p<0.05; ** p<0.01; ***p<0.005 versus shCTR.

Figure 5. Pharmacological inhibition of SYK prevents growth, invasion, and dissemination of prostate cancer cells. (a) Bright field images showing PC3 spheroids 6 days post-injection into collagen gels in the absence or presence of the indicated concentrations of BAY-61-360 (one of three experiments is shown). (b) Representative images and quantification of expansion (mean spheroid area) and ECM invasion (MCD) for PC3 cell spheroids measured at 6 days post-injection in collagen gels and treated with indicated concentrations of R-406 (blue, Hoechst; red, Phalloidin). (c) Brightfield images showing C4-2B cell spheroids at 6 days post formation in the absence or presence of the indicated concentrations of SYK inhibitors. Arrowheads point to ECM
invading strands seen under control conditions, which are not observed in presence of inhibitors. (d) Representative images and quantified MCD for dissemination in zebrafish of PC3 cells in the absence or presence of indicated concentrations of R-406. Combined data from 2 independent experiments using >70 embryos per condition are shown. Arrows point to cells disseminated to tail region. Scale bars, 120\(\mu\)m; ns, not significant; *\(p<0.05\); ***\(p<0.005\).
**a**

Adenoviral shRNA transduction

PC3 → CMDil labeling

Intra-yolk injection

Automated alignment of images & calculation of cumulative distance of tumor foci from injection point per embryo >> determination of mean cumulative distance for all embryos in one group (MCD)

**b**

![Graph showing MCD values](image)

- **GFP_v5**: MCD values
- **SRC_v3**: MCD values
- **SYK_v7**: MCD values
- **CD44_v3**: MCD values

**c**

- **GFP_v5**
- **SYK_v3**
- **SYK_v7**
- **CD44_v3**

**d**

SYK mRNA expression

- **Mock**
- **shCTR**
- **shSYK #1**
- **shSYK #2**

**e**

- **Mock**
- **shCTR**
- **shSYK #1**
- **shSYK #2**

**f**

- **ShCTR**
- **shSYK**

**g**

- **shCTR**
- **shSYK #2**

**h**

- Mean spheroid area
- MCD
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**Figure 2**

**Panel a:** Bar graph showing SYK/GAPDH (log 10 scale) for various cell lines.

**Panel b:** Bar graph illustrating the dependency and sensitivity of androgen on SYK/GAPDH levels.

**Panel c:** Table comparing EMC dataset and Taylor dataset for PCa Met / PCa ratio and TTEST results.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Ratio</th>
<th>TTEST</th>
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<tbody>
<tr>
<td>EMC dataset</td>
<td>1.85</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Taylor dataset</td>
<td>1.62</td>
<td>&lt;0.00001</td>
</tr>
</tbody>
</table>

**Panel d:** Graph showing the percentage of SYK positive cells for different groups of PCa.

**Panel e:** Images depicting immunohistochemical staining for PCa with arrows indicating positive staining locations.
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Veerander Paul Singh Ghotra, Shuning He, Geertje van der Horst, et al.

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