Src controls castration recurrence of CWR22 prostate cancer xenografts

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Castration-recurrent prostate cancer, CWR22, dasatinib, KXO1, shRNA, Src, xenografts

Abstract
Recurrence of prostate cancer (CaP) after androgen-deprivation therapy continues to have the greatest impact on patient survival. Castration-recurrent (CR)-CaP is likely driven by the activation of androgen receptor (AR) through multiple mechanisms including induction of AR coregulators, AR mutants or splice variants, and AR posttranslational modification such as phosphorylation by Src-family and Ack1 tyrosine kinases. Here, we address whether Src is required for the CR growth of human CWR22 CaP xenografts. The shRNA-mediated Src knockdown or treatment with the Src inhibitors, dasatinib or KXO1, reduced CaP recurrence over controls and increased time-to-recurrence following castration. Moreover, CR-CaP [Src-shRNA] tumors that recurred had similar Src protein and activation levels as those of parental cells, strengthening the notion that Src activity is required for progression to CR-CaP. In contrast, the ability of dasatinib or KXO1 to inhibit Src kinase activity in vitro did not correlate with their ability to inhibit serum-driven in vitro proliferation of CR and androgen-dependent stable cell lines derived from CWR22 tumors (CWR22Rv1 and CWR22PC, respectively), suggesting that the in vitro proliferation of these CaP lines is Src independent. Taken together, these findings strongly suggest that Src is a potent and specific therapeutic target for CR-CaP progression.

Introduction
Prostate cancer (CaP), the second leading cause of cancer deaths in U.S. men (http://seer.cancer.gov/statfacts/html/prost.html), progresses from localized to invasive disease associated with metastasis to local lymph nodes and bones. Unlike the high cure rate of early, localized disease, the so-called lethal clinical phenotype of CaP relates to recurrence following androgen-ablation therapy, producing castration-recurrent (CR) CaP that responds poorly to standard chemotherapy and radiation [1]. Multiple studies indicate that the vast majority of CR-CaP cases present with increased protein and activation levels of WT-AR (wild-type androgen receptor) [2]. This is thought to facilitate AR-driven tumor progression in response to the postcastration expression of low tissue androgen levels [3]. AR mutations and splice variants have been described that can facilitate AR activity in the CR setting [4, 5], yet other mechanisms leading to the activation of AR in CR-CaP have been described, including AR stabilization [6], induction of AR coregulators and posttranslational modification [7].
Several Src-family tyrosine kinases (SFK) are overexpressed and activated in a wide variety of human cancers, including metastatic- or CR-CaP [8–10]. Progression to androgen independence in human (prostate cancer cells) LNCaP is associated with an increased interaction of activated Src with AR [11–13]. Additionally, increased levels of activated receptor tyrosine kinases in CaP progression, known to activate downstream SFK, is associated with increased androgen-independent AR activation [14]. Src activity is also required for the increased expression and secretion of neutrophil specific for Src (Src-1; Oncogene Sciences). Secondary antibodies included horseradish peroxidase-labeled anti-rabbit or anti-mouse Ig (EMD-Millipore, Billerica, MA). All antibodies included horseradish peroxidase-labeled anti-rabbit or anti-mouse Ig (EMD-Millipore, Billerica, MA). All antibodies included horseradish peroxidase-labeled anti-rabbit or anti-mouse Ig (EMD-Millipore, Billerica, MA). All antibodies included horseradish peroxidase-labeled anti-rabbit or anti-mouse Ig (EMD-Millipore, Billerica, MA). All antibodies included horseradish peroxidase-labeled anti-rabbit or anti-mouse Ig (EMD-Millipore, Billerica, MA). All antibodies included horseradish peroxidase-labeled anti-rabbit or anti-mouse Ig (EMD-Millipore, Billerica, MA). All antibodies included horseradish peroxidase-labeled anti-rabbit or anti-mouse Ig (EMD-Millipore, Billerica, MA).

**Antibodies and reagents**

The following primary antibodies (Ab) were used: rabbit polyclonals specific for GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), Lyn, Paxillin, Paxillin[Y118], Src[Y416] (Cell Signaling Technology, Beverly, MA), and mouse monoclonal specific for Src (Src-1; Oncogene Sciences). Secondary antibodies included horseradish peroxidase-labeled anti-rabbit or anti-mouse Ig (EMD-Millipore, Billerica, MA). All reagents were from Sigma (St. Louis, MO) unless stated otherwise.

**Material and Methods**

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**Cell culture and virus production**

HEK293T cells (ATCC CRL-11268) were maintained in Dulbecco’s modified Eagle’s media (DMEM) supplemented with 10% heat-inactivated bovine serum (BS). Lentiviruses were produced by transient transfection of HEK293T cells with shRNA-encoding lentivirus vector DNA plus DNAs encoding VSV (vesicular stomatitis virus)-G envelope (pMD2G) and HIV-based Gag, Pol and regulatory proteins (pCMV-R8.74), gifts of Didier Trono (École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland).

**Proliferation assay**

CWR22Rv1 and CWR22PC plated in triplicate in RPMI-1640 plus 10% fetal calf serum (FCS) at a density of 2000/well in 96-well plates were treated for 72 h with increasing concentrations of KXO1 or dasatinib (from 1 nmol/L to 100 μmol/L), or vehicle (dimethyl sulfoxide, DMSO), then stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide using the Vybrant® MTT Cell Proliferation Assay Kit (Promega, Madison, WI).

**Growth of CWR22 xenografts**

One week before tumor cell inoculation, sustained-release testosterone (T) pellets (12.5 mg/kg in silastic tubing) were placed s.c. between the shoulder blades in castrated athymic nude male mice. Each mouse was injected s.c. with 10⁶ cells in 100 μL of Matrigel (5 mg/mL; Bedford, MA) in phosphate buffered saline (PBS). Tumor volumes and mouse weights were measured weekly, and the tumor volumes were calculated using the formula, L x L x W x 0.5234. When the tumors reached ~250 mm³, the T pellets were removed and treatment (orally once daily, sid po) started the next day for 28 days with dasatinib (15 mg/kg), KXO1 (10 mg/kg) or vehicle (80 mmol/L sodium citrate/citric acid buffer, pH 3.0). Tumor volumes were measured every 2 weeks for recurrence. To assess the ability of dasatinib or KXO1 to inhibit Src kinase activity in tumors, CWR22-tumored (250–400 mm³), T-pelleted male nude mice were treated for 2 weeks with sid po doses of dasatinib, KXO1 or vehicle, as above. Mice were sacrificed 3 h after the final dosing, and tumor lysates were prepared immediately.

**Transduction of CWR22**

AD CWR22 primary tumors grown in T-pelleted nude male mice were harvested at volumes of 250–400 mm³, and tumor tissues were digested with collagenase type I (Invitrogen-Life Technologies, Grand Island, NY). After washing the cells twice with sterile PBS, 10⁶ cells/6-cm
dish were plated and then infected after 4 h of attachment with 1 mL of DMEM containing lentiviruses (scrambled-shRNA or Src-shRNA [17]; gifts of Zhiyong Guo, University of Maryland School of Medicine) at a multiplicity of 2.5 (based on expression of viral Green Fluorescent Protein [GFP]). Infection was facilitated by centrifugation at 2500 \( g \) for 30 min in a swinging bucket rotor.

**Immunoblotting**

Immunoblotting (IB): The tumor tissues were homogenized and lysed in RIPA (radioimmunoprecipitation assay) buffer (10 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA (ethylenediaminetetraacetic acid), 8% glycerol, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with 10 mmol/L Na_3VO_4, 1 mmol/L NaF, and Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). A quantity of 40 \( \mu \)g total proteins per sample was separated by SDS-PAGE (sodiumdodecyl sulfate polyacrylamide gel electrophoresis), blotted onto PVDF (polyvinylidene difluoride) membranes, which were blocked for 30 min with 5% BS albumin in \( 1 \times TBS/T \) (0.1% Tween-20 in Tris-buffered saline) probed with primary and secondary Abs (including three TBS/T washes after each Ab step), and then imaged after incubation with Lumi-Light chemiluminescence reagent (Roche) as described previously [33]. Digital imaging and signal quantification were performed using a Chemi-Genius2 Bio-Imager (Syngene, Frederick, MD) and GeneTools software.

**Statistical analyses**

Differences in time to tumor recurrence between treatment and vehicle control were compared using Kaplan–Meier curves and the log-rank test. Mean time to recurrence is computed from the area under the curve truncated at 220 days (restricted mean life). The \( P \)-value for the comparison of Src shRNA is based on a chi-square test.

**Results and Discussion**

**Src inhibitors decrease CWR22 tumor recurrence**

The well established human CWR22 xenograft model [18] can recapitulate initial AD CaP growth in vivo followed by castration-induced regression and the recurrence in 40–50% of hosts of CR-CaP that express and are driven by AR [32] (Fig. 1A). Recurrence is defined by the growth of the primary-site (s.c.) tumor after a postcastration regression to volumes comparable to (and eventually, greater than) precastration levels (Fig. 1A). Nonrecurrence is defined as no net growth over regressed tumor volumes during the 7-month postcastration period. It should be noted that CR-CaP lesions as well as a cell line derived from these lesions, CWR22Rv1, express mutated version of AR (H874Y) from the parental CWR22 [34], as well as a truncated AR which exhibits constitutive nuclear localization and DNA-binding activity [35]. Importantly, these forms are still likely regulated by Src as CWR22Rv1 growth in castrated nude mice requires Src [17].

In order to determine whether Src is required for the spontaneous generation of CR-CaP in the CWR22 model, CWR22 xenografts were grown to \( \approx 250 \) mm\(^3\) in T-pel-
lated, castrated male nude mice, then treated for 28 days (starting 1 day before T-pellet removal) with doses of dasatinib or KXO1 (vs. vehicle) (Fig. 1A) previously shown to inhibit Src-driven tumor growth in vivo [10, 36]. Compared to controls, dasatinib and KXO1 had no effect on postcastration tumor regression (Table 1), suggesting that this process is SFK independent. In contrast, KXO1 and dasatinib decreased overall CR-CaP formation by 60% or 50%, respectively (Table 1), and although these decreases may not be statistically significant, the ability of KXO1 and dasatinib to delay the time-to-recurrence of CR-CaP (Fig. 1B) by 1 or 2 months, respectively, showed strong statistical power (Table 1).

The ability of dasatinib and KXO1 to inhibit Src activation in cultured CWR22Rv1 cells was demonstrated by comparing total Src protein to autophosphorylation levels, marked by Src\textsuperscript{poY416} (Fig. 2A, upper panel), previously established as a surrogate marker for Src kinase activity levels in cells [37]. Src activation levels in CWR22Rv1 cells were inhibited by 1 \( \mu \)mol/L dasatinib or KXO1; however, dasatinib suppressed Src activation levels in CWR22PC, an AD cell line derived from primary CWR22 xenografts [38], KXO1 induced Src activation levels in these cells, both at the level of Src autophosphorylation and transphosphorylation of the substrate, paxillin, on Y118 (Fig. 2A). This may reflect different mechanisms of action by the two compounds: dasatinib is an ATP-competitive kinase inhibitor [39] whereas KXO1 is a non-ATP-competitive peptide binding-site inhibitor [40, 41]. How this is manifest remains unclear because Src activation is equally inducible by serum in both CWR22Rv1 and CWR22PC cells, and equally unaffected by androgen alone (Fig. 2B). Importantly, though, ("AD": CWR22) and CR (CWR22Rv1) tumors exhibited inhibition of Src activity 3 h after the last dose in a 14-day treatment regimen with dasatinib or KXO1 (Fig. 2C), indicating that Src was targetable by these drugs in the in vivo setting. Additionally, unlike dasatinib, KXO1 may uniquely be able to bind Src complexes and/or conformations that occur only in androgen-sensitive cells in the in vitro setting.

Given the discordance between the ability of dasatinib or KXO1 to inhibit Src activity in vitro versus in vivo, we tested the ability of these drugs to inhibit in vitro proliferation of the AD versus CR CaP lines. Even though dasatinib inhibited Src autophosphorylation level \( >90\% \) at 1 \( \mu \)mol/L (Fig. 2A), it was poor at inhibiting proliferation in vitro based on growth inhibitory (GI\textsubscript{50}) values of 26 \( \mu \)mol/L or 56 \( \mu \)mol/L in CWR22Rv1 or CWR22PC cells, respectively (Table 2). In contrast, even though KXO1 inhibited Src activity in the CWR22Rv1 cells only (Fig. 2A), it was more potent than dasatinib at inhibiting in vitro proliferation, with GI\textsubscript{50} values of 232 nmol/L in CWR22Rv1 or 21 nmol/L in CWR22PC cells. Taken together with the data above, this suggests that CR growth in vivo is Src dependent whereas the serum-induced in vitro proliferation of CWR22Rv1 or CWR22PC is either Src independent or the broad specificity of dasatinib negates inhibitory pathways required downstream of Src for proliferation in vitro. It is also likely that the increased ability of KXO1 to inhibit the in vitro proliferation of CWR22Rv1 or CWR22PC might be due to additional non-Src targeting functions, such as pretubulin inhibition, as has been described recently (http://www.kinexpharma.com/drug-pipeline/kx01).

If CR growth of the CWR22 tumors is Src dependent, then the CR tumors that arise spontaneous or after dasatinib/KXO1 treatment should continue to express activated Src. Thus, relative levels of activated Src were determined in the CR-CaP tumors that arose months after treatment with vehicle, dasatinib or KXO1. All CR-CaP lesions recurring after dasatinib or KXO1 treatment exhibited similar relative Src\textsuperscript{poY416} levels to vehicle-treated CR-CaP tumors (Fig. 2D). This contrasts with data in Figure 2C showing that acute treatment with dasatinib or KXO1 could inhibit relative Src\textsuperscript{poY416} levels. Given that expression of activated Src is sufficient to induce CR-CaP in a tissue recombination model of CaP [24], our results suggest that continued Src activation promotes CR-CaP in the CWR22 model. It remains unclear whether longer drug treatment might be sufficient to suppress CR-CaP generation more completely; however, the finding of sustained Src activation levels in CR-CaP lesions from dasatinib- and KXO1-treated mice strongly suggests that drug resistance in this context is not due to non-Src compensatory mechanisms.

### Table 1. Effect of KXO1\textsuperscript{1} and dasatinib\textsuperscript{2} on tumor occurrence.

| Group   | Recurrence | Mean time to recurrence (SE)\textsuperscript{4} | Log-rank test vs. vehicle\textsuperscript{5} |
|---------|------------|-----------------------------------------------|------------------------------------------|
| Vehicle | 10/20 (50%)| 164.3 (13.4)                                  |                                          |
| KXO1    | 4/20 (20%) | 206.0 (6.3)                                   | \( P = 0.0494 \)                          |
| Dasatinib | 5/20 (25%)  | 200.0 (7.8)                                   | \( P = 0.0225 \)                          |

\textsuperscript{1}Ten milligram per kilogram po sid for 28 days after castration.

\textsuperscript{2}Fifteen milligram per kilogram po sid for 28 days after castration.

\textsuperscript{3}Days postcastration for maximal regression or growth arrest (SE).

\textsuperscript{4}Values for the recurrence percentages are vehicle versus KXO1 (\( P = 0.097 \)) and vehicle versus dasatinib (\( P = 0.191 \)) with standard error (SE) based on the standard chi-square tests.

\textsuperscript{5}The Log-rank tests compare the difference between individual survival curves. The overall test (any difference between the three) is \( P = 0.0261 \).

**siRNA-mediated Src knockdown suppresses recurrence of the CWR22 tumor**

Although dasatinib was originally described as a Src/Abl-specific inhibitor [39], there is appreciation that it...
functions as a pan-tyrosine kinase inhibitor [42]. Additionally, KXO1 (also called KX2-391) targets several SFK members as well as Abl, and newer data indicate that it also targets tubulin polymerization (D. Hangauer, Kinex Pharmaceuticals LLC, pers. comm.). Thus, to more specifically address the role of Src in the generation of CR-CaP, we sought to knock down Src expression using lentivirus transduction of Src-shRNA. We first showed that compared to control vector-infected cells, CWR22Rv1 transduced with Src shRNA (“shSrc”) showed significant knockdown of Src protein levels (Fig. 3A) compared to another SFK family member, Lyn. Primary CWR22 tumors grown to ∼250 mm³ in T-pelleted male nude mice were excised, converted into single-cell suspensions by collagenase treatment, and after washing, plated and
rapidly infected with high-titer lentivirus encoding control- or Src-shRNA at efficiencies close to 100% infection, based on expression of the surrogate lentivirus-encoded GFP marker (Fig. 3B, left panel). Indeed, GFP expression was sustained in vitro even as the cells senesced in culture (after three passages; Fig. 3B, right panel) and after AD tumor growth following reinoculation into T-pelleted male nude mice (Fig. 3C). All recurrent tumors exhibited strong GFP expression in androgen-dependent and recurrent tumors formed after reinjection of CWR22 cells transduced with control- or Src-shRNA GFP-expressing lentiviruses (a–c for control-shRNA; A–E for shSrc), and in recurrent tumors (d–g for control-shRNA; F and G for shSrc). (D) Lysates of androgen-dependent (a–c for control-shRNA; A–E for shSrc) or recurrent CWR22 tumors (d–g for control-shRNA; F and G for shSrc) were analyzed by immunoblotting for Src versus GAPDH protein levels.

Table 3. Effect of Src shRNA on tumor occurrence.

| Group (n = 10) | Recurrence | χ² test vs. vehicle |
|---------------|------------|-------------------|
| Control-shRNA | 4/10 (40%) | P = 0.622 |
| Src-shRNA     | 2/10 (20%) |                   |

loss of Src had no effect on the rates of primary tumor growth or postcastration regression (data not shown), in agreement with our data given above that neither dasatinib nor KXO1 affected postcastration regression (Table 1). The levels of Src protein in five primary, AD shSrc-expressing tumors (Fig. 3D, lanes A–E) was uniformly lower than in primary control tumors (lanes a–c), indicating a sustained effect of the Src shRNA in vivo. In contrast, Src protein levels in the two shSrc CR-CaP lesions (lanes F and G) were similar to those in control primary and recurrent lesions. Although these numbers
are small, these data strengthen the concept that Src is required for CR-CaP generation in this system.

This study is the first to demonstrate a role for Src in the spontaneous generation of CR-CaP using a model that starts with an AD human CaP xenograft. The growing acceptance that Src plays a pivotal role in CaP progression to recurrence and even more specifically, to the formation of bone metastases [43], has spawned multiple clinical studies in CR-CaP using Src inhibitors in conjunction with chemotherapies, such as docetaxel [1, 31, 44–47]. Initial Phase II and Phase I/II studies indicate efficacy for dasatinib alone or in combination with docetaxel using prostate-specific protein (PSA) level and boney metastasis monitoring as therapeutic markers [48, 49]. Data are pending from a current multicenter Phase II trial with KXO1 in CR-CaP cases with boney metastases (NCT01074138).

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Conflict of Interest

B. S., B. G., and L. G. have no conflicts of interests; I. H. G. is on the Scientific Advisory Board of Kinex Pharmaceuticals, LLC.

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