Lyn Is a Target Gene for Prostate Cancer: Sequence-Based Inhibition Induces Regression of Human Tumor Xenografts

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ABSTRACT

The Src-related protein kinase Lyn plays an important role in B-cell activation. However, several lines of evidence suggest that it is also involved in the control of cellular proliferation and the inhibition of apoptosis. We have discovered that Lyn is expressed in normal prostate epithelia, in 95% of primary human prostate cancer (PC) specimens examined, and in all of the PC cell lines that we assayed. Moreover, Lyn knockout mice display abnormal prostate gland morphogenesis, which suggests that Lyn plays an important role in prostate epithelium development and implies that Lyn is a candidate target for specific therapy for PC. Using a drug-design strategy to construct sequence-based peptide inhibitors, a Lyn-specific inhibitor, KRX-123, targeting a unique interaction site within Lyn, was synthesized. KRX-123 was found to inhibit cellular proliferation in three hormone-refractory PC cell lines, DU145, PC3, and TSU-Pr1 with IC50 values of 2–4 μM. In vivo, tumor volume of DU145 explants in nude mice was significantly reduced after one-once-a-week injections of KRX-123, at a dose of 10 mg/kg, for a period of 5 weeks. Histological analyses of the treated tumors indicated extensive apoptosis. Thus, we suggest that Lyn inhibition may serve as a prime target for the treatment of hormone-refractory PC.

INTRODUCTION

Prostate cancer (PC) is the most common malignancy detected in men. Although hormone therapy may be effective in the early stages of PC, virtually all metastatic PCs become refractory to hormonal treatment (1). The emergence of hormone resistance eventually results in the patient’s death because, currently, there is no effective treatment for hormone-refractory PC (HRPC). Protein kinases (PKs) control major events related to cell proliferation, and their deregulated activity can lead to cellular transformation and is associated with tumor maintenance and progression (2). Therefore, the identification of key regulatory PKs involved in PC could be an initial step in developing new therapeutic options in which the activity of the identified kinases is modulated by novel designer agents. Site-specific phosphorylation by PKs acts as a chemical relay that turns intracellular signaling cascades or between integrins and cytoskeletal elements (3). Lyn, a nonreceptor protein tyrosine kinases (PTKs) mediate signaling between transforming growth factor β levels and Lyn expression in PC (11) demonstrated a role for Lyn in mediating the neutral endopeptidase inhibition of focal adhesion kinase in PC cell lines.

Because of the indication of an involvement of Lyn in cancer in general, and of PC in particular, we conducted a series of studies designed to characterize the role of Lyn in PC. We first demonstrated that Lyn is expressed in normal prostatic epithelia and that Lyn expression is extensively manifested in the vast majority of primary human PCs. Our examination of Lyn knockout mice revealed a compromised prostate gland development. To further facilitate these studies, we have developed a sequence-based peptide inhibitor of Lyn that targets a unique interaction site within Lyn. Our studies show that inhibition of Lyn signaling in HRPC cell lines by the Lyn-derived peptide inhibits cell proliferation. Moreover, Lyn appears to be an outstanding therapeutic target for HRPC because the administration of the Lyn-specific inhibitor to nude mice bearing HRPC tumor explants induces both cell apoptosis and tumor regression.

MATERIALS AND METHODS

Reagents

Synthetic Peptides. Peptides were synthesized by Fmoc [N-(9-fluorenyl)-methoxycarbonyl] solid-phase peptide synthesis (NovoTide Ltd. and Genemed Synthesis Inc.) and were purified by reverse-phase high-performance liquid chromatography. Identity and purity were confirmed by high-performance liquid chromatography–mass spectroscopy. For cell-based assays, peptides were formulated (with approved excipients only) as follows: 15 mg peptide were dissolved in 0.25 ml of 4% benzyl alcohol, 4% pluronic L44, and 2% benzyl benzoate in propylene glycol. To this, 0.125 ml of 2.2% glycine in double-distilled water and 0.125 ml of 50 mM sodium bicarbonate (pH 7.5) were added while vigorously stirring the tube. The preparation was heated to 100°C for 15 min and then was homogenized with a Polytron (Kinematica) for 2 min, during which 0.5 ml of 0.3 M lactose were gradually added. Heating and homogenizing were repeated, and the preparation was sterilized by heating to 100°C for 30 min. No change in the peptide high-performance liquid chromatography–mass spectroscopy profile was noted after this treatment. The resulting preparation, designated BBlaC, is a very fine emulsion, suitable also for i.v. injection.

Antibodies. Anti-Lyn (clone 44), for immunoblotting, immunoprecipitation, and immunohistochemistry, anti-Syk (clone N-19), anti-Fyn (clone FYN3), anti-Lck (clone c2012), and anti-mitogen-activated protein kinase (anti-MAPK, C-14) were purchased from Santa Cruz Biotechnology; anti-CD19...
antibodies (clones MB19-1 and 1D3) were purchased from PharMingen; anti-phospho-tyrosine monoclonal antibody (mAb; clone 4G10), and anti-Lyn (catalogue no. 06-207), used for immunohistochemistry and immunoblotting, were purchased from Upstate Biotechnology; and anti-phospho-MAPK (clone MAPK-YT) was purchased from Sigma.

Expression Constructs. To generate expression constructs for Syk and Lyn, we cloned reverse transcription-PCR fragments containing the entire open reading frames (GeneBank accesses L28824 and M16038, respectively) in pGEX-2T (Amersham) and pET-21b (Novagen), respectively. To introduce a lysine-to-leucine substitution at position 275 in the kinase domain of Lyn (kinase-dead Lyn, designated Lyn-K275L), in vitro mutagenesis was performed using GeneEditor in vitro site-directed mutagenesis system (Promega). All of the plasmid constructs were verified by sequencing. Syk was expressed in bacteria as a glutathione S-transferase fusion protein, which was isolated from lysates by chromatography on glutathione-agarose. Lyn-K275L was refolded from bacteria insoluble fraction by dialysis against decreasing pH gradient. Protein concentrations were calculated from the 280-nm UV absorbance.

Lyn−/− Mice, Prostate Whole-Mount, and Histological Examination

The Lyn−/− mice were those generated by Chan et al. (6), using a targeting vector in which exons 3–7 of Lyn were replaced with a pol2s-neo cassette. Age-matched C57BL/6 wild-type mice were used as controls. Mice were kept in a specific pathogen-free barrier facility, and all procedures were approved by the Animal Care and Use Committee of the Hebrew University. For analysis of prostate morphology, mice were 10 weeks of age, whereas for proliferation assays, mice aged 3 and 7 weeks were examined. Mice were given injections of bromodeoxyuridine (Amersham; 100 μl/10 g body weight) 1 and 24 h before sacrifice. Microdissection of prostate glands was performed essentially as described previously (12). Histological analysis of prostate glands was performed on en bloc specimens of male mouse urogenital systems marked with India ink to correctly identify the ventral, ventrolateral and dorsal prostate lobes. Prostates, fixed in 4% formaldehyde and embedded in paraffin, were sectioned at 4 μm and were either stained with H&E or immunohistochemically stained with anti-bromodeoxyuridine (NeoMarkers; clone BRD.3).

Immunohistochemistry and Tissue Microarrays

Initial sections were stained for H&E to verify histology. Sections of formalin-fixed paraffin-embedded specimens were stained with anti-Lyn antibody using a standard biotin-avidin complex immunohistochemistry (Zymed). Control staining for specificity was carried out in the presence of a Lyn-specific blocking peptide (sc-15P; Santa Cruz Biotechnology). In all of the experiments, negative control, excluding primary antibody, was included. Tissue microarrays (including specimens of normal human prostate tissue) were purchased from Clonetics Inc. (PC tissue arrays PR200). Specimens of human PC metastasis to lymph node or bone were obtained from the archives of Shoshana Ravid, The Hebrew University-Hadassah Medical Center. The use of human tissues was approved by the Institutional Review Board.

Northern Blot Hybridization

Total RNA was prepared with Tri-Reagent (Sigma). Human spleen total RNA and human prostate total RNA were purchased from Ambion. RNA samples were denatured with glyoxal and electrophoresed through a 1% agarose gel. RNAs were transferred onto a nylon-based membrane and were hybridized with a human Lyn cDNA probe, labeled with 32P by randomly labeling assays, 4000 cells/well were plated in 96-well plates. Four h after plating, serial dilutions of peptides formulated in BBlac (see “Reagents” section), were added in hexaplicate, for an additional 72 h. Samples were fixed in 4% buffered formaldehyde, and the relative cell numbers were determined as described previously (13).

Analysis of Intracellular Penetration

To facilitate intracellular permeabilization of the peptides, their NH2 terminus was acetylated with a large hydrophobic tail of myristic acid. To enable visualization, we used a COOH terminus-biotin-tagged peptide, Myristyl-G-LVTVKIK®-NH2 and a control peptide with an identical sequence except for K& in its COOH terminus (where k = D-Lys, K@ = Lys-ε-amino-biotin, and K& = Lys-ε-amino-benzoyle). DU145 cells were grown in chamber slides (30,000 cells/slide) and were incubated for 2 h with 10 μM biotin-conjugated peptide or the control peptide. Slides were fixed with 4% formaldehyde and permeabilized by 0.2% Triton X-100; avidin-FITC (Calbiochem), diluted in...
Treatment of Human HRPC Xenografts in Nude Mice

CD1 nude mice were inoculated with DU145 cells (4 × 10^6/mouse) s.c. Peptide treatment was initiated when tumors reached ~100 mm^3. The animals were treated once a week by i.v. injection of 10 mg/kg peptide, formulated in BBlac, for a period of 5 weeks. Tumor volume was calculated according to the equation: volume = 0.52 × (width)^2 × length. Vehicle and KRX-123.719-treated groups contained five mice each, whereas the KRX-123.302-treated and nontreated groups contained six mice each. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) analysis was performed on sections obtained at the end of treatment, using the DeadEnd fluorometric TUNEL system (Promega). To quantify the proportion of TUNEL-positive cells, at least three arbitrarily chosen high-power fields from four tumors of control and peptide-treated groups were photomicrographed, and the ratio of green/blue nuclei in each tumor was calculated.

RESULTS

Lyn Is Expressed in Normal and Malignant Prostatic Epithelium. As a first step in determining the role of Lyn in PC, the pattern of Lyn expression in normal prostate glands was investigated. In an earlier study, Allard et al. (14) have demonstrated the presence of Lyn in whole extracts of normal and hyperplastic dog prostate glands. To explore the relevance of their results to the prostatic epithelium, immunohistochemical studies using specific anti-Lyn antibody were performed on either normal human or mouse prostate glands. Two commercially available anti-Lyn antibodies, directed against different epitopes, were initially used, both showed specific staining of prostatic epithelium. Staining was abolished by a competing Lyn-specific blocking peptide (Fig. 1, A and B), thus proving the specificity of our staining procedure. To verify it further, we performed immunoblotting analysis on protein extracts from various human PC cell lines with the anti-Lyn antibody. Only a doublet of M_1, 53,000 and 56,000 was detected in all of them, consistent with a specific staining of the two isoforms of Lyn (Fig. 5B). Using Lyn-specific antibody, we found that Lyn is expressed in the prostatic epithelia of both humans and mice (Fig. 1, A and C). In an overall survey, all of the normal human (11 of 11) and mouse (5 of 5) prostate epithelia examined were Lyn positive. Previous studies detected Lyn transcripts, in addition to lymphoid tissues, in a variety of adult mouse tissues, including liver, kidney, brain, heart, ovary, and testes (15, 16). Our immunohistochemical staining of human tissues confirms that Lyn expression can be detected in lymphoid tissues as well as in the kidney, brain, ovary, placenta, and liver (Kupffer cells). However, we did not detect positive cells in the myocardium. In addition, breast, stomach, and colon epithelia showed clear staining with anti-Lyn antibodies (data not shown).

We then used immunohistochemistry to assess the expression of Lyn in human primary PC tissue arrays (Fig. 1, D–F), and in PC metastasis (Fig. 1, G and H). Lyn expression was evident in 137 (95%) of 145 samples of primary PC and in all of the analyzed PC metastasis to lymph node or bone samples (5 of 5 and 6 of 6, respectively). In all cases, Lyn staining in the neoplastic tissue was at least as intense as that of the adjacent normal prostate epithelium, when present (e.g., see Fig. 1D). Notably, in cancers showing two Gleason patterns in different parts of the same tumor, Lyn expression tended to be higher in the less differentiated regions. In addition, Lyn mRNA was detected using Northern blot analysis in human WISH-PC2 tumor xenografts [derived from an androgen-independent neuroendocrine small cell prostate carcinoma (17)], cultured DU145 HRPC cells, and normal whole human prostate RNA (Fig. 1F).

The Prostate Gland in Lyn-Deficient Mice Is Underdeveloped. To find out whether Lyn expression is important for normal prostate gland development, we analyzed prostate morphology in Lyn-deficient (Lyn<sup>−/−</sup>) mice. To that end, whole-mount prostate gland
specimens from six 10-week-old Lyn−/− mice, and six age-matched wild-type (Lyn+/+) controls were prepared according to Sugimura et al. (12). It clearly showed that the prostate glands of Lyn−/− mice were uniformly smaller and had a much lower degree of branching of the ductal networks, as compared with those of Lyn+/+ mice (Fig. 2, top panels). To study more carefully the morphology of the epithelial cells in Lyn−/− mice, we prepared en bloc preparations from four 10-week-old Lyn−/− mice, and four age-matched controls. Histological analysis of the prostate gland in Lyn−/− mice revealed an overall reduction in the epithelium complexity with marked attenuation of the thickness of the epithelial layer. This difference is accentuated in the dorsal lobes, which normally have a more complex architecture than their ventral counterparts (Fig. 2, middle panels). Prostate morphology of heterozygous Lyn+/− mice was indistinguishable from that of Lyn+/+ mice (data not shown). To quantify the difference, serial sections of the entire gland were prepared from Lyn+/+ and Lyn−/− mice. The calculated area of the largest cross-section in the Lyn+/+ mice was 6.74 mm² ± 0.34 mm² (mean ± SE; n = 10) as compared with 2.83 mm² ± 0.44 mm² (n = 7) in Lyn−/− mice (P < 0.001 by Student’s t test). To further analyze the role of Lyn in normal prostate development, we examined whether Lyn deficiency affected epithelial proliferation at early stages of prostate development. For this, Lyn+/+ and Lyn−/− mice, aged 3 and 7 weeks, were given injections of bromodeoxyuridine, and their prostates were dissected. Staining for bromodeoxyuridine revealed that the proliferation rate is markedly higher in Lyn+/+ prostates compared with Lyn−/− prostates, at both ages (Fig. 2, bottom panels, and data not shown), suggesting that Lyn is involved in the signaling cascade responsible for the regulation of epithelial growth. Collectively, our findings suggest that Lyn plays an important role in the development of prostate epithelium and its physiology. This led us to explore the possibility that Lyn inhibition may prove beneficial for the treatment of prostate cancer.

A Lyn-Derived Designer Peptide Specifically Inhibits Lyn-Dependent Phosphorylation. Currently, most available inhibitors of PKs target the ATP binding site of the enzyme, which is common to all PKs, a feature that entails an inherent lack of specificity by this type of compounds (18). On the other hand, it was shown that short peptides derived from the substrate interaction domain of MAPK-kinase 1 (MEK1) can inhibit the phosphorylation of its substrate MAPK2, presumably by abrogating the association of the kinase with its substrate (19). With respect to Lyn, a detailed structural analysis, based on nuclear magnetic resonance data and high resolution modeling, pointed to the loop between its kinase domain in kinase-substrate interaction and the region that determines substrate specificity (21–24). This loop is fully exposed and available for external interactions (see the three-dimensional illustration in Fig. 4A). Comparative analysis among different PKs revealed that PK subfamilies are typified by an exclusive combination of amino acids (aa) within the HJ-loop that are unique to each subfamily. We, therefore, hypothesized that peptide copies of that...
region might serve as specific inhibitors, acting via disruption of the interaction between the kinase and its substrates.

To that end, we synthesized a series of short peptides derived from the HJ-loop of Lyn and used an in vitro kinase assay to test the efficacy and specificity of these peptides against Lyn and other members of the Src family. The activity of one of these peptides, designated KRX-123.302, is shown in Fig. 3. As a first step, we determined whether KRX-123.302 affects Lyn transphosphorylation in vitro. Incubation of active Lyn with kinase-dead Lyn (Lyn-K275L) and ATP resulted in robust phosphorylation of Lyn. Addition of KRX-123.302 to the reaction mixture resulted in a significant inhibition of Lyn transphosphorylation in a dose-dependent manner, showing 50% inhibition at around 1 μM (Fig. 3A). We further assessed the activity of KRX-123.302 toward two related PTKs, Lck and Fyn. Whereas Lyn (and KRX-123.302 accordingly) contains Tyr at position P4 of the HJ-loop (see Fig. 4B for details), in Lck and Fyn, the corresponding residues are His and Lys, respectively. In addition, Lyn has Lys, whereas Lck and Fyn have Arg at position P6. As shown in Fig. 3A, even 20 μM KRX-123.302 did not inhibit the transphosphorylation of either Lck or Fyn, thus suggesting that KRX-123.302 is a selective Lyn inhibitor. The next set of experiments was designed to test whether KRX-123.302 can inhibit the phosphorylation of Syk, a physiological substrate of Lyn (6). We expressed Syk in bacteria as a glutathione S-transferase fusion protein and incubated purified glutathione S-transferase-Syk with active Lyn and ATP, in the presence of KRX-123.302. KRX-123.302 markedly inhibited the phosphorylation of Syk in a dose-dependent manner showing 50% inhibition at 0.3 μM. The addition of a control nonrelated myristylated peptide designated KRX-107.110 (see “Materials and Methods”), did not inhibit Lyn-dependent phosphorylation at up to 5 μM (Fig. 3B). The phosphorylation of an additional Lyn target, Vav (25), was also inhibited by KRX-123.302 in a dose-dependent manner (data not shown). Thus, we have found that a specific sequence of 8 aa derived from a unique interaction site of Lyn, specifically inhibits target phosphorylation by Lyn, but not by closely related Src family members Lck and Fyn in vitro. We hypothesize that the synthetic peptide behaves like a pseudokinase and inhibits Lyn via binding to Lyn substrates.

The inhibition of Lyn activity by KRX-123.302 in the cell-free assays prompted us to examine its effect on Lyn-dependent phosphorylation and signaling in intact B lymphocytes. We chose B-cell antigen receptor (BCR) activation as a model system because the signaling pathways mediated by Lyn have been thoroughly studied in intact B lymphocytes. We chose B-cell assays prompted us to examine its effect on Lyn-dependent phosphorylation and signaling in intact B lymphocytes. We hypothesized that the synthetic peptide behaves like a pseudokinase and inhibits Lyn via binding to Lyn substrates.

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HJ-loop (the loop between the nonbiotinylated control peptide (left panel) or with the biotin-tagged peptide (alone. Highlighted in blue, the seven-amino-acid (aa) residues corresponding to the HJ-loop of Lyn. Lyn, yet expresses high levels of Src, was refractory to the treatment (Fig. 5). On the other hand, a human colon cancer cell line (HT29) that barely expresses Lyn, is strongly inhibited by the Lyn-derived peptide (Fig. 5).

The Lyn-Derived Designer Peptides Inhibit HRPC Cell Proliferation in Vitro. To study the possibility that Lyn inhibition compromises prostate cancer cells, we tested the activity of the different synthetic peptides derived from the HJ-loop of Lyn, vis à vis the proliferation of the HRPC cell lines, DU145 and PC3, as detailed below (Fig. 4B). Our initial study showed that a synthetic peptide, composed of a seven-aa sequence of the Lyn parental HJ-loop, IV-TYGKI (designated KRX-123.101; see Fig. 4B), significantly inhibits HRPC cell proliferation. We further modified this peptide structure to optimize its biological activity, and we refer to this group of peptides collectively as KRX-123 (Fig. 4B). The covalent attachment of myristic acid, through additional Gly at the NH$_2$ terminus, enables intracellular-peptidylization of the peptide, as demonstrated by fluorescent labeling. The internalized peptide is evenly distributed in the cytoplasm and is excluded from the nucleus (Fig. 4C).

As can be seen in Fig. 4B, the peptide activity is sequence dependent and a nonrelated control peptide, KRX-683.112 (a myristylated derivative of the HJ-loop of G-protein-coupled receptor kinase-2), had no effect on the proliferation of either DU145 or PC3. Likewise, some single aa modifications abolish the inhibitory activity (e.g., KRX-123.156 and KRX-123.124). Other modifications of the original KRX-123.101 were associated with increased inhibitory efficacy; in particular, the di-iodo substitution of Tyr (KRX-123.135), and the replacement of the native Gly by D-Lys (KRX-123.134) resulted in enhancement of the inhibitory activity. In addition, the peptide activity can be enhanced by aromatic adducts at the COOH terminus (Fig. 4B, KRX-123.302 and KRX-123.719). It is noteworthy that the inverted peptide sequence gave a response similar to the parental one (KRX-123.202 versus KRX-123.101). This phenomenon, of a significant activity of the retro sequence of a short peptide, has been described in other systems (26), indicating the greater importance of the side-chain residues location relative to each other, rather than to the backbone.

To determine whether the Lyn inhibitor was effective in other cell types, we tested human cancer cell lines from other epithelial tissues for the inhibition of their proliferation by KRX-123.302. We also tested Lyn and Src expression levels in these cell lines using Western blot analysis. All three of the PC lines tested (DU145, PC3, and TSU-Pr1), express considerable levels of Lyn and Src and were strongly inhibited by the Lyn-derived peptide (Fig. 5). On the other hand, a human colon cancer cell line (HT29) that barely expresses Lyn, yet expresses high levels of Src, was refractory to the treatment (Fig. 5).

In Vivo Administration of Lyn-Derived Peptides Induces Regression of HRPC Xenografts. After structural optimization of the Lyn-derived peptide in HRPC cell lines in vitro, we chose the two most active derivatives, KRX-123.302 and KRX-123.719, for testing their efficacy in vivo. To that end, nude mice were inoculated s.c. with DU145 cells. When the tumors reached a volume of $\sim 100\ mm^3$, we initiated i.v. treatment with an inhibitory peptide or vehicle only (a single injection once a week, for a period of 5 weeks). An i.v. injection of KRX-123.302 or KRX-123.719, at a dose of 10 mg/kg resulted in significant tumor regression (Fig. 6A). On the other hand, in all of the control animals (vehicle-treated plus nontreated), the tumors continued to grow, with more than doubling in tumor volume within 1 month (Fig. 6B). Statistical analysis of tumor volume among the various groups showed a significant difference between KRX-123.719-treated tumors and vehicle ($P < 0.01$, Mann Whitney test) or no treatment ($P < 0.01$). Tumor volume of KRX-123.302-treated mice was also significantly smaller than that of nontreated animals ($P < 0.01$); however, the difference from the vehicle-treated group did not reach statistical significance ($P = 0.16$). In an additional set of experiments with various modifications of the peptides, 56 animals were treated with inhibitory peptides, whereas 26 animals received vehicle only or no treatment. Cumulatively, complete regression of the tumors was noted in 25% (17 of 67) of the treated mice, after once-a-week injections of the inhibitory peptide for a period of 5–8 weeks, whereas no regression was observed in the controls (0/37; $P < 0.005$, $\chi^2$ test with one degree of freedom).

To study the morphological changes induced in the tumors as a
result of the treatment, we removed tumors several weeks after treatment initiation, before complete regression was achieved, and submitted them for histological evaluation. H&E staining of the PC xenografts depicted more apoptotic bodies in the KRX-123-treated tumors. Accordingly, TUNEL assay demonstrated massive apoptosis in xenografts of the KRX-123-treated mice (Fig. 2). Second, Lyn is extensively expressed in human PC specimens (Fig. 1). Third, the inhibition of Lyn activity using a specific sequence-based inhibitor decreases proliferation of HRPC cell lines (Figs. 4, 5), and significantly reduces tumor growth in a tumor explant model (Fig. 6A). Fourth, Lyn inhibition induced apoptosis in HRPC cells in vivo, as demonstrated by TUNEL analysis (Fig. 6B). In addition, RNA interference-mediated specific inhibition of Lyn expression significantly suppressed the proliferation of DU145 cells. Our finding that the known B-cell-specific PTK, Lyn, plays an important role in prostate epithelium physiology is an example for different assignments of allegedly cell-specific PTKs, in alternate tissues or cell types. Yet another such example is the PTK Syk, which, until recently, was considered an exclusive B-cell signaling molecule and was now shown to play a regulatory role in the breast epithelium (30).

It is well known that Lyn activation plays a critical role in BCR signaling, in which it leads to attenuation of the immune response (5, 6). However, several lines of evidence suggest that Lyn regulates cell survival through the stimulation of proliferation and the inhibition of apoptosis in various hematopoietic cell lineages (31–33). In addition, overexpression of dominant-active Lyn in colon cancer cells conferred chemoresistance through activation of the phosphoinositide 3-kinase/protein kinase B pathway (7). Similarly, the expression of wild-type, but not kinase-inactive, Lyn was found to protect human embryonic kidney 293 and HeLa cells from drug or ionizing radiation-induced apoptosis (8). Importantly, Grishin et al. (8) have also shown that interaction between Lyn and growth arrest–DNA damage protein 34 negatively regulates genotoxic apoptosis.

Several publications reported expression of Lyn in PC cell lines, and in dog’s normal and hyperplastic prostate (10, 11, 14). Lyn was shown to be ubiquitously expressed in a panel of human PC cell lines; however, its role in this system remained unclear (11). Another study...
has shown that not only is Lyn expressed in a PC cell line, but that it is down-regulated within 30 min after transforming growth factor \( \beta \) treatment (10). This finding, in concert with data illustrating that transforming growth factor \( \beta \) is a negative regulator of PC cell growth (34), suggests that transforming growth factor \( \beta \) may exert its negative regulatory role in PC cells, at least partially, via the inhibition of Lyn expression. The above findings, in conjunction with our own data, collectively support the view that Lyn may play a role in the evolution of PC and, therefore, can serve as a target for the development of anti-PC therapy. It could be argued that Lyn inhibition in PC patients will prove to be a double-edged sword because Lyn-deficient mice develop autoimmune disease and show defective mast cell function. However, our preliminary results based on treating dozens of immune-competent animals with high doses of KRX-123.302 detected only slight reductions in platelet numbers that did not fall below the physiological level, and minimal focal liver inflammation that did not result in altered liver function tests (data not shown). In addition, no signs of autoimmune kidney disease were noted after 5 weeks of KRX-123.302 treatment. Obviously, additional studies are needed to identify more subtle effects of reversible Lyn inhibition in immune-competent animals before human studies are contemplated.

Protein-protein interactions govern many important cellular functions and constitute a major challenge for drug discovery attributable to their complexity. We have devised a rational strategy for inhibiting the binding of PKs to their respective substrates. This process involves first identifying a key kinase-substrate interaction site, and then developing sequence-based peptide inhibitors to interfere with this interaction. Strong emphasis is placed on the structure–activity relationship with the peptide being modified in a manner that enhances efficacy. This strategy of sequence-based modulation is widely applicable for multiple PKs because the protein–protein interaction site targeted is easily identifiable in most PKs, yet is distinct for each kinase subfamily, so that selective inhibitors can be specifically devised.

Our structure-based approach, contrasts with the more common high-throughput screening of random combinatorial-chemistry libraries. In this study, we targeted the putative substrate-binding site of Lyn. This region within the kinase domain is composed of a short linear sequence, the HJ-loop, residing between a helix F and a helix G (Fig. 4A). Several studies point to the direct involvement of this region in substrate binding by PKs (21–24). For example, it is sufficient to exchange a short sequence that includes the HJ-loop, between Jun NH2-terminal kinase 1 and 2, to confer Jun NH2-terminal kinase 2 substrate specificity to an otherwise Jun NH2-terminal kinase 1 construct (22). Our comparative analysis across dozens of available PK sequences revealed that there is a consistent pattern of distinct composition of aa at the HJ-loop, unique to each PK subfamily and individual kinase within. Thus, each PK subset displays a unique combination of contact residues, embedded within a conserved framework. We thus postulated that peptides derived from this region can serve as kinase-specific inhibitors and would interfere with PK signaling in a predictive manner by serving as a kinase decoy. In agreement with this notion, we showed here that short peptides derived from this region of the Lyn kinase are able to (a) inhibit Lyn transphosphorylation and Lyn kinase activity in cell-free assays (Fig. 3, A and B); (b) inhibit Lyn transphosphorylation and interfere with Lyn-dependent signaling in intact B cells (Fig. 3, C–E); (c) inhibit the proliferation of Lyn-expressing HRPC cells (Figs. 4 and 5); (d) induce apoptosis in such cells (Fig. 6B); and (e) inhibit growth and even induce regression of tumors after injections to mice bearing
HRPC xenografts (Fig. 6A). Although our results suggest that the Lyn-derived peptides inhibit PC explants in vivo through Lyn inhibition, at present we are unable to study the effect of the inhibitor on downstream Lyn targets in PC, because these targets are not yet identified.

Several key issues still require further investigation. The identity and biochemical function of the immediate molecular interactors upstream of Lyn in the normal prostate and PC cells are yet unknown. It will be interesting to characterize them and analyze their phosphorylation status under different physiological and pathological conditions. Another intriguing question is related to the specificity of the inhibitor on several key issues still require further investigation. The identity and biochemical function of the immediate molecular interactors upstream of Lyn in the normal prostate and PC cells are yet unknown. It will be interesting to characterize them and analyze their phosphorylation status under different physiological and pathological conditions. Another intriguing question is related to the specificity of the inhibitor on downstream Lyn in the normal prostate and PC cells are yet unknown. It will be interesting to characterize them and analyze their phosphorylation status under different physiological and pathological conditions. Another intriguing question is related to the specificity of the inhibitor on Lyn kinase defining a family of enzymes. Philos. Trans. R. Soc. Lond. B. Biol. Sci., 315–312, 1993.


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