Na/K-ATPase signaling: from bench and going to bedside

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FINAL APPROVAL OF DISSERTATION
Doctor of Philosophy in Biomedical Sciences

Na/K-ATPase Signaling: from Bench to Bedside

Submitted by:
Zhichuan Li

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biomedical Sciences

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Na/K-ATPase Signaling: from Bench, and going to Bedside

Zhichuan Li
University of Toledo
College of Medicine
2008
I dedicate this work to my wife Xiaochen Zhao for all her support and love.
ACKNOWLEDGEMENTS

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INTRODUCTION

Na/K-ATPase is ubiquitously expressed in most eukaryotic cells and is essential for maintaining the ion gradient by transporting Na\(^+\) and K\(^+\) across the cell membrane. Since its discovery about half a century ago (Skou 1957), extensive advancements have been achieved in the aspects of its structure, molecular mechanism of its enzymatic ion pumping functions as well as physiological significance (Kaplan 2002; Morth et al. 2007; Schoner et al. 2007). Within the last decade, researchers from this institution and other independent labs have demonstrated that in addition to the classical ion transporting, this membrane protein can also relay extracellular ouabain binding signaling into the cell through regulation of protein tyrosine phosphorylation (Kometiani et al. 1998; Contreras et al. 1999; Haas et al. 2000; Aydemir-Koksoy et al. 2001; Kometiani et al. 2005). The downstream signals through ouabain-triggered protein phosphorylation events include the activation of mitogen-activated protein kinase (MAPK) signal cascades, mitochondrial reactive oxygen species (ROS) production as well as activation of phospholipase C (PLC) and inositol triphosphate (IP3) receptor (IP3R) in different intracellular compartments (Haas et al. 2000; Haas et al. 2002; Yuan et al. 2005; Tian et al. 2006; Zhang et al. 2006). Most of these pathways are independent of changes in intracellular ion concentrations (Liu et al. 2000) and more interestingly, diminished after Src inhibition or knockout of Src family kinases (Haas et al. 2000; Aydemir-Koksoy et al. 2001; Yuan et al. 2005), which suggests that Src activation is the earliest event in the ouabain-regulated signaling pathways. These observations have led to the hypothesis that Na/K-ATPase may directly form a complex with Src and then convert
Na/K-ATPase to a signaling receptor for ouabain through regulation of non-receptor tyrosine kinase Src.

Towards this direction, important insights into the signaling mechanism of ouabain-triggered Src activation have been recently reported (Tian et al. 2006). First, Na/K-ATPase and Src colocalized in the plasma membrane in several cell lines. Fluorescence resonance energy transfer (FRET) analysis indicated that both proteins were in close proximity, suggesting a direct interaction. Second, in vitro GST pull-down assay demonstrated direct interaction between the $\alpha_1$ subunit of Na/K-ATPase and Src. While the Src homology 2 domain (SH2) bound the second cytosolic domain (CD2) of the $\alpha_1$ constitutively, the interaction between the Src kinase domain and the third cytosolic domain (CD3) of the $\alpha_1$ was regulated by ouabain. Third, binding of Src to either the Na/K-ATPase or GST-CD3 inhibited Src activity. Ouabain released the kinase domain from the Na/K-ATPase and then activated the Na/K-ATPase-associated Src. Accordingly, FRET efficiency between the Na/K-ATPase and Src was decreased after exposure of LLC-PK1 cells to ouabain. Concomitantly, it stimulated cellular Src activity and subsequently the tyrosine phosphorylation of multiple proteins that were either associated with or in close proximity of the Na/K-ATPase/Src complex.

However, the mechanism that is responsible for the inhibition of Src by the CD3 domain is still not clear. So herein, the first aim of this dissertation is to identify the binding and regulatory motif in the CD3. With the in vitro GST pull-down assay as well as the reference to the three dimensional structure of the Na/K-ATPase, we were able to map the binding motif to the N-terminal 56 amino acids in the nucleotide-binding domain of Na/K-ATPase. Furthermore,
NaKtide, a peptide containing 20 residues, was identified to have inhibitory effect on Src activity. Accordingly, cell permeable NaKtide was able to block ouabain-induced signaling through inhibition of Src in the neonatal cardiac myocytes and then could function as a potential ouabain antagonist.

In addition to the study about the mechanism by which Na/K-ATPase inhibits Src, an important notion is that Na/K-ATPase expression level is down-regulated in many cancer cells as well as human tumors (Akopyanz et al. 1991; Espineda et al. 2003; Espineda et al. 2004; Sakai et al. 2004). We have previously shown that Na/K-ATPase amount is inversely correlated to intrinsic c-Src activity (Liang et al. 2006). Because Src activity is up-regulated in many cancer cells, we tested whether the nearly developed NaKtide is effective in inhibiting tumor growth in vivo.
LITERATURE

Na/K-ATPase

As a membrane protein, Na/K-ATPase belongs to a family of P-type ATPase found in most eukaryotic cells. It is essential for maintaining the ion gradient by transporting Na\(^+\) and K\(^+\) across the cell membrane (Kaplan 2002). The ion gradient across the membrane provides driving force for the secondary transport of nutrients (i.e., amino acids, glucose), ions (i.e., Ca\(^{2+}\), H\(^+\), Cl\(^-\), SO\(_4\)^{2-}\) etc.), as well as neurotransmitters.

Structure of Na/K-ATPase

The Na/K-ATPase is composed of two major subunits termed as \(\alpha\) and \(\beta\) subunit. The \(\alpha\) subunit is composed of about 1000 amino acids and is generally believed to have 10 transmembrane domains with both N- and C-termini located in the cytoplasm, which is homologous to two other P-ATPases, gastric H/K-ATPase and sarcoplasmic reticulum Ca-ATPase (SERCA). There are four well-characterized domains in the P-ATPases: A (Actuator), P (Phosphorylation), N (Nucleotide-Binding) and transmembrane domains. The A domain consists of the N-terminus and CD2, while the P and N domains are located in the CD3, with the conserved motif D-K-T-G-T-[L/I/V/M]-[T/I/S], a hallmark of the P-ATPase family.

As a type II transmembrane protein, the \(\beta\) subunit is composed of approximately 300 amino acids and has a single transmembrane domain with its N-terminus in the cytoplasm. The \(\alpha\) subunit contains the binding sites for ATP, Na\(^+\), K\(^+\) and cardiotonic steroids such as ouabain. It is responsible for
transporting 2 K\(^+\) into and 3 Na\(^+\) out of the cells by utilizing the energy from hydrolysis of ATP, so it is also named as the catalytic subunit. However, without co-expression of the β subunit in Xenopus oocytes or insect cells, this catalytic subunit cannot perform normal enzymatic activity (Noguchi et al. 1987; DeTomaso et al. 1993). Further study showed that the β subunit may be also involved in the trafficking and delivery of the α subunit to the cell membrane (Geering 2001).

In mammals, at least four distinct isoforms of the α subunit and three isoforms of the β subunit have been cloned and sequenced. The α1 is the major isoform in many tissues and the dominant isoform in the kidney and most epithelia. The α2 is expressed in skeletal muscle, brain, heart tissue. The α3 isoform is mainly found in nerves and brain but also found in both neonatal and aging rat heart. The α4 isoform is only localized in spermatozoa. Although there are only minor sequence differences (α1, α2 and α3 have about 87% identity, while α1 and α4 have 78% identity), they present different affinities to the enzyme substrates and ouabain (Jewell et al. 1991; Horisberger et al. 2002). This may indicate different physiological roles among these isoforms.

The β1 is expressed ubiquitously in mammalian cells. The β2 is mainly expressed in neuronal tissues but also detectable in adult heart (Wang et al. 1996). The β3 is found in skeletal, lung, muscle, and brain tissues. The β\(_m\) is a recently identified member of β subunit family with predominant expression in skeletal muscle (Pestov et al. 1999; Pestov et al. 2001). There are three glycosylated asparagines and 3 S-S bridges in the extracellular domain. The
S-S bridges, but not glycosylation, are essential for the catalytic activity of the α subunit.

The γ subunit has been identified in kidney tissue with molecular weights ranging from 6.5 kDa to 7.5 kDa. It belongs to a group of short single-transmembrane proteins termed the FXYD proteins. A FXYD motif in the extracellular N-terminus and two conserved glycines and a serine residue in the transmembrane domain are characteristic for this family. Recent studies showed that at least 4 members of FXYD proteins interact with the Na/K-ATPase and function as the modulator of the Na/K-ATPase in a tissue-specific manner (Therien et al. 2001; Garty et al. 2006).

Crystal structure information from the rabbit SERCA1a in the E1 and E2 conformation and the Na/K-ATPase α1 subunit in E2 conformation has greatly advanced our knowledge about the structure-function relationship of the Na/K-ATPase (Sweadner et al. 2001; Morth et al. 2007). The E1 and E2 crystal structure of SERCA1a clearly showed a significant movement of A and N domain during the ion pumping cycle. This is consistent with early observations that many modifications in the A domain and CD3 through mutations or protein-protein interactions may affect the activity of the catalytic subunit (Devarajan et al. 1994; Daly et al. 1997; Blostein et al. 1998). Also, the N domain structure obtained from homology modeling is similar to the NMR and crystallographic structure (Hakansson 2003; Hilge et al. 2003).

**Oligomerization of Na/K-ATPase**

It is generally accepted that the α and β subunits form αβ heterodimers in equal molar amounts, while the γ subunit is associated with αβ
heterodimers in a tissue-specific manner. Like many other membrane transporter proteins, the Na/K-ATPase may form oligomers in the plasma membrane. Many studies through chemical cross-linking, FRET and electron microscopy have shown that Na/K-ATPase may exist as \((αβ)_{2}\) and some \((αβ)_{4}\) (Askari et al. 1980; Hayashi et al. 1989; Yokoyama et al. 1999; Martin et al. 2000).

Later studies with the overexpression of the \(α\) subunit in insect cells demonstrated the specific association between the Na/K-ATPase \(α\) subunit itself (Blanco et al. 1994). Further study showed that either \(α1T\) (ΔGly554 to C-terminus) or delete mutant in the CD3 (ΔArg350-Pro785) failed to associate with the full-length \(α\) subunit, which suggesting part of the CD3 (Gly554 to Pro 785) is necessary for specific \(α/α\) association (Koster et al. 1995; Koster et al. 1997; Laughery et al. 2004). Interestingly, both GST and His tagged CD3 can associate with purified dog kidney Na/K-ATPase in the presence of Mg\(^{2+}\) and ATP(Costa et al. 2003). It is still not known whether this interaction is involved in \(αβ\) oligomerization. If it does, then specific region from either \(α/α\) association or disassociation may be formed for some Na/K-ATPase binding proteins. As an example, does the \(α/α\) or \(αβ\) oligomerization affect Src binding and its kinase activity?

**Src family tyrosine kinases**

Over the past few decades, a few protein families have achieved the preeminence of the protein kinases. The protein kinases play important roles in many signaling pathways by which growth factors, hormones, neurotransmitters and toxins exert their effects and regulate cellular functions.
Most importantly, malfunction of some kinases directly contributes to the development of many human diseases. Thus inhibitors of some kinases have been served as drugs for treating human diseases during the past few years.

Src family kinases (SFKs) are nonreceptor tyrosine kinases. The prototype member of the Src family protein tyrosine kinases was first identified as v-Src, the molecule responsible for the cell-transforming ability of Rous sarcoma virus (Brugge et al. 1977). The identification of c-Src proto-oncogene in the vertebrate leads to extensive studies to understand the structure and function of the Src tyrosine kinase (Stehelin et al. 1976; Shalloway et al. 1981; Takeya et al. 1983). So far, at least nine Src family members have been identified and divided into two groups: tyrosine kinases with a broad expression range (Src, Fyn, Yes) and those with limited expression mainly in hematopoietic cells (Fgr, Lyn, Hck, Lck, Blk, Yrc and Yrk) (Martins-Green et al. 2000; Martin 2001).

**Localization of SFKs**

As a group of non-receptor kinases, SFKs are important regulators of various signal transduction pathways. It is well documented that Src, for example, plays an important role in the signal transduction pathways of many extracellular stimuli, i.e. cytokines, growth factors and stress responses (Thomas et al. 1997). SFKs have been found to be cytosolic proteins, while many signaling components including receptors (Integrin, EGFR, PDGFR, FGFR, etc.) and Src substrates (paxillin, histone deacetylase 3) (Longworth et al. 2006) are membrane-associated proteins. To achieve efficient signal transduction and amplification, Src may associate with the cell membrane
through “protein translocation” machinery which was also adopted by PKC and PI3K. Indeed, Src can be translocated from the cytosol and perinuclear region to the plasma membrane and membranes of intracellular organelles, including the endoplasmic reticulum, endosomes and secretory vesicles. Cell-specific localization patterns of SFKs have also been observed.

Further studies have revealed that N-terminal residues are critical to program SFKs for the association with membranes. Src, for instance, has a Glycine residue in its N-terminus, which can be recognized by N-myristoyltransferase and then Src undergoes a posttranslational modification called myristoylation. Results from studies by using the myristoylation minus mutation of Src or knocking down of N-myristoyltransferase have demonstrated myristoylation is necessary for the membrane localization of Src (Song et al. 1997; Ducker et al. 2005). However, myristoylation of Src does not guarantee its membrane association because a large number of myristoylated Src molecules is found to be free in the cytosol (Buss et al. 1984). Also, Src is synthesized and myristoylated on polyribosomes (Levinson et al. 1981), so myristoylated Src could be in the process of transmitting to the plasma membrane, or kicking on and off the plasma membrane. The myristoylation process is also found to play similar role in the membranes association of Lck, Fgr, Hck (Robbins et al. 1995; Zlatkine et al. 1997; Baker et al. 1998; Yasuda et al. 2000).

Palmitoylation is another type of posttranslational lipid modification found in some SFKs, including Hck, Fyn, Yes, Fgr, Lck (Koegl et al. 1994; Shenoy-Scaria et al. 1994; Robbins et al. 1995; Wolven et al. 1997; Carreno et al. 2000). Different from myristoylation, palmitoylation can be reversible and
transient. In the studies of the locations of two forms of Hck, it is found that palmitoylation of p59^{hck} requires prior myristoylation. Moreover, the following palmitoylation will enhance its association with cell membrane as well as the association with the caveolae, which is also applied to Fyn. Since palmitoylation is readily reversible through enzymatic machinery (Linder et al. 2007), depalmitoylation of SFKs may lead to their rapid dissociation from caveolae, which could provide another way to regulate the signaling pathway as found in Ras (Choy et al. 1999).

In addition to lipid modifications in SFKs, which are important for the membrane association of SFKs, there are some early observations which suggested that electrostatic interactions might provide additional binding energy. For example, incorporating 33% acidic lipids into phosphatidylcholine vesicles increases the binding of c-Src to phospholipids vesicles 1000-fold, while mutation of basic residues in the N-terminus of c-Src decreases the membrane binding and consequently reduces v-Src’s transforming ability (Sigal et al. 1994). It has been implicated that the hydrophobic interactions resulted from the myristoylation and/or palmitoylation and electrostatic interactions can act together to anchor not only Src, but also myristoylated alanine-rich C kinase substrate (MARCKS) and endothelial nitric oxide synthase (eNOS) to the phospholipid membrane (McLaughlin et al. 1995).

Another recently described player in affecting Src distribution is its binding partner (Collin et al. 2007). For instance, when Src associates with TOM1L1 - clathrin heavy chain complex, Src is dissociated from the cholesterol-enriched microdomain caveolae.
**Structure of SFKs**

Generally, each member of SFKs contains several distinct functional regions: (1) the N-terminal Src homology 4 (or SH4) domain followed by a unique region. The SH4 domain is believed to be involved in its membrane localization through myristoylation and/or palmitoylation. The unique region is important for each family member to specifically interact with signaling proteins; (2) the SH3, which contains about 50 amino acids and binds with the polyproline motifs; (3) the SH3-SH2 linker region, where more flexibility introduced by triple Glycine mutation results in activation of Src and Hck (Young et al. 2001); (4) the SH2 domain, which interacts with the phosphorylated tyrosine residues; (5) SH2-Kinase linker regions, which can form intra-molecular interaction with SH3 and regulate its activity; (6) the conserved kinase domain (SH1), which contains the autophosphorylation site (Tyr418, human c-Src numbering) and is required for full kinase activation; (7) the short C-terminal regulatory tail. The tyrosine residue (Tyr529) in this tail can bind to SH2 after phosphorylated usually by cytoplasmic c-Src kinase (Csk).

The phosphotyrosine binding pocket in the SH2 domain contains four positive charged amino acids: Arg157, Arg177, His203, and Lys205. The most critical amino acid is Arg177, which actually can form H-bonds with the phosphate oxygens of the phosphor-Tyrosine side chain. Mutation of Arg177 in FLVRES sequence abolishes the SH2 binding ability to pTyr containing ligands (Bibbins et al. 1993).
The kinase domain of SFKs is essential for the catalytic function. The overall structure of SFKs kinase domain closely resembles that of protein kinase A (Hanks et al. 1988; Knighton et al. 1991), which consists of an N-terminal lobe and a larger C-terminal lobe linked by the activation loop (A-loop) (405-433). The antiparallel \( \beta \) sheets in the N-lobe and the cleft region formed between the lobes provide the ATP binding pocket. In the case of Src, ATP binding pocket includes Arg387. Studies have shown that R387A/K v-Src mutant can not bind ATP which results in inactivation of v-Src (Chan et al. 1996). According to the modeled structure, Arg387 may bind with \( \gamma \)-phosphate of ATP.

The A-loop generally begins with the conserved Asp-Phe-Gly (DFG) motif and ends with Ala-Pro-Glu (APG) motif. Specifically, the Asp residue in the DFG motif plays an important role in the coordinating the Mg\(^{2+}\) ion which interacts with the phosphate group of ATP (Adams et al. 1993), while the Phe residue interacts with the \( \alpha \)C helix located after the \( \beta \)3 through hydrophobic interaction, helping to position the \( \alpha \)C helix (Williams et al. 1997). Additionally, the conserved region in the kinase domain is the glycine-rich phosphate-binding loop (P-loop) (c-Src residues 275-284) located between the \( \beta \)1 and \( \beta \)2.

Comparison of the active and inactive forms of the kinase domains (Yamaguchi et al. 1996; Xu et al. 1997) shows Glu (c-Src residue 312) in the \( \alpha \)C helix and Lys (c-Src residue 297) are in close proximity and form salt bridge, then allows the interaction between Lys side chain and the phosphate of ATP, while improper positioning of \( \alpha \)C Helix in the inactive form either by the interaction between SH3 and SH2-Kinase domain linker or by other inhibitors prevents formation of salt bridge.
Functions of SFKs

As fundamental enzymes for cell proliferation, replication, growth, adhesion and motility, SFKs have been involved in the signal transduction pathways provoked by many extracellular stimuli, including growth factors, hormones, etc. (Thomas et al. 1997). Even though extensive studies from cell cultures have demonstrated that SFKs play a very important role in the signaling cascades and that blocking of SFKs affects many cellular functions, the unexpected physiological significance of Src family proteins has been disclosed by using SFKs knockout mice. For instance, deficiency of Src alone or Src/Hck in the mouse cause a bone remodeling defect called osteopetrosis (Soriano et al. 1991; Lowell et al. 1996); Lyn-/- mice have smaller and less developed prostate glands (Goldenberg-Furmanov et al. 2004).

Some new roles of Src family kinases have been disclosed recently. For example, accumulating evidence has shown that Src family kinases are involved in fat metabolism. Fyn knock-out mice display a reduced percentage of adipose mass associated with decreased adipocyte cell size and markedly improved glucose tolerance due to increased peripheral tissue insulin sensitivity (Bastie et al. 2007); Src-family tyrosine kinase inhibitors decreased the degree of adipogenesis of 3T3-L1 preadipocytes. Deficiency of Src-family tyrosine kinases blocked the adipogenesis of fibroblast cells (Sun et al. 2005).

Src has been implicated to be a key intermediary in the case of stroke, where Src deficiency or blockade of Src activity in mice provides cerebral
protection following stroke (Paul et al. 2001). Therefore Src may serve as a novel therapeutic target in the pathophysiology of cerebral ischemia.

Src is also involved in the noise-induced hearing loss (NIHL) and inhibitors of Src, including KX1-004, KX1-005 and KX1-174, may be used as therapeutic intervention for NIHL (Harris et al. 2005). Src kinase inhibitors, Dasatinib (BMS-354825, Sprycel) and AZD0530, prevent Dengue Virus infection (Chu et al. 2007).

Regulation of SFKs

Tyrosine phosphorylation is well-known to play very important roles in the regulation of SFKs. There are at least two tyrosine phosphorylation sites regulating Src activity: Tyr418 whose auto-phosphorylation contributes to Src activation and Tyr529, whose phosphorylation by Csk facilitates intramolecular interaction between the C-terminal tail (Tyr529) and the SH2 and then reduces the potential to be fully activated (Waksman et al. 1992; Yamaguchi et al. 1996; Xu et al. 1997; Young et al. 2001). The intramolecular interaction between the SH3 and kinase domain also stabilizes Src to form a closed conformation which reduces the accessibility of ATP to the kinase domain. Varieties of Src-interacting proteins which can compete for the binding to these domains and disrupt the intramolecular interactions have been shown to regulate Src kinase activity inside of cells. For example, the platelet-derived growth factor receptor (PDGFR) (Alonso et al. 1995) and FAK (Cobb et al. 1994) can bind to the SH2 and activate Src. Nef (Moarefi et al. 1997; Trible et al. 2006) and Sin (Alexandropoulos et al. 1996) can activate Hck and Src respectively through direct interaction with the SH3 domain. Crk-
associated substrate, p130Cas, can interact with both the SH3 and SH2 domain and then activate Src (Burnham et al. 2000), while G\textsubscript{\alpha}s and G\textsubscript{\alpha}i can activate Src through the interaction with the kinase domain (Ma et al. 2000). RACK1 also has been reported to inactivate Src through its binding to the SH2 domain (Chang et al. 1998; Chang et al. 2001). Recently, the Wiscott-Aldrich syndrome protein (WASP) was found to interact with the kinase domain of Src and Abl, and inhibit their kinase activities (Schulte et al. 2003) through an unknown mechanism.

**Src kinase inhibitors**

Of particular interests, rational design of small molecule inhibitors of protein kinases has been increasingly performed due to the connections between kinases activity and human diseases (see Table 1). Since ATP serves as the essential element for the kinase activation, most of the kinase inhibitors directly bind the ATP binding sites in a competitive manner, i.e. PP1, PP2, Dasatinib and Imatinib (STI-571, Gleevec) (Hanke et al. 1996; Shah et al. 2004; Seeliger et al. 2007). However, due to the overall structural similarity of ATP binding sites in more than 500 kinases, small molecules targeting the ATP binding sites may be not specific for just one kinase. For example, although widely used to investigate the role of SFKs in multiple signaling pathways and cellular activities, PP1 and PP2 have been shown to affect the kinase activity of p38 MAPK, Btk, c-Kit, Csk and CK1 (Bain et al. 2003; Tatton et al. 2003; Bain et al. 2007). Imatinib has been shown to be clinically successful in targeting the nonreceptor tyrosine kinase, c-Abl. Recent studies have demonstrated that several other kinases, including c-Kit, PDGF receptor,
and c-Src, are also inhibited by Imatinib (Seeliger et al. 2007), although this may expand its indications and have some beneficial effects for patients. Similar observations from many other kinase inhibitors (Davies et al. 2000) suggest that relatively poor specificity is a rather common occurrence for the ATP analog inhibitors. Another disadvantage for the ATP-competing inhibitors is the acquired resistance which, in some cases, is conferred by a secondary mutation in the catalytic cleft regions (Iqbal et al. 2004; Kobayashi et al. 2005; Ray et al. 2007). This mutation dramatically reduces the binding ability of inhibitors. A recent study clearly demonstrated that, in some patients who carry no mutation in the BCR-ABL gene, activation of Lyn kinase is associated with the imatinib-resistance in CML patients (Wu et al. 2008).

It is interesting to note that some newly designed non-ATP competitive inhibitors have been demonstrated to override the aforementioned acquired resistance (Gumireddy et al. 2005; Adrian et al. 2006) and that combination with other agents has been clinically proven to be a more comprehensive therapeutic strategy (Kopetz et al. 2007). Structurally, these non-ATP competitive inhibitors are designed to target relatively specific regions other than the ATP-binding pocket in the kinase domain (Burke et al. 1993; Marsilje et al. 2000; Milkiewicz et al. 2000; Sawyer 2007; Schenone et al. 2007). Experimentally, these non-ATP competitive inhibitors equally inhibit kinase activity under varying concentrations of ATP in vitro. One of the best example in this category will be KX-01 (Kinex Pharmaceuticals 2007) under a phase I trial. What is unique about KX-01 is that it targets Src substrate binding sites other than ATP site and has high potency to tumor cell lines from all major cancer types. Also, since correct positioning of the $\alpha$C helix and P-loop is
necessary for the activation of Src, it is plausible that some compounds may associate with these regions and to produce subsequent steric hindrances that may prevent their correct positioning.
Figure 1. Schematic Presentation of the Structure of Src Kinase Domain and the ATP Binding Pocket.
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<th>Target</th>
<th>Stage of development (as of Jun 2008)</th>
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<td>Dasatinib</td>
<td>SFKs, Abl, Kit, PDGFR, Eph receptors</td>
<td>Approved: CML/Ph+ALL&lt;br&gt;Phase II: prostate cancer, breast cancer</td>
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<tr>
<td>(BMS-354825,</td>
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<tr>
<td>Sprycel)</td>
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<tr>
<td>Imatinib</td>
<td>c-Kit, PDGFR, Src</td>
<td>Approved: CML, gastrointestinal cancer&lt;br&gt;Preclinical: systemic sclerosis&lt;br&gt;Phase I: uterine papillary serous carcinoma&lt;br&gt;Phase I: advanced thymic carcinoma&lt;br&gt;Phase II: melanoma&lt;br&gt;Phase II: thyroid carcinoma&lt;br&gt;Phase II/IV: leukemia</td>
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<tr>
<td>AZD-0530</td>
<td>Src, Abl</td>
<td>Preclinical: breast cancer&lt;br&gt;Phase I/II: colorectal cancer, pancreatic cancer&lt;br&gt;Phase II: prostate cancer</td>
</tr>
<tr>
<td>XL999</td>
<td>Src, VEGFR2, PDGFR, Kit, FGFR1</td>
<td>Phase I: solid tumors&lt;br&gt;Phase II: lung cancer, renal cell carcinoma, colorectal cancer, ovarian cancer</td>
</tr>
<tr>
<td>Bosutinib</td>
<td>SFKs, Abl</td>
<td>Phase I: solid tumors&lt;br&gt;Phase II: breast cancer, CML/Ph+ALL</td>
</tr>
<tr>
<td>(SKI-606)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>Src, many other tyrosine kinases</td>
<td>Phase I/II: breast cancer, prostate cancer</td>
</tr>
<tr>
<td>KX-01 (KX2-391)</td>
<td>Src</td>
<td>Phase I: advanced solid tumor</td>
</tr>
<tr>
<td>(NS-187)</td>
<td></td>
<td></td>
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<tr>
<td>INNO-406</td>
<td>Lyn, Abl</td>
<td>Phase I: CML/ALL</td>
</tr>
<tr>
<td>(XL-228)</td>
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<tr>
<td>PD173955</td>
<td>Src, Yes, Abl, Kit</td>
<td>Preclinical</td>
</tr>
<tr>
<td>CGP76030</td>
<td>SFKs, EGFR, VEGFR, Abl</td>
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<td>CGP77675</td>
<td>SFKs, EGFR, VEGFR, FAK</td>
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<td>AP22161</td>
<td>Src</td>
<td>Preclinical</td>
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<td>AP22408</td>
<td>Src</td>
<td>Preclinical as bone-targeted agents</td>
</tr>
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<tr>
<td>AZM475271</td>
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</tr>
<tr>
<td>SMI-11958</td>
<td>Src</td>
<td>Preclinical</td>
</tr>
<tr>
<td>UC15A</td>
<td>Src</td>
<td>Preclinical</td>
</tr>
<tr>
<td>PP1</td>
<td>SFKs</td>
<td>Research tool</td>
</tr>
<tr>
<td>PP2</td>
<td>SFKs</td>
<td>Research tool</td>
</tr>
<tr>
<td>SU6656</td>
<td>SFKs, PDGFR</td>
<td>Research tool</td>
</tr>
</tbody>
</table>

CML, Chronic Myeloid Leukemia; Ph+ALL, Philadelphia-positive Acute Lymphocytic Leukemia
Na/K-ATPase as a Signaling Transducer

Cardiotonic Steroids

Chemically, cardiotonic steroids (CTS) include plant-derived digitalis drugs such as digoxin and ouabain, and vertebrate-derived aglycones such as bufalin and marinobufagenin (MBG) (Akera et al. 1976; Schoner et al. 2007). While digoxin has been used for more than 200 years to manage congestive heart failure and/or atria fibrillation, bufalin and MBG represent the major active components of a traditional Chinese medicine called Chan’su that is prescribed as a cardiotonic, diuretic and analgesic agent. Unlike many other steroids, CTS have one or several sugar molecules attached to its steroidal genin (Melero et al. 2000). Although several studies suggest that the sugar group is only loosely associated with extracellular binding sites, the removal of ouabain’s rhamnose moiety dramatically decreased the ligand binding affinity (Middleton et al. 2000; Paula et al. 2005). So far, the binding sites between CTS and the Na/K-ATPase have not been fully disclosed.

Although CTS have been considered only as drugs since their discovery, several studies clearly identified both ouabain and MBG as endogenous steroids that exist in human circulation and can be synthesized in cultured mammalian cells as well (Hamlyn et al. 1982; Hamlyn et al. 1991; Kawamura et al. 1999; Komiyama et al. 2001). Recent studies have also found that their production and secretion are regulated by multiple physiological stimuli including adrenocorticotropic hormone (ACTH); and angiotensin II (Hamlyn et al. 1991; Laredo et al. 1997; Fedorova et al. 1998; Komiyama et al. 2005; Schoner et al. 2007). Interestingly, the increase in the endogenous digitalis level has been observed in many patients with renal
failure and correlates with hypertension and congestive heart failure (Pierdomenico et al. 2001; Wang et al. 2003). Increases in plasma CTS have also been observed in human with chronic high-salt diet (Hamlyn et al. 1982). Moreover, 50% of patients with essential hypertension have substantially elevated levels of endogenous ouabain (Manunta et al. 1999). Plasma CTS are also high in several salt-dependent hypertensive animals (Fedorova et al. 2000). Furthermore, both the antibody to digoxin, marinobufagenin, and the antagonist to ouabain, PST 2238, have been indicated to antagonize the hypertensive effect of endogenous digitalis (Ferrandi et al. 2004; Fedorova et al. 2005).

Functions of CTS

CTS are found to play an important role in regulation of renal salt handling, vascular and cardiac contractions. Because CTS, as ligands for Na/K-ATPase, specifically bind and inhibit Na/K-ATPase, the physiological and pharmacological functions of CTS are through the changes of intracellular ion concentrations. Specifically, Na/K-ATPase pumping activity is inhibited after ligand binding, thus the intracellular Na$^+$ concentration is increased. With the involvement of the membrane Na$^+$/Ca$^{2+}$ exchanger, the intracellular Ca$^{2+}$ is increased, which results in a positive effect on the contractility of the cardiac muscle (Blaustein et al. 1969; Reuter et al. 2002; Iwamoto et al. 2004).

In addition to the aforementioned cardiac and vascular effects, CTS play an important role in the regulation of cell growth. In fact, the effects of ouabain on gene expression and the mitogen-induced differentiation and proliferation of lymphoblasts were first noted in the 1970s (Cuff et al. 1975;
Kaplan 1978; Pollack et al. 1981). Recent studies have greatly expanded the diversity of CTS-affecting cells. Moreover, they showed that CTS activated multiple growth pathways and stimulated either differentiation / apoptosis or hypertrophic / proliferative growth in a cell type-specific manner (Griffiths et al. 1991; Nakagawa et al. 1992; Golomb et al. 1994; Peng et al. 1996; Feschenko et al. 1997; Huang et al. 1997; Kawazoe et al. 1999; Haas et al. 2000; McConkey et al. 2000; Chueh et al. 2001; Lee et al. 2001; Haas et al. 2002; Dmitrieva et al. 2003; Ferrandi et al. 2004; Trevisi et al. 2004; Kometiani et al. 2005; Lopez-Lazaro et al. 2005; Bielawski et al. 2006; Golden et al. 2006; Li et al. 2006; Liang et al. 2006; Ramirez-Ortega et al. 2006; Kulikov et al. 2007; Li et al. 2007; Mijatovic et al. 2007). Significantly, the growth regulatory effects of CTS could occur at nano- and sub-nanomolar concentrations that exhibited no inhibition of cellular Na/K-ATPase pumping capacity (Aydemir-Koksoy et al. 2001; Saunders et al. 2004; Khundmiri et al. 2006; Kulikov et al. 2007; Li et al. 2007; Qiu et al. 2007). Finally, recent studies have demonstrated the potential physiological and pathological significance of CTS-mediated cell growth regulation (Ferrandi et al. 2004; Kennedy et al. 2006; Nguyen et al. 2007). To this end, it is important to note the following important studies: First, the effect of sub-nanomolar concentrations of ouabain on cell growth was first observed in cultured canine smooth muscle cells (Aydemir-Koksoy et al. 2001). This observation was subsequently confirmed in several other types of cells including endothelial cells and cardiac fibroblasts (Saunders et al. 2004; Elkareh et al. 2007; Li et al. 2007). Second, a recent study from Blanco’s laboratory suggested that endogenous CTS might play an important role in the pathogenesis of
autosomal dominant polycystic kidney disease (ADPKD) (Nguyen et al. 2007). It is known that cyst formation and enlargement require proliferation of mural renal epithelial cells. Blanco and colleagues found that a basal lateral application of sub-nanomolar concentrations of ouabain was sufficient to stimulate the proliferation of ADPKD cells, but not the normal human kidney cells, via the activation of an ERK pathway (Nguyen et al. 2007). Finally, recent in vivo studies have confirmed a role of CTS in control of cardiac hypertrophy and fibrosis observed in uremic cardiomyopathy (Ferrandi et al. 2004; Kennedy et al. 2006; Elkareh et al. 2007).

**Src and CTS-Induced Stimulation of Protein Tyrosine Phosphorylation**

The discovery of a class of proteins (e.g., v-Src and v-Abl) associated with the polyomavirus that phosphorylates tyrosine had revealed the importance of protein tyrosine phosphorylation in control of cell growth (Courtneidge et al. 1983). Accordingly, many studies had been conducted to test whether ouabain and other CTS affected protein tyrosine phosphorylation (Kometiani et al. 1998; Contreras et al. 1999; Haas et al. 2000; Aydemir-Koksoy et al. 2001; Kometiani et al. 2005; Kotova et al. 2006). These studies demonstrated that sub-toxic concentrations of ouabain indeed stimulated tyrosine phosphorylation of multiple proteins in cardiac myocytes, A7r5 cells, HeLa cells, and LLC-PK1 cells. These ouabain effects were rapid and dose-dependent. Moreover, addition of a non-specific tyrosine kinase inhibitor such as genistein or herbimycin A was sufficient to block ouabain-induced tyrosine phosphorylation, and subsequently the growth effect of ouabain on cultured
cells (Haas et al. 2000; Aydemir-Koksoy et al. 2001). More recently, these early observations were confirmed not only in vitro but also in vivo (Ferrandi et al. 2004; Kennedy et al. 2006). Interestingly, studies have further revealed that increases in protein tyrosine phosphorylation are also important for CTS-induced cellular actions other than cell growth regulation (Valente et al. 2003; Yuan et al. 2005; Kotova et al. 2006; Thundathil et al. 2006).

In principle, increases in protein tyrosine phosphorylation can occur through stimulation of protein tyrosine kinases or inhibition of tyrosine phosphatases or both. The Na/K-ATPase has no intrinsic tyrosine kinase activity. Thus, it is unlikely that the Na/K-ATPase can serve as a receptor tyrosine kinase to convert the ouabain binding to increases in protein tyrosine phosphorylation. Although the α subunit contains a consensus tyrosine phosphatase motif, preliminary studies have shown that it does not have significant tyrosine phosphatase activity. There are many precedents where ligands stimulate protein tyrosine phosphorylation employing receptors that lack intrinsic kinase activity (Ihle et al. 1995; Clements et al. 1999; McGarrigle et al. 2007). Examples of this mode of signal transduction include cytokine receptors (Ihle et al. 1995) and G protein-coupled receptors (GPCRs) (McGarrigle et al. 2007). While the formation of so-called “binary” cytokine receptors involves non-covalent association of JAK members with the receptor, the GPCRs employ Src family kinases. Thus, it is plausible that non-receptor tyrosine kinases such as Src could also be responsible for ouabain-induced protein tyrosine phosphorylation. Indeed, our initial studies revealed that ouabain increased translocation of cytosolic Src to a Triton-insoluble fraction and stimulated Src activity in cultured cardiac myocytes, A7r5 and
LLC-PK1 cells (Haas et al. 2000). The stimulation was apparently due to an increase in tyrosine phosphorylation of Src Tyr418, but not the dephosphorylation of Tyr529. Moreover, inhibition of Src by several inhibitors blocked ouabain-induced tyrosine phosphorylation of cellular proteins and abolished many ouabain-activated down-stream signal pathways including the activation of ERK. These findings were further confirmed in SYF mouse fibroblasts where Src family kinases were knocked out (Haas et al. 2002). Functionally, activation of Src is not only important for CTS to regulate cell growth, but also other cellular activities (Liu et al. 2004; Jung et al. 2006; Kotova et al. 2006; Khundmiri et al. 2007; Liu et al. 2007; Pasdois et al. 2007). To this end, it is of interest to mention that the activated Src appears to be able to phosphorylate the Na/K-ATPase and then regulate the pump activity (Bozulic et al. 2004; Ferrandi et al. 2004; Bozulic et al. 2005).

**Na/K-ATPase/Src Complex as a Functional CTS Receptor**

The following evidence supports the notion that the Na/K-ATPase directly interacts with Src to form a functional receptor complex in live cells (Liu et al. 2003; Wang et al. 2004; Kotova et al. 2006; Liang et al. 2006; Tian et al. 2006): First, several laboratories, including ours, observed that the Na/K-ATPase and Src were co-enriched in caveolar fractions in different types of cells. Second, immunofluorescence imaging analysis showed co-localization of these two proteins in the plasma membrane. Third, both proteins could be co-immunoprecipitated by either anti-α1 or anti-Src antibodies. Fourth, FRET analysis indicated that both proteins were in close proximity, providing further support of a direct interaction in live cells. Finally,
in vitro GST pull-down assay demonstrated direct interactions between the $\alpha_1$ subunit of Na/K-ATPase and Src. The interaction between the $\alpha_1$ and Src involves at least two contacting sites: one being the CD2 domain of $\alpha_1$ and Src SH2, and the other consisting of the CD3 domain of $\alpha_1$ and the Src kinase domain (Fig. 1). Based on the available structure of $\alpha_1$, the CD2 and CD3 constitute the A and N domains, respectively, and they are highly exposed in the E1 conformation. Interestingly, both domains are known to interact with structural, membrane and soluble proteins. Examples of these interactions include ankyrin, IP3Rs, phosphoinositide 3' kinase (PI3K), PLC-$\gamma$ and cofilin (Jordan et al. 1995; Zhang et al. 1998; Yudowski et al. 2000; Lee et al. 2001; Barwe et al. 2005; Yuan et al. 2005; Zhang et al. 2006).

Functionally, the interaction between the Na/K-ATPase and Src keeps Src in an inactive state. When the molecular mechanism of this inhibition was probed, we found that the CD3/kinase domain interaction was responsible for this inhibition (Tian et al. 2006). This is reminiscent of Wiscott-Aldrich syndrome protein - induced Src inhibition (Schulte et al. 2003), but in contrast to the Src kinase domain interaction with $G_{\alphaS}$ and $G_{\alphai}$, which results in Src activation (Ma et al. 2000). Interestingly, even though the RACK1/SH2 interaction inactivates Src (Chang et al. 1998; Chang et al. 2001), the CD2/SH2 interaction appears to have no effect on the kinase activity. When the effect of ouabain on these domain interactions was examined, we observed that ouabain reduced the binding of the Src kinase domain, but not the SH2 or full length Src, to the Na/K-ATPase. Thus, we suggest that binding of ouabain to the Na/K-ATPase/Src receptor complex may free the Src kinase domain, resulting in activation of the Na/K-ATPase-associated Src kinase.
While the Na/K-ATPase provides the ligand binding site, the Na/K-ATPase-associated Src functions as a signal transducer, converting as well as amplifying the ligand binding signal to increases in protein tyrosine phosphorylation. It is of interest to point out that this mode of Src activation is unique and different from other known mechanisms of Src activation induced by either receptor tyrosine kinases or GPCRs. Specifically, it does not require dephosphorylation of pY529 or binding of the Src SH2 to a phosphorylated tyrosine (Moarefi et al. 1997; Thomas et al. 1997; Ma et al. 2000). Moreover, the interaction between the Na/K-ATPase and Src represents a novel regulation of cellular Src activity because of the involvement of both the SH2 and the kinase domains.

Additionally, there are many ion transporters and channels that are capable of forming signaling complexes and functioning as signaling receptors or integrators. Examples for such roles of ion transporting proteins include, but are not limited to, the Na/H-exchanger (Baumgartner et al. 2004), the transient receptor potential channels (Wang et al. 2005), the red blood cell anion exchanger band 3 (Bruce et al. 2003), IP3Rs (Patterson et al. 2004) and plasma membrane Ca-ATPase (PMCA) (Buch et al. 2005; Oceandy et al. 2007). For instance, PMCA4b regulates cardiac contractility through its interaction with neuronal nitric oxide synthase (nNOS) mediated by the PDZ domain in PMCA4b (Oceandy et al. 2007). PMCA4b also directly interacts with the catalytic domain of the calcium dependent phosphatase calcineurin and functions as a negative modulator of calcineurin-mediated signaling pathways in mammalian cells (Buch et al. 2005).
Figure 2. Schematic Presentation of the Ouabain-Regulated Interaction between Na/K-ATPase and Src.

**Regulation of Src through Na/K-ATPase/Src complex**
Significance of the Signaling Function of Na/K-ATPase

The physiological and pathological significance of the newly appreciated signaling function of Na/K-ATPase is still not understood. However, the fact that Na/K-ATPase regulates Src activity could also link the CTS to many cellular functions regulated by Src. For example, CTS level changes under pathological conditions may affect multiple kinases activity, which could further regulate L-calcium channels as well as many ion transporters through direct phosphorylation (Bence-Hanulec et al. 2000; Davis et al. 2001; Hernando et al. 2007).

Changes in the total Na/K-ATPase amount (see Table 2) have been documented in many clinical cases (Charlemagne et al. 1994; Larsen et al. 1997; Schwinger et al. 1999; Lecuona et al. 2007). For example, Na/K-ATPase expression level in hypertrophic heart is decreased (Larsen et al. 1997). Studies from another group showed that both Src and MAPK were significantly activated after cardiac hypertrophy (Takeishi et al. 2001). Downregulation of Na/K-ATPase is also found in certain cancers, including human colorectal cancer tissues (Sakai et al. 2004), human bladder cancer tissues (Espineda et al. 2003), and kidney cancer cells (Espineda et al. 2004).
Table 2. Changes of Na/K-ATPase Expression under Pathological Conditions.

<table>
<thead>
<tr>
<th>Syndrome and Tissue</th>
<th>Na/K-ATPase Expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Failure Heart</td>
<td>α1 ↓ 38%</td>
<td>(Schwinger et al. 1999)</td>
</tr>
<tr>
<td>Rat Failure Heart</td>
<td>α1 ↓ 20%&lt;br&gt;α2 ↓ 70%</td>
<td>(Swift et al. 2008)</td>
</tr>
<tr>
<td>Human Hypertrophied Heart</td>
<td>α1 ↓ 60%</td>
<td>(Larsen et al. 1997)</td>
</tr>
<tr>
<td>Rat Hypertrophied Heart</td>
<td>α1 ↓ 42%</td>
<td>(Larsen et al. 1997)</td>
</tr>
<tr>
<td>Rat Hypertrophied Heart</td>
<td>α2 ↓</td>
<td>(Charlemagne et al. 1994)</td>
</tr>
<tr>
<td>Rat Diabetic Brain</td>
<td>α1 ↓ 61%&lt;br&gt;α3 ↓ 20%</td>
<td>(Ver et al. 1995)</td>
</tr>
<tr>
<td>Rat Diabetic Kidney</td>
<td>α1 ↑</td>
<td>(Ver et al. 1995)</td>
</tr>
<tr>
<td>Rat Diabetic Heart</td>
<td>α1 ↑&lt;br&gt;α2 ↓</td>
<td>(Gerbi et al. 1997)</td>
</tr>
<tr>
<td>Human Acute Lung Injury</td>
<td>α1 ↓</td>
<td>(Lecuona et al. 2007)</td>
</tr>
<tr>
<td>Rat Lung after Renal Injury</td>
<td>α1 ↓</td>
<td>(Rabb et al. 2003)</td>
</tr>
</tbody>
</table>

Cell-penetrating Peptides (CPPs)

Accumulating progress in functional genomics and molecular biology in recent years has led to the discovery of many potential new therapeutic macromolecules. They include, for example, monoclonal antibodies [EGFR antibodies: Panitumumab, Cetuximab, Matuzumab (Diasio et al. 2006)], peptides [36-mer Enfuvirtide (Robertson 2003)], antisense oligonucleotides [Fomivirsen (Taylor et al. 1999)], aptamers [Pegaptanib, (Ng et al. 2006)] as well as recombinant proteins and plasmid DNA. However, because of their low biomembrane permeability as well as relatively low in vivo bioavailability, it is still a challenge to efficiently deliver these macromolecules to intracellular compartments.
In my study, NaKtide, a 20-mer polypeptide derived from Na/K-ATPase α1 subunit, was identified as the binding motif for Src and functionally inhibited Src \textit{in vitro}. NaKtide is membrane-impermeable. To test the biological activity of NaKtide, delivery of it across the cell membrane is the first concern. Interestingly, many studies have demonstrated that the conjugation of various polycationic polymers is capable of promoting the movement of macromolecules across cell membrane (Joliot et al. 2004). Basically, the majority of these polymers share the same feature where strong basic amino acids, lysine and arginine, are repeatedly present.

CPPs and their applications

Based on their origins, CPPs can be divided into three categories (see Table 3): First, synthetic short peptides containing repeating basic residues (lysine or arginine). It has been shown that polybasic peptides, such as poly-L-lysine or poly-L-arginine, significantly enhanced protein uptake (Ryser 1967; Shen et al. 1978). These peptides are still used in delivering many macromolecules. For instance, chemical conjugation of proteins / peptides / oligonucleotides to either poly-L-lysine or poly-L-arginine allowed significantly improved internalization in various cells (Shen et al. 1978; Mitchell et al. 2000; Matsushita et al. 2001).

Another group, representing the most studied CPPs, is derived from naturally occurring proteins with cell-penetrating ability: the \textit{Drosophila} Antennapedia protein and the human immunodeficiency virus (HIV) – coded trans-activating transcriptional (Tat) protein. Penetratin peptide (originally 60 amino acids) derived from the homeodomain of Antennapedia protein was
first discovered to efficiently transduce Rab 3 polypeptide across the membrane of myoblasts, myotubes and neurons (Perez et al. 1992). After 2 years, shorter peptide (AP) from the third helix of Antennapedia homeodomain was identified to be sufficient to translocate through biological membranes (Derossi et al. 1994). Tat protein was identified as a cell-penetrating protein encoded by human immunodeficiency virus (HIV) about 20 years ago (Frankel et al. 1988). The TAT peptide, containing 13 amino acid residues from Tat’s basic domain, was first reported as a CPP about 10 years later (Vives et al. 1997). So far, among more than 300 different applications using different CPPs, TATs and APs were among the most widely used CPPs, accounting for 44% and 23%, respectively (Vives 2005).

Since the discovery of TAT and AP in the mid-1990s, many other CPPs from natural proteins have been identified. They include, for instance, pVEC from vascular endothelial cadherin (Elmquist et al. 2001), kFGF from fibroblast growth factor (Lin et al. 1996), integrin peptide from β3-integrin (Liu et al. 1996) and buforin 2 from buforin (Takeshima et al. 2003). In the cases of kFGF and integrin peptide, they have zero net charge. The detailed mechanism by which they penetrate through cell membranes is still not clear.

There are also some chimeric CPPs, such as MPG peptide (Morris et al. 1997), Pep-1 (Morris et al. 2001), Transportan (Pooga et al. 1998) and Transportan 10 (Soomets et al. 2000). For example, Transportan has 12 amino acids derived from galanin and 14 amino acids from the bee venom mastoparan linked via a lysine. Pep-1 consists of a hydrophobic tryptophan-rich motif containing five tryptophan residues (KETWWETWWTEW), a hydrophilic lysine-rich domain (KKKRKV) derived from the nuclear localization
sequence of simian virus 40 (SV-40) large T antigen and a spacer domain (SQP), separating the aforementioned two domains. The tryptophan-rich domain is required for efficient targeting to the cell membrane and for forming hydrophobic interactions with proteins, while the hydrophilic lysine-rich domain may improve intracellular delivery and solubility of the peptide vector. The linker region containing a proline residue provides the flexibility and the integrity of both the hydrophobic and the hydrophilic domains.
Table 3. List of well-known CPPs

<table>
<thead>
<tr>
<th>Category</th>
<th>Name</th>
<th>Sequence</th>
<th>Net charge</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic CPPs</td>
<td>Poly-L-lysine</td>
<td>(K)n</td>
<td>+n</td>
<td>(Shen et al. 1978)</td>
</tr>
<tr>
<td></td>
<td>Poly-L-arginine</td>
<td>(R)n</td>
<td>+n</td>
<td>(Mitchell et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>KLALKLALKALKAALKLA</td>
<td>+5</td>
<td>(Oehlke et al. 1998)</td>
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<tr>
<td></td>
<td>KALA</td>
<td>WEAKLAKALAKALAKHLAKALAKALKACEA</td>
<td>+5</td>
<td>(Wyman et al. 1997)</td>
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<td>(Hong et al. 2000)</td>
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<td>Pep-7</td>
<td>SDLWEMMMVSLACQY</td>
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<td>(Kim et al. 2005)</td>
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<td>SynB4</td>
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<td>SynB5</td>
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<td>Naturally occurring protein-derived CPPs</td>
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<td>(Liu et al. 1996)</td>
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<td>Buforin</td>
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<td></td>
<td>CaE</td>
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<td>(Gorodetsky et al. 2004)</td>
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<tr>
<td></td>
<td>Cβ</td>
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<td>+5</td>
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<td></td>
<td>Cγ</td>
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<td>DPV3</td>
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<td>GAYDLRRERQSRRLRRERQSS</td>
<td>+6</td>
<td>(De Coupade et al. 2005)</td>
</tr>
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<td>Chimeric CPPs</td>
<td>kFGF</td>
<td>AAVALLPAVLLALLAP</td>
<td>0</td>
<td>(Lin et al. 1996)</td>
</tr>
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</tr>
<tr>
<td>NF-κB P50</td>
<td>VQKRKQLM</td>
<td>+4</td>
<td></td>
<td>(Ragin et al. 2002)</td>
</tr>
<tr>
<td>pVEC</td>
<td>LIIILRRIRKQAHAS</td>
<td>+8</td>
<td>(Elmquist et al. 2001)</td>
<td></td>
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<tr>
<td>pISL</td>
<td>RVIRVWFQNKRCDDK</td>
<td>+6</td>
<td>(Karlsson et al. 1990)</td>
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<tr>
<td>SV-40 NLS</td>
<td>PKKKRRKV</td>
<td>+5</td>
<td></td>
<td>(Beven et al. 1997)</td>
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<tr>
<td>TAT</td>
<td>GRKKRRQRRRPPQ</td>
<td>+8</td>
<td>(Vives et al. 1997)</td>
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<tr>
<td></td>
<td>DPV3/10</td>
<td>RKKRRRESRRARRSPRH</td>
<td>+11</td>
<td>(De Coupade et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>DPV10/6</td>
<td>SRRARRSPREGKKKRKR</td>
<td>+11</td>
<td>(De Coupade et al. 2005)</td>
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<td></td>
<td>DPV1047</td>
<td>VKRGLKLHVRPRVTRMDV</td>
<td>+7</td>
<td>(De Coupade et al. 2005)</td>
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<td></td>
<td>MPG</td>
<td>GALFLGWLGAAGSTMGAPKKRKV</td>
<td>+5</td>
<td>(Morris et al. 1997)</td>
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<td>Pep-1</td>
<td>KETWETWTEWSQPKKKRKV</td>
<td>+3</td>
<td>(Morris et al. 2001)</td>
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<td>Transportan</td>
<td>GWTLNSAGYLLKINLAAALAKKIL</td>
<td>+5</td>
<td>(Pooga et al. 1998)</td>
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<td>Transportan 10</td>
<td>AGYLLKINLAAALKKIL</td>
<td>+4</td>
<td>(Soomets et al. 2000)</td>
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</table>
The abovementioned CPPs have been widely applied in the delivery of synthetic organic chemicals (Jensen et al. 2003), peptides (Jasmin et al. 2006; Senatus et al. 2006; Zhou et al. 2006; Mamidipudi et al. 2007; Qi et al. 2008), proteins/antibodies (Rojas et al. 1998; Jo et al. 2005; Shin et al. 2005), and antisense oligonucleotides / plasmids (Turner et al. 2005; Moschos et al. 2007). They are also applicable in the delivery of larger cargos, such as liposomes (Torchilin et al. 2001; Tseng et al. 2002; Torchilin et al. 2003), polymeric nanoparticles (Lewin et al. 2000), phages (Eguchi et al. 2001) and adenoviruses (Gratton et al. 2003; Kida et al. 2006; Han et al. 2007). Even though a few of them have been already approved by FDA for clinical I/II trials, there are no CPPs-conjugated drugs approved on the market.

Mechanism of cellular CPPs uptake

Generally, extracellular molecules can be internalized through 1) transmembrane channels or transporters; or 2) membrane proteins or sugars at the cell surface via endocytosis. Although the applications of CPPs-mediated cellular delivery of various bioactive macromolecules are emerging, the detailed mechanism behind these phenomena is still under investigation. Some early evidence has suggested that endocytosis is less likely involved. For example, both TAT and AP are still able to penetrate through the cell membrane at low temperature (Derossi et al. 1996; Vives et al. 1997). Receptor recognition is not required since unnatural D-isoforms of CPPS or insertions of proline residues in CPPs do not impair their cellular uptake (Derossi et al. 1996). Finally, the fact that substituting one or three residues by proline does not impair internalization suggests that helical structure of
CPPs is not required either. Meanwhile, replacement of two tryptophan residues in AP by phenylalanines abolishes its internalization (Derossi et al. 1994). Results from alanine-scanning studies confirmed several basic residues are essential (Fischer et al. 2000; Drin et al. 2001). These led to the proposed model of CPPs internalization. CPPs bind to negatively charged phospholipids or proteoglycans through an electrostatic interaction. Tryptophan residues destabilize the lipid bilayer and allow inverted micelles to associate with CPPs trapped inside. Eventually, part of the formed micelles may release the trapped CPPs into the cytoplasm. This model is applicable to many TAT / AP-mediated internalizations. It is also supported by recent finding that cell surface proteoglycans mediate the uptake of polycationic CPPs (Poon et al. 2007).

Although CPPs translocation process was thought to be energy-independent for many years, several recent findings suggest that part of CPPs translocation is energy-dependent and reduced by endocytosis inhibitors (Drin et al. 2003; Vives et al. 2003; Moss et al. 2005). First, some of the CPPs accumulate inside vesicle structures. Second, the internalization of some CPPs is in the temperature- and energy-dependent manner demonstrated by the decreased internalization of CPPs at low temperature or ATP-depleted cells. Third, various endocytosis inhibitors can partially block the internalization of CPPs. All these recent studies suggest that the endocytosis pathway is at least partially involved. Furthermore, in the majority of early studies in the fixed cells, the localization of CPPs in the nucleus and cytosol was found to be artificial effect due to the fixation method (Pichon et al. 1999; Leifert et al. 2002; Lundberg et al. 2002; Drin et al. 2003).
membrane distribution and punctuated vesicle patterns of FITC-labeled CPPs in live cells also support this notion that endocytosis might play a role in CPPs-mediated translocation.

Some recent studies did clearly demonstrate that lipid raft-dependent macropinocytosis is required for TAT-mediated transduction (Wadia et al. 2004). Macropinocytosis is a rapid receptor-independent form of endocytosis. It requires actin membrane protrusions that envelope into vesicles, termed macropinosomes (Jones 2007). Amiloride, a specific inhibitor of the Na$^+$/H$^+$ exchanger, and cytochalasin D, an F-actin elongation inhibitor, inhibit TAT transduction in a dose-dependent manner, while a dominant-negative mutant of dynamin, Dyn$^{K44A}$, fails to block TAT transduction (Wadia et al. 2004).

**Disadvantage of CPPs-mediated macromolecules uptake**

Stability of these CPPs is questionable since they may undergo cleavage or degradation in the presence of furin as well as various intracellular proteases (Tikhonov et al. 2004; Chauhan et al. 2007). On the other hand, since CPPs are permeable to cell membranes as well as the blood-brain barrier, studies from whole animals have shown that TAT peptides administrated intravenously are removed from circulation instantaneously due to rapid uptake into almost every organ, including spleen, liver, lung, heart and brain (Lee et al. 2001; Cai et al. 2006). Conjugating of bioactive macromolecules will significantly decrease the plasma clearance rate as well as the organ uptake. Overall, the specific targeting through the whole body is still a problem, which may be another disadvantage for future drug application.
It has been suggested that the TAT / AP conjugating peptides mainly localize to the nucleus (Astriab-Fisher et al. 2002; Guelen et al. 2004; Ziegler et al. 2005). They may also be associated with the plasma membrane (Lundberg et al. 2002; Richard et al. 2003), or cytosolic compartments (Astriab-Fisher et al. 2002; Guelen et al. 2004). It is also interesting to mention that several studies have shown the fixation methods may affect the localization of TAT conjugating peptides (Leifert et al. 2002; Lundberg et al. 2002). Anyway, localization of CPPs in a cell type-specific manner suggests that it is necessary to check whether the bioactive molecules can reach their targets inside of cells.

**Prostate Cancer**

After its first diagnosis about one and a half centuries ago (Adams 1853), prostate cancer has been identified as a major health problem worldwide. It is the second most frequently diagnosed cancer in men, with over 780,000 new cases estimated in 2007. Near 75% of these cases are expected to be diagnosed in the developed countries, including the United States, Canada and Western European countries. For example, in the United States, 1 man in 6 will get prostate cancer during his lifetime, making it the most common cancer among men and the second leading cause of cancer-related mortality. Locally, in the state of Ohio, prostate cancer has the highest incidence rate among all cancers in men during 2000-2004 (Collin et al. 2008; Jemal et al. 2008).

This remarkable health problem in men may be attributed to several risk factors; age, being the most significant one. The mean age of prostate
cancer patients is about 72-74 years, and about 85% of total patients are over age 65 in Sweden (Gronberg 2003). Studies in Detroit, MI also show that 25% of men in the 30s and 40% in the 50s have signs of prostate cancer in its latent stage (Sakr et al. 1994). Prostate cancer occurs about 60% more often in African American men than in white American men, suggesting that race is another risk factor. Dietary factors may also play an important role in developing prostate cancer. Migrants from low incidence rate areas, such as China and Japan to high incidence rate areas, including the United States and United Kingdom, adopt a higher prostate cancer risk within 6 months (Nelson et al. 2002). Hereditary prostate cancer accounts for 5 to 10% of total cases and as much as 30 to 40% of early onset cases (Carter et al. 1993), but so far the genes responsible for hereditary prostate cancer have not been fully understood. Other possible risk factors include sedentary lifestyle and exposure to metal cadmium etc. (Bostwick et al. 2004).

The normal prostate consists of glandular epithelial and fibromuscular stroma compartments. The glandular structure can be divided into peripheral, transition, and central zones. More than 80% of prostate cancers arise in the peripheral zone, which contains about 70% of the prostate glandular elements. Cell proliferation is normally slow in the adult prostate as well as in a cancerous prostate; however, an enlarged prostate has been correlated to low frequency of apoptosis. Androgens, such as testosterone, play an important role in stimulating the proliferation of prostate epithelial cells, while androgen ablation induces apoptosis in most prostate cancer cells. Accordingly, inhibiting androgen production has been useful for prostate cancer therapy (Denmeade et al. 2002). However, many prostate cancers in clinical patients
will transform into hormone-resistant prostate cancers. This may be due to changes of ligand binding function of androgen receptor (Han et al. 2005) or loss of androgen and estrogen receptors by transcriptional silencing through hypermethylation (Sasaki et al. 2002). Most importantly, prostate cancer lethality is caused by an increased resistance to apoptosis rather than enhanced proliferation rates. So clinically, patients developing hormone-resistance prostate cancer have low response to commonly used chemotherapeutic agents, which target cells with high proliferation rates (Berges et al. 1995).

**Role of SFKs in Prostate Cancer**

As discussed before, some members of SFKs (Src, Fyn, Yes) are expressed in a broad range of tissues including prostate. Systematic scanning of the kinase profile showed that Lck, previously thought as a lymphocyte-specific tyrosine kinase, is present in prostate carcinoma xenograft, but not in commonly used prostate cancer cells (Robinson et al. 1996). Lyn was first discovered in many hematopoietic cells and present throughout B cell development (Bolen et al. 1992). It was also found in normal dog prostate gland (Allard et al. 1997) as well as normal and malignant human prostate epithelium (Goldenberg-Furmanov et al. 2004). Since SFKs have been shown to be up-regulated in multiple types of human tumors, with Src activity increasing proportionally to the progressive stages of the disease (Summy et al. 2003), the presence of SFKs in prostate tumors raises a question about whether SFKs are involved in the development process of normal and malignant prostate.
In prostate cancer cells, inhibition of SFKs decreases cell proliferation mainly through inhibition of mitogenic pathways and induction of apoptosis (Lombardo et al. 2004; Chang et al. 2007; Fizazi 2007). Block of Src also inhibits cell invasion and migration (Nam et al. 2005) through selective inhibition of phosphorylation of Src substrates, such as focal adhesion kinase (FAK) (Parsons 2003) and p130Cas (Brabek et al. 2005). More recently, administration of both Dasatinib, a potent inhibitor for both Src and Lyn (Park et al. 2008) and KRX-1323, a small peptide inhibitor specific for Lyn (Goldenberg-Furmanov et al. 2004) to nude mice bearing prostate tumor xenografts induces cell apoptosis and inhibits tumor growth. It has also been suggested that Lyn may affect prostate cancer cell proliferation, while Src may primarily affect cell migration (Park et al. 2008).

Xenograft mice tumor models

Before development of the nude mouse model, human tumors were grafted and grown in immune-privileged sites, such as the anterior chamber of the eye and the cheek pouch. These sites are inconvenient for experimental procedure, and the tumors are eventually rejected. The nude mouse is a hairless and immunodeficient mutant (Flanagan 1966). It lacks a thymus, which is essential for the production of T-cells, lymphocytes that are essential to the immune system. Thus the nude mouse does not reject tumor transplantations from other species. The nude mouse is widely used for preclinical research in cancer (van Weerden et al. 2000; Sausville et al. 2006). The major advantage of the nude mouse model is that the tumor can be
formed and then studied in a whole animal system after transplanting an actual human tumor or injecting human tumor cells.

The SCID (severe combined immunodeficient) mice (Bosma et al. 1983) have been introduced as a more immunodeficient model for xenograft tumor studies. The advent of NOD/SCID mice presents a new immunodeficient model obtained by crossing the SCID and NOD (non obese diabetic) mouse strains (Shultz et al. 1995). The NOD strain is characterized by a functional deficit in nature killer cells (NKCs), an absence of circulating complement and defects in the differentiation and function of antigen-presenting cells. The NOD/SCID model combines multiple functional defects of adaptive and innate immunity. It is very suitable for xenografts of many human tumors. A comparative study of tumor growth rate of various hematopoietic cancer cell lines in different animal models (Nude, Rag1, SCID and NOD/SCID mice) has shown a better growth rate in the NOD/SCID model (Hudson et al. 1998). In the case of human PC-3 and DU145 prostate cancer cells, NOD/SCID mice produced more metastatic sites than normal nude mice (Bastide et al. 2002).
MATERIALS AND METHODS

Materials

Chemicals of the highest purity and culture media were purchased from Sigma (St. Louis, MO). PP2, a Src kinase inhibitor, and staurosporine, a non-specific PKC inhibitor, were obtained from Calbiochem (San Diego, CA). The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): monoclonal anti-Src antibody (B12), polyclonal anti-ERK antibody, monoclonal anti-phosphor-ERK antibody, goat anti-rabbit and goat anti-mouse secondary antibodies. The monoclonal anti-His antibody was from GE Healthcare (Buckinghamshire, England). Polyclonal antibodies against phosphor-Akt (Ser473) and Akt were purchased from Cell Signaling Technology (Danvers, MA). The monoclonal anti-α1 antibody (α6F) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). To purify GST-fused proteins and His-tagged proteins, we obtained Glutathione beads from Amersham Bioscience (Uppsala, Sweden) and ProBond Purification System from Invitrogen (Carlsbad, CA). Recombinant human Src, Lyn and IGF-1 were obtained from Upstate Biotechnology (Lake Placid, NY). Plasmids pEYFP-C1 and pECFP-N1 were purchased from Clontech (Palo Alto, CA), and pGEX-4T-1 and pTrc-His A vector were from Invitrogen (Carlsbad, CA). The Optitran nitrocellulose membranes used for Western blotting were obtained from Schleicher and Schuell (Dassel, Germany). All the peptides were synthesized from Genemed Synthesis (San Antonio, TX) with the purity of at least 95%. Identity and purity
were confirmed by high-performance liquid chromatography - mass spectroscopy in the Proteomic Core Lab at University of Toledo.

**Plasmid Constructs**

The preparation of plasmid constructs expressing GST fusion proteins were prepared as described previously (Tian et al. 2006). GST-CD3 (amino acid residue 350–785), GST-ND (amino acid residue 379–594), GST-ND2 (amino acid residue 379–475), GST-ND2R (amino acid residue 476–594), GST-ND1 (amino acid residue 379–435) and GST-ND1R (amino acid residue 436–594) expression vectors were subcloned in frame into pGEX-4T-1 expression vector (Amersham Bio sciences) based on the sequence of pig kidney Na/K-ATPase α1 subunit. Different GST-Src kinase domain and His-tagged Src constructs were generated by excising the corresponding Src cDNA from the GST-Src vector (Ma et al. 2000) and then inserting them into pGEX-4T-1 and pTrc-His A vector, respectively. Plasmids including pEYFP-ND1, pEYFP-ND and pEYFP-CD3 were made by directional subcloning the corresponding cDNAs from the GST-Src vector into pEYFP-C1 vector. All constructs were verified by DNA sequencing.

**Cell Preparation, Culture and Transient Transfections**

Pig kidney proximal LLC-PK1, mouse fibroblast SYF and SYF + Src cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in DMEM medium containing 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 µg/ml) in a 5% CO₂-humidified incubator. Na/K-ATPase knockdown cells PY-17 and TCN23-19
were generated from LLC-PK1 cells as previously described (Liang et al. 2006) and cultured in DMEM containing 10% fetal bovine serum, penicillin (100 units/ml)/streptomycin (100 μg/ml) and 1 μg/ml puromycin. LLC-PK1, PY-17 and TCN23-19 cells were serum-starved for 24 h, whereas SYF and SYF + Src cells were cultured in the medium containing 0.5% FBS for 24 h and used for the experiments. Human prostate cancer cells (DU145, LNCaP), breast cancer cells (BT-20, MCF-7), colon cancer cells (DLD-1, HT-29) and neuroblastoma cells (BE(2)-C, IMR32, SK-N-DZ, SMS-SAN) were obtained from ATCC and maintained in medium recommended by ATCC. Transient transfections were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

Primary cultures of neonatal rat cardiac myocytes were prepared as described previously with minor modifications (Peng et al. 1996). Myocytes were dispersed from ventricles of 1- to 2-day-old Sprague-Dawley rats by digestion with 0.04% collagenase II (Worthington) and 0.05% pancreatin (Sigma) at 37°C. Noncardiomyocytes were eliminated by preplating for 1.5 h at 37°C. Myocytes were plated at a density of 8 x 10^{2} cells/mm^{2} in 100-mm Corning cell culture dishes in Dulbecco's modified Eagle's medium-M199 (4:1) containing 10% (vol/vol) fetal bovine serum (24 h, 37°C) and then incubated in serum-free medium for 48 h before experiments were carried out. All research on rats was done according to procedures and guidelines approved by the Institutional Animal Care and Use Committee of the University of Toledo.
Preparation of Src, Na/K-ATPase, GST-fused Proteins, and His-tagged Proteins

Src, without the first 85 amino acid residues, was purified from sf-9 cells as described previously (Ma et al. 2000) and used in the initial binding assays to ensure that Src binds to the Na/K-ATPase. In the Src phosphorylation experiment, purified recombinant full-length Src from Upstate Biotechnology was used. Na/K-ATPase was purified from pig kidney outer medulla using the Jorgensen method as we previously described (Tian et al. 2006) and the preparations with specific activities between 1200 and 1400 µmol Pi/mg/h were used in this work. GST-fused proteins or His-tagged proteins were expressed in *Escherichia coli* BL21 (Invitrogen) and purified using glutathione beads or ProBond Purification System (Invitrogen). Soluble GST-fused proteins were eluted from the glutathione beads with elution buffer [10 mM reduced glutathione, 0.1% Triton X-100, 50 mM Tris-HCl, (pH8.0)] and then dialyzed in the buffer containing 0.1% Triton X-100, 50 mM Tris-HCl (pH8.0) to remove remnant glutathione.

**In vitro Na/K-ATPase/Src Kinase Domain Binding and GST Pulldown Assay**

*In vitro* binding assay was performed as described previously (Tian et al. 2006). Briefly, to test if native Na/K-ATPase binds His-kinase domain (His-KD), the purified Na/K-ATPase was incubated with purified His-KD in PBS for 30 min at 37°C. The complex was then collected by centrifugation at 100,000 x g for 30 min. Control experiments showed that the Na/K-ATPase-bound, but not the free, His-KD could be copelleted by centrifugation and visualized by
the Colloidal Blue Stain Kit (Invitrogen) after SDS-PAGE analysis. To further confirm this direct interaction, GST pulldown assay were also performed as following: 5 µg GST-fused proteins were conjugated on glutathione beads and incubated with 100ng purified his-Src in 500 µl PBS in the presence of 0.5% Triton X-100 at room temperature for 30 min. The beads were washed with the same buffer four times. The bound his-Src was resolved on 10% SDS-PAGE and detected by Western blot with anti-His antibody.

**Kinase Activity Assay of Src and Lyn**

To determine how Na/K-ATPase constructs or peptides affect Src/Lyn kinase activity, the purified Src (4.5 U) or Lyn (20 ng) was incubated with different amounts of the purified GST-fused Na/K-ATPase constructs or peptides in phosphate-buffer saline (PBS) for 30 min at 37°C. Afterward, 2 mM ATP/Mg\(^{2+}\) was added. The reaction continued for 5 min at 37°C and was stopped by addition of SDS sample buffer. Afterward, the Src pY418 and Lyn pY396 were measured by anti-pY418 antibody to indicate Src/Lyn activation (Ma et al. 2000). For the Src activity assay in PY-17 cells and primary rat neonatal cardiac myocytes, cells were lysed in ice-cold RIPA buffer containing 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 50 mM Tris-HCl (pH 7.4). Cell lysates were cleared by centrifugation at 16,000 x g for 15 min, and the supernatants were separated by SDS-PAGE (60 µg/lane) and transferred to an Optitran membrane and were analyzed with anti-pY418 antibody. The pY418 signal was detected using the enhanced chemiluminescence kit.
(Pierce) and quantified using a Bio-Rad GS-670 imaging densitometer as previously described (Haas et al. 2002).

**PKC kinase activity assay**

Activity of PKC was measured by PepTag phosphorylation assay for non-radioactive detection of PKC (Promega) as described in the product instructions. Briefly, 40 ng PKC were incubated for 30 min at 30°C with the reaction mixture containing 5 μl reaction buffer, 5 μl PepTag C1 (0.4 μg/μl), 5 μl PKC activator solution, 1 μl peptide protection solution and 10 μM staurosporine or peptide. The reaction mixture was then subjected to electrophoresis on a 0.8% agarose gel at 100 V for 20 min. After electrophoresis, negatively-charged phosphorylated PepTag C1 peptides migrated toward the anode (+), while positively-charged non-phosphorylated PepTag C1 peptides migrated toward cathode (-). Quantitative data were presented as the percentage of PKC activity of control group with PepTag C1 peptide.

**Immunoblot analysis**

Following the indicated treatment, the incubation medium was rapidly replaced by ice-cold PBS. The washed cells were then lysed in ice-cold RIPA buffer, and subjected to Western blot analysis with anti phosphor-MAPK antibody as described above. Then the same membrane was stripped and reprobed with anti MAPK antibody to probe for total MAPK.

**Cell Viability Assay**
After confluency reaches 60%, various cells were exposed to peptides with indicated concentration and time. Cells were then trypsinized and the cell suspensions were mixed with trypan blue at room temperature for 5 m. The numbers of viable cells (trypan blue negative) were counted with hemocytometer.

**FRET Analysis by Acceptor Photobleaching in Fixed Cells**

FRET analysis by acceptor photobleaching was performed as described before (Tian et al. 2006). Briefly, Src-ECFP and EYFP-rat α1 plasmids were cotransfected into LLC-PK1 cells. After 24 h, cells grown on glass coverslips were exposed to peptides for 1 h. After washing with PBS solution, cells were fixed with ice-cold methanol for 15 min at -20°C. The mounted coverslip was then used for FRET measurement with the Leica DMIRE2 confocal microscope (Wetzlar, Germany). The laser lines of 456 nm and 515 nm were used to illuminate fluorescence, and the emission intensities were recorded at 465–509 nm for Src-ECFP and 530–570 nm for EYFP-rat α1. The cells that expressed both Src-ECFP and EYFP-rat α1 were chosen to perform the FRET analysis. A membrane region of interest (ROI 1) was selected and photobleached by applying 100% intensity of 515 nm laser. The emission intensities of Src-ECFP and EYFP-rat α1 before and after the photobleaching process in the selected ROI 1 region were used to calculate the FRET efficiency. The FRET efficiency was also calculated at a nonphotobleached region (ROI 2) and used as a control.

**Localization Analysis of permeable NaKtide in Live Cells**
Cells were cultured on 35mm dishes and then subjected to the indicated treatment of FITC-labeled permeable NaKtide at 37 °C. Cells were washed twice with PBS and localization of permeable NaKtide was assessed by directly monitoring FITC fluorescence with a Leica DMIRE2 confocal microscope.

Establishment of Human DU-145 Xenograft Tumors in NOD/SCID Mice

NOD/SCID mice nude mice (NCI) were housed in laminar airflow cabinets under pathogen-free conditions with a 12 h light / 12 h dark schedule and fed autoclaved standard chow and water. Xenografts of DU-145 were initiated by subcutaneous injection of $5 \times 10^6$ DU-145 cells into the left and right flanks of female nude mice at age of 4-6 weeks. Tumor volume was estimated by caliper measurements of the length (L) and width (W) as $V = \frac{L \times W^2}{2}$. Treatment was started after tumors reached an average volume of 100 mm$^3$. Mice were injected subcutaneously near the tumor site with permeable NaKtide in saline at the dose of 10 mg/kg (body weight) every other day for one week. All research on NOD/SCID mice was done according to procedures and guidelines approved by the Institutional Animal Care and Use Committee of University of Toledo Health Science Campus.

Analysis of Data

Data are given as the mean ± SE. Statistical analysis was performed using the Student t test, and significance was accepted at $p < 0.05$. 

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RESULTS

Identification of ND1 as a Src-interacting domain from the Na/K-ATPase α1 subunit

Our previous findings have demonstrated that CD3 of Na/K-ATPase α1 subunit directly interacts with Src Kinase domain and inactivates Src (Tian et al. 2006). As depicted in Fig. 3A, the Na/K-ATPase CD3 consists of both P and N domains. The 3D structure of Na/K-ATPase indicates that the N domain is exposed, whereas the P domain is relatively close to the membrane (Toyoshima et al. 2000; Sweadner et al. 2001; Morth et al. 2007). Thus, it is likely that the N domain interacts with the kinase domain and inhibits Src. To test this hypothesis, we constructed three GST-fusion proteins (GST-PD-N, GST-ND and GSP-PD-C) and determined the binding of these proteins to the purified His-Src. These studies confirmed that the N, but not the P, domain of α1 subunit interacted with Src (Fig. 3B). As depicted in Fig. 3A, the N domain contains over 200 amino acid residues. Based on the published structural data (Hilge et al. 2003; Morth et al. 2007), the N-terminus of the N domain is less structural and may undergo an induced-fit movement. To further map the binding motif in the N domain, we constructed ND2 and ND2 remaining (ND2R) GST fusion proteins as illustrated in Fig. 3A. GST pull-down assay showed that GST-ND2, but not GST-ND2R, bound Src (Fig. 3C). Further structural analysis of the ND2 reveals that the N-terminus of ND2 is highly unstructured (Hilge et al. 2003). Moreover, this domain is also highly exposed and is less important for ATP binding (Imagawa et al. 2003), and thus the catalytic function of the Na/K-ATPase. Finally, it is known that phospholamban
binds to the corresponding flexible domain of SERCA. Thus, it is plausible that this flexible region in Na/K-ATPase α1 could be involved in the interaction with the Src kinase domain. To test this hypothesis, we constructed two more GST-fusion proteins (ND1 and ND1R), and assessed their binding to Src. As depicted in Fig. 3C, we observed that the ND1, but not the ND1R, interacted with Src.

We have shown that in the Na/K-ATPase/Src receptor complex, the CD2 and CD3 domains bind the Src SH2 and kinase domain, respectively. Binding of ouabain to the Na/K-ATPase releases the kinase domain from the CD3, but not the SH2 from the CD2 (Tian et al. 2006), thus keeping the receptor complex intact as a scaffolding protein to recruit other signaling partners. This suggests that the affinity of CD3/kinase domain interaction must be lower than that of CD2/SH2 interaction. Indeed, as shown in Fig. 3D, GST pull-down assay showed GST-CD2 binds Src with a much higher affinity than that of GST-ND1.

**ND1 as a potent Src inhibitor can target to the Na/K-ATPase/Src complex in cultured cells**

To test whether the newly identified ND1 inhibits Src as CD3 did, we incubated Src with 100 ng soluble GST fusion proteins in the test tube and measured Src pY418 levels by Western blot. GST-CD3 was used in the experiment as a positive control. As depicted in Fig. 4A, both GST-ND1 and GST-ND were as effective as the positive control in inhibiting Src activity. Moreover, the inhibitory effect of ND1 on Src was dose-dependent (Fig. 4B) and the IC\textsubscript{50} is about 50 nM. To further test whether ND1 can be used as a
minigene product to inhibit Src in live cells, we measured Src pY418 level changes in LLC-PK1 cells transfected with different YFP expression vectors. We found that expression of YFP-ND1, but not YFP, reduced Src activity in cell lysates as did by YFP-ND and YFP-CD3 (Fig. 4C).

The above studies demonstrate the effectiveness of ND1 as a Src inhibitor in cultured cells. To further test whether ND1 can target the plasma membrane Na/K-ATPase/Src complex, we performed the following three sets of experiments. First, as depicted in Fig. 5A, we observed that YFP-ND1 was expressed as a soluble protein. However, we did detect a pool of YFP-ND1 residing near the plasma membrane. Second, to test whether this pool of YFP-ND1 interacts with Src, we co-transfected LLC-PK1 cells with YFP-ND1 and Src-CFP, and then performed FRET analysis. A significant FRET was detected in the cotransfected cells (13.4±2.4%), which suggested that YFP-ND1 and the plasma membrane Src-CFP were likely to associate. Finally, when cell lysates were immunoprecipitated by an anti-\(\alpha_{1}\) antibody, we found that YFP-ND1 was co-precipitated (Fig. 5B). Thus, we conclude that YFP-ND1 is most likely capable of interacting with the Na/K-ATPase-associated Src.

**Identification of KD1 as the target of ND1**

The N-lobe of Src kinase domain has been implicated to be involved in the regulation of Src kinase activity through intramolecular interactions as well as substrate binding. Also, the two-pair interactions (N-lobe and ND1; SH2 and CD2) were possible when Na/K-ATPase/Src complex was modeled. To test the possibility, we purified GST-KD3 (266L-419Q), which covers the full-
length N-lobe. As depicted in Fig. 6B, GST-KD (266L-519F) as well as GST-
KD3, but not GST, was capable to bind with purified pig kidney Na/K-ATPase.
Since no significant changes were observed in GST-ND1-induced Src
inhibition under ATP concentration ranging from 0.1 to 2 mM, we contended
that GST-KD2 (266L-370A) which lacks the ATP binding site will still bind with
Na/K-ATPase. Indeed, as shown in Fig. 6B, GST-KD2 has similar binding
capability as GST-KD3. It is known that E312 in the αC helix and K297 in the
N-terminus of KD2 form a salt bridge which is essential for Src activation. To
test the hypothesis that ND1 may target the αC helix and then prevent the
formation of the salt bridge between E312 and K297, we constructed GST-
KD1 (266L-319E). As depicted in Fig. 6B, GST-KD1 was capable of
interacting with the Na/K-ATPase. Functionally, addition of KD1, like the full-
length KD (Tian et al. 2006), was effective in blocking GST-ND1-induced Src
inhibition (Fig. 6C). These findings led us to propose that ND1 interacts
directly with the αC helix. To test this, K297M mutation was introduced to
release αC helix from the intramolecular interaction. As expected, GST-KD1
(K297M) significantly increased the capacity of binding Na/K-ATPase (Fig.
6D).

**Peptide derived from the Na/K-ATPase ND1 inhibits Src**

Because the above data suggest that ND1 binds and inhibits Src, it is
tempting to test whether we could develop a peptide Src inhibitor from ND1.
We synthesized four 20 mer peptides that cover the entire ND1 and tested
which one acts like ND1, capable of inhibiting Src (Fig. 7). As depicted in Fig.
8 A and B, 1 μM P3 caused almost a 100% inhibition of Src while P4
produced a partial inhibition. When the dose-response curve was constructed, we observed that P3 was quite potent in inhibiting Src with an IC<sub>50</sub> of 70 nM (Fig. 8B), comparable to that of GST-ND1 (Fig. 4B). On the other hand, P1 showed no effect. P2 peptide, corresponding to a largely disordered, solvent-accessible region similar to the phospholamban binding motif in SERCA, also showed no inhibitory effect on Src. Previously, we showed that SERCA N-domain did not interact with Src (Tian et al. 2006). Consistently, SERCA and the Na/K-ATPase α1 shared less than 30% homology in the P3 region and the SERCA peptide failed to inhibit Src up to 10 μM (data not shown). In short, we have developed a α1-specific peptide Src inhibitor P3. We named this peptide NaKtide (Na/K-ATPase derived peptide). Because P1 showed no effect on Src, this peptide will be used as a negative control.

To test whether NaKtide acts as an ATP analog as a generic Src inhibitor PP2, we measured its effect on Src in the presence of different concentrations of ATP. As shown in Fig. 8C, changes in ATP concentration from 0.1 to 2 mM did not affect NaKtide-induced Src inhibition. This result was consistent with that KD1, target of ND1, does not possess ATP binding sites.

To probe whether NaKtide is relatively specific to Src, we measured the dose-dependent effect of NaKtide on Lyn kinase. As shown in Fig. 8D, NaKtide produced a dose-dependent inhibition of Lyn. However, the IC<sub>50</sub> is about 2.5 μM, 40 times higher than that of Src inhibition.

To test whether NaKtide affects the PKC family of kinases, a PKC family kinase cocktail was exposed to P1 and NaKtide, and then measured for the activity. As shown in Fig. 8E, unlike staurosporine (a non-specific PKC family kinase inhibitor), NaKtide as well as P1 showed no effect up to 10 μM.
Development of permeable NaKtide as a cell-permeable Src inhibitor

To further test whether the identified peptide inhibitor works in live cells, we loaded LLC-PK1 cells with 10 μM NaKtide using a saponin-based protocol as previously described (Johnson et al. 1996). Then cells were fixed with ethanol and immunostained with anti-pY418 antibody. Loading the cells with NaKtide, but not the control peptide P1, decreased the basal Src pY418 fluorescence intensity (data not shown), suggesting NaKtide could inhibit endogenous Src activity in live cells. Even though the saponin-based protocol was useful for us to verify the effect of NaKtide in live LLC-PK1 cells, its disadvantages limited us expanding our studies to varieties of cells, animal tissues or whole animals. Recent studies have demonstrated that the coupling of biological molecules to varieties of positively CPPs can facilitate their uptake into cultured mammalian cells as well as animal tissues (Shokolenko et al. 2005; Mae et al. 2006). Accordingly, we synthesized a couple of permeable NaKtides by tagging NaKtide with several positively charged peptides. As listed in Table 4, pNaKtide is produced by coupling GRKKRRQRRRPPQ to the N-terminus of NaKtide while pNaKtide1 is composed of RQIKIWFQNRRMKWKK and NaKtide. In vitro kinase assay showed that both permeable NaKtides were highly potent Src inhibitors while the control C-P1 (GRKKRRQRRRPPQ-P1) was inactive (Fig. 9A, pNaKtide1 data not shown).

To assess the cell permeability, pNaKtide was labeled with FITC. As depicted in Fig. 9B, confocal imaging analysis of live cells indicated that pNaKtide was cell-permeable. Maximal loading was achieved after 30 to 60
min of incubation with 1 μM pNaKtide in almost every LLC-PK1 cell in culture. Moreover, unlike YFP-ND1, pNaKtide resided mainly in the plasma membrane with some distribution to the intracellular membrane compartments. We also observed its nuclear localization in some fibroblasts (data not shown) and membrane association in neonatal cardiac myocytes (data not shown). Together, these studies further confirmed that pNaKtide was highly permeable to various cells and also displayed different localization patterns which may provide additional cell specificity for pNaKtide.

To test the effectiveness of pNaKtide, both LLC-PK1 and TCN23-19 cells were exposed to 1 μM pNaKtide for different times. Cell lysates were then subjected to Western analysis of active Src and ERKs. The Na/K-ATPase knockdown TCN23-19 cells were derived from LLC-PK1 cells and exhibited higher basal Src and ERK activity (Liang et al. 2006). As depicted in Fig. 10A, pNaKtide caused a significant inhibition of Src and ERK in TCN23-19 cells, indicating that pNaKtide is an effective Src inhibitor. Interestingly, the effect of pNaKtide on Src and ERK in LLC-PK1 cells was much less than those in TCN23-19 cells, suggesting that pNaKtide may selectively target the Na/K-ATPase-interacting pool of Src. This notion is supported by the fact that PP2, a generic Src inhibitor, produced a significant inhibition of Src in LLC-PK1 cells (Table. 5). To be sure that the effect of pNaKtide on ERK is due to its inhibition of Src, we repeated the above experiments in SYF + Src and SYF cells. As shown in Fig. 10B, pNaKtide had no effect on ERK in SYF cells where Src family kinases (Src, Yes, and Fyn) were knocked out. However, pNaKtide reduced ERK activity in SYF + Src cells where Src was rescued.
Permeable NaKtide as a functional ouabain antagonist

Because ouabain stimulates many cellular signaling events by activating the Na/K-ATPase-associated Src, the above findings led us to conduct the following three sets of studies, testing whether permeable NaKtide can act as a functional ouabain antagonist.

First, we performed FRET analysis to determine the effect of pNaKtide on the formation of Na/K-ATPase/Src receptor complex. LLC-PK1 cells were co-transfected with EYFP-α1 and Src-ECFP, and then exposed to different concentrations of pNaKtide. As depicted in Fig. 11A, both EYFP-α1 and Src-ECFP were targeted to the plasma membrane. Moreover, a significant FRET efficiency (11.1 ± 1.9%) was detected as reported before (Tian et al. 2006). Addition of pNaKtide produced a significant reduction in the FRET efficiency as well as the percentage of cells with FRET in a dose-dependent manner (Fig. 11 B and C), indicating that pNaKtide is effective in blocking the formation of a stable Na/K-ATPase/Src complex. Since the maximal effect was observed when the cells were exposed to 1 to 5 μM pNaKtide, 1 μM will be used in the following experiments to test its effectiveness in blocking ouabain-induced signal transduction in cell cultures.

Second, we measured the effect of pNaKtide in ouabain-induced activation of Src and ERK in live cells. As expected, we found that 1 μM pNaKtide completely abolished ouabain-induced ERK activation in LLC-PK1 cells (Fig. 12A). To be sure that this is not a cell-specific effect, we repeated the same experiments in primary cultures of cardiac myocytes. As shown in Fig. 12B, ouabain-induced activation of Src and ERK was also blocked by pNaKtide.
Finally, we compared pNaKtide and PP2. As shown in Table 5, both pNaKtide and PP2 have a similar IC\textsubscript{50} on Src kinase. However, PP2 had more inhibition on basal Src activity than that of pNaKtide in both LLC-PK1 and cardiac myocytes. Moreover, when cardiac myocytes were stimulated by IGF-1, PP2, but not pNaKtide, caused a significant inhibition of ERK activation (Fig. 12C). Taken together, the above findings indicate that pNaKtide can block the formation of Na/K-ATPase/Src receptor complex, thus functions as a ouabain antagonist.

**Downregulation of Na/K-ATPase in cancer cells**

Downregulation of Na/K-ATPase in some tumor tissues has been indicated for a long time (Verna et al. 1983; Yamanaka et al. 1989; Seligson et al. 2008). The decrease in Na/K-ATPase amount correlates with the degree of tumor malignance. However, the mechanism behind this phenomenon is not well characterized. Our previous data have shown that in kidney epithelial cells, the amount of Na/K-ATPase is inversely correlated with the activity of Src as well as Src effectors, including ERK and FAK (Liang et al. 2006). It is plausible that downregulation of Na/K-ATPase in certain cancer cells may be accompanied by the increase of Src activity, which will facilitate the cell growth. To test that, several cancer cells, including prostate cancer cell DU145, breast cancer cell MCF-7, colon cancer cells (DLD-1, HT-29) and neuroblastoma cells, were harvested when density reached 95% and Na/K-ATPase \(\alpha_1\) level in cell lysates were compared with that in LLC-PK1 cell lysate by Western blot. As depicted in Fig. 13A, all cancer cells expressed less Na/K-ATPase \(\alpha_1\) than LLC-PK1 except two colon cancer cells DLD-1 and
HT-29. To test the possible relationship between Na/K-ATPase expression level and cell proliferation rate, LLC-PK1 cells with different densities were analyzed. As shown in Fig. 13B, increase of Na/K-ATPase amount was accompanied by the increase of cell density. Also as expected, Src pY418 level was decreased, which was in line with what was observed before (Liang et al. 2006).

**Effects of ND1 on cancer cells**

Our data demonstrated ND1 as the functional domain in the Na/K-ATPase to inhibit Src in LLC-PK1 cells. Together with the downregulation of Na/K-ATPase and upregulation of Src activity in cancer cells, we hypothesize that introduction of ND1 to cancer cells may inhibit Src activity and lead to cell death. To test that, DU145 cells were transfected with pEYFP-ND1 expression plasmid. After 36 h, significant amount of cells with YFP fluorescence were dead in the YFP-ND1 overexpressing cells, but not in YFP expressing cells (Fig. 14A). Meanwhile, some detached cells or debris with YFP fluorescence were detected in the medium. Further efforts to establish a stable cell line expressing YFP-ND1 also failed because no YFP fluorescence was detected in the remnant live cells after subculture. These observations suggest that overexpression of ND1 could induce cell death through inhibiting Src. We also checked the effect of overexpression of ND1 in MCF-7 cells. As shown in Fig. 14B, MCF-7 cells overexpressing YFP-ND1 were still viable after 48 h of transfection. However, these cells were unable to attach to dishes after subculturing.
pNaKtide induces cell death in various cancer cells

Since NaKtide is identified to be the region involved in Src inhibition and pNaKtide is capable of transducing into LLC-PK1 cells, we chose to address whether pNaKtide has any effect on the growth of DU145 cells. First, as shown in Fig. 15A, FITC-labeled pNaKtide was capable of penetrating the cell membrane within 60 min. However, the number of viable cells decreased over the time, corresponding to the loss of FITC fluorescence. These data suggest that pNaKtide may induce cell death in DU145 cells. Second, consistent with the imaging analysis, cell death was observed in DU145 cells after exposure to pNaKtide in a dose- as well as time-dependent manner (Fig. 15B). However, pNaKtide had no effect on the growth of non-cancerous LLC-PK1 cells and minimal effect on SYF and SYF + Src cells (Fig. 15B).

We further checked the pNaKtide effect on the viability of various cancer cells. All cancer cells were maintained in serum-containing medium and exposed to peptides with indicated concentrations for 24 h. As shown in Fig. 15B, several neuroblastoma cells were highly sensitive to pNaKtide, while two colon cancer cells tested were resistant to pNaKtide.

Involvement of necroptosis in pNaKtide-induced cell death

Among the types of cell death induced by kinase inhibitors, apoptosis is the most common one. The term “apoptosis” first appeared in the literature in 1972, to delineate a mode of cell death within living tissues featuring distinctive morphological changes such as cell shrinkage, membrane blebbing and condensation of chromatin (Kerr et al. 1972). So we first checked whether apoptosis was induced after cells were exposed to pNaKtide. Morphologically,
no cell shrinkage or membrane blebbing was observed, indicating apoptosis is not involved (Fig. 16A) in both DU145 and BE(2)-C cells. A conserved family of caspases is normally activated during apoptosis. Preliminary data showed that pan-caspase inhibitor Z-VAD-FMK failed to block pNaKtide-induced cell death. Bcl-2 has been shown to be able to inhibit apoptosis (Youle et al. 2008). However, overexpression of Bcl-2 in BE(2)-C cells failed to rescue the cell death induced by pNaKtide (data not shown). All these data underscore the notion that pNaKtide may trigger a non-apoptotic and non-caspase-dependent cell death in these cancer cells. This is also in line with the observation that pNaKtide can induce rapid cell death in neuroblastoma BE(2)-C cells which can easily develop resistance to certain apoptosis inducers.

There are many other types of cell death, including PARP1-mediated cell death, autophagic cell death, and necroptosis etc. (Degterev et al. 2008). PARP1 cleavage was not detectable by Western blot using commercial available antibody which can recognize both the full-length (116 kDa) and cleaved (85 kDa and smaller sizes) forms of PARP1 (data not shown). The phase-contrast imaging results indicated pNaKtide may trigger the formation of membrane-enclosed vesicles, which are indicative of autophagy (Kabeya et al. 2000). The occurrence of autophagy in pNaKtide-induced cell death is still under investigation.

Necroptosis is a recently appreciated type of cell death identified by Dr. Yuan’s group, wherer small molecule necrostatin-1 has been shown to inhibit receptor-interacting protein 1 (RIP1) kinase and then specifically block necroptosis (Degterev et al. 2005; Degterev et al. 2008). Several naturally occurred small molecules have been shown to trigger necroptotic cell death
To test whether pNaKtide triggers necroptosis, DU145 cells were exposed to necrostatin-1 for 24 h, and then cell death effect of pNaKtide was evaluated. As shown in Fig. 16B, necrostatin-1 was partially effective in blocking pNaKtide-induced cell death in DU145 cells.

**pNaKtide inhibits DU145 xenograft tumor growth in NOD/SCID mice**

The observation that pNaKtide induces rapid cell death in the DU145 cells leads us to further test its efficacy on the xenograft tumor in NOD/SCID mice. Two groups of 4- to 6-week-old NOD/SCID mice were injected subcutaneously in the flank with 5 x 10^6 DU145 cells. The treatment with pNaKtide (in saline) or vehicle only was initiated after the tumors reached an average volume of about 100 mm^3. Subcutaneous injection of pNaKtide at the dose of 10mg/kg resulted in significant inhibition of DU145 xenograft tumor growth, indicated by decreased averaged tumor volume (Fig. 17 B and C) and actual tumor weight (63.4% reduction, Fig. 17A). Second, treatment of pNaKtide had a modest effect on DU145 tumor incidence (33.3% reduction, Fig. 17A). Meanwhile, there was severe metastasis observed in one mice of the vehicle group, while no metastasis found in pNaKtide treated group. It is important to note that pNaKtide treatment did not affect the whole body weight as well as the levels of cholesterol, or high density lipoprotein (HDL) in blood plasma. There are no significant changes in the enzyme activity of alanine aminotransferase (ALT) or creatinine level in the plasma, indicating no severe hepatic or nephritic toxicity.
To check pNaKtide’s effect on Src activity in the xenografts, we removed the tumors after the treatment and checked Src pY418 phosphorylation in the tumor homogenates. Consistently, as shown in the cultured cells, pNaKtide injection caused significant decrease in Src pY418 phosphorylation (Fig. 17D), suggesting pNaKtide was capable of reaching the tumor sites and inhibiting Src activity.

To further study the morphological changes in the xenograft tumors, we did formalin-fixed, paraffin-embedded sections and then submitted them for histological evaluations. Hematoxylin & Eosin (H&E) staining of DU145 xenograft tumors showed compact cell distribution patterns with dark blue nuclear staining in the vehicle group, suggesting these tumor cells were highly proliferating. Accordingly, there were some cells with prominent nucleoli under stages of mitosis (arrowhead in Fig. 18). H&E staining of xenografts depicted less cell population and nuclear division in pNaKtide-treated tumors. In addition, TUNEL assay with these tumors did not show any positive DNA fragmentation in pNaKtide group, which is in line with the previous observation that apoptosis may not be involved in the cell death observed in the cultured cells.
FIGURE 3. Identification of the Na/K-ATPase motifs involved in the interaction with Src. A, schematic presentations of different GST-fusion proteins. B, C, and D, binding of GST-ND1 to Src. Each experiment was repeated three times. Coomassie blue staining of purified GST-fused proteins was shown in the upper panel and Western blot of bound His-Src was shown in the lower panel.
FIGURE 4. Regulation of Src by ND1. A, GST fusion proteins (100 ng) were incubated with recombinant Src (4.5 U) for 15 min in PBS, then 2 mM ATP/Mg²⁺ was added and incubated for an additional 5 min. After resolving by SDS-PAGE, the membranes were probed with anti-Src pY418 antibody. ** p<0.01. B, dose-dependent inhibition of Src by GST-ND1 in the test tubes. C, effects of ND1 overexpression on Src activity in LLC-PK1 cells. ** p<0.01.
FIGURE 5. Targeting of YFP-ND1 to Na/K-ATPase/Src complex in live cells. A, localization of YFP-ND1 in LLC-PK1 cells. LLC-PK1 cells were transfected with pEYFP-ND1 and localization of YFP-ND1 was detected with a Leica DMIRE2 confocal microscope. Arrow indicated the membrane localization of YFP-ND1. B, Lysates from LLC-PK1 cells with overexpressing YFP proteins were immunoprecipitated with anti-Na/K-ATPase α1 antibody and then analyzed by Western blot using anti-GFP antibody and anti-Na/K-ATPase α1 antibody.
FIGURE 6. KD1 as the target of ND1. A, Schematic presentation of Src kinase domain. B, binding of KD1 to Na/K-ATPase. Pull-down assay was performed with purified pig kidney Na/K-ATPase as described in “Materials and Methods”. Coomassie blue staining of purified GST-fused proteins was shown in the upper panel and Western blot of bound Na/K-ATPase was shown in the lower panel. C, KD1 abolished ND1-induced Src inhibition. 100ng GST-ND1, 500ng GST-KD1 or mixture of both were incubated with Src in the presence of 2mM ATP/Mg\textsuperscript{2+} and then analyzed for Src pY418 level. N=3, ** p<0.01. D, K298M mutation increases the binding capacity of KD1. The purified Na/K-ATPase (1μg) was incubated with 2 μg GST-KD1 or GST-KD1 (K298M) in PBS for 15 min. The pull-down products were analyzed by Western using anti-α1 antibody. N=3.
FIGURE 8. Effect of ND1-derived peptides on Src activity. A, 1μM of each peptide was incubated with recombinant Src (4.5U) for 15min, then 2 mM ATP/Mg$^{2+}$ was added and incubated for another 5 min. Src activity was measured accordingly. Quantitative data were collected as percentage of control and presented as mean ± S.E. of three independent experiments. **, p<0.01. B, dose-dependent inhibition of Src by P3. Curve fit analysis was performed by GraphPad software. C, NaKtide (P3) is not an ATP competitive inhibitor of Src. 0.1 μM NaKtide was incubated with Src, then mixed with different concentrations of ATP/Mg$^{2+}$. D, effects of NaKtide on Lyn kinase activity. E, effects of NaKtide on kinase activity of PKC. N=3, ** p<0.01.
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FIGURE 9. Characterization of pNaKtide. A, dose-dependent inhibition of Src by pNaKtide. Curve fit analysis was performed by GraphPad software. B, pNaKtide loading in LLC-PK1 cells. LLC-PK1 cells were serum-starved for 12 h and then exposed to 1 μM of FITC-pNaKtide at 37°C for 60 min. Cells were washed twice with PBS, and location of pNaKtide was assessed by directly monitoring FITC fluorescence. The scale bar represents 20 μM.
FIGURE 10. Effect of pNaKtide on Src and Src-mediated signaling pathway. A, Cells were serum-starved for 12 h and were exposed to 1μM C-P1 or pNaKtide for 1 h. Cell lysates in RIPA buffer were assayed by Western blot. N=3. * p<0.05; ** p<0.01. B, SYF + Src and SYF cells were cultured in the medium containing 0.5% FBS for 24 h before exposure to 1 μM pNaKtide. Cell lysates were analyzed by Western blot. N=3. * p<0.05.
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<td>Neonatal cardiac myocytes</td>
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GST-ND1 was used *in vitro* for Src activity assay (a). Cells were transfected with either YFP as control or YFP-ND1 for 24 h (b) or pre-treated with 1 μM pNaKtide for 1 h (c) or 1 μM PP2 for 30 min (d). Src pY418 in lysates was analyzed and data are means ± SE, % of control, N=3 to 5.

LLC-PK1 cells were transfected with EYFP-rat α1 (yellow) and Src-ECFP (cyan). Cells were then fixed and FRET analysis was performed. The boxed area (ROI 1) was photobleached and analyzed for FRET. We also measure area (ROI 2) that was not photobleached (A). The same FRET analysis was performed in transfected LLC-PK1 cells pretreated with 1 μM C-P1 or different concentrations of pNaKtide for 1 h. Average FRET efficiency (B) and percentage of cells showing FRET efficiency (C, cut-off value is 4.0%) were calculated. At least 20 cells from three experiments were measured for each.

\[
FRET_{eff} = \frac{D_{post} - D_{pre}}{D_{pre}} \quad \text{for all} \quad D_{post} > D_{pre}
\]

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FIGURE 12. Effect of pNaKtide on ouabain-induced signal transduction. LLC-PK1 (A) and primary cultured cardiac myocytes (B) were pre-incubated with 1 μM peptides for 1 h and then exposed to 100 nM (LLC-PK1) or 100μM (myocytes) ouabain. Cell lysates were analyzed by Western blot. ** p<0.01. C, primary cultured cardiac myocytes were pre-incubated with 1 μM peptides for 1 h or PP2 for 30 min, and then exposed to 20ng/ml IGF-1 for 5 min. N=3. ** p<0.01.
FIGURE 13. Changes of Na/K-ATPase α1 amount in cancer cells as well as non-cancerous cells with different cell densities. A, Cells were harvested at 95% density and lysates were analyzed by Western blot with anti-Na/K-ATPase α1 antibody. B, Lysates of LLC-PK1 cells under indicated cell densities were analyzed with anti-Na/K-ATPase α1, pY418 and Src antibodies. N=3. ** P<0.01.
FIGURE 14. Effects of overexpressing ND1 on cell viability in cancer cells. DU145 cells (A) or MCF-7 cells (B) were transiently transfected with plasmid constructs expressing YFP or YFP-ND1 using LipofectAMINE 2000. Fluorescence and phase-contrast images were collected at indicated time after transfection and then merged with SPOT Version 4.6 software (Diagnostic Instruments). Original magnifications, × 400. The same experiments were repeated four times.
FIGURE 15. Effects of pNaKtide on cell viability in various cancer cells. A, loading of pNaKtide in DU145 cells. FITC-labeled pNaKtide was incubated with DU145 cells for the indicated times, and then images were collected as described in Fig. 9B. The scale bar represents 20 μM. B, effects of pNaKtide on cell viability in various cells. Cells were incubated to pNaKtide for 24 h, and then cell numbers were counted. N=4. * p<0.05; ** p<0.01.
FIGURE 16. Mechanism of pNaKtide-induced cell death in cancer cells. A, morphologic changes induced by pNaKtide. DU145 or BE(2)-C cells were exposed to 10 μM CPP-conjugated peptides for 6 h, then phase-contrast images were collected. Original magnifications, ×900. B, necrostatin-1 partially rescued pNaKtide-induced DU145 cell death. DU145 cells were exposed to necrostatin-1 for 24 h, and then pNaKtide was loaded for 24 h. Viable cells were counted as described in Fig 15B. N=6
**TABLE 1**

<table>
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<th>Administration</th>
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<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>pNaKtide</td>
<td>4/6</td>
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**FIGURE 17. Effects of pNaKtide on the growth of DU145 xenograft tumors.**

5 × 10^6 DU145 prostate cells were injected subcutaneously in the flank of NOD/SCID mice. Tumor volume was estimated by caliper measurements of the length (L) and width (W) as V=(L × W^2)/2. After tumor volume reaches 100mm^3, mice were treated by injecting 10mg/kg pNaKtide formulated in saline. A, tumorigenicity of DU145 cells in NOD/SCID mice. Tumors were removed and weighted after 44 days. N=6. *, P<0.05. **, P<0.01. B, mice bearing xenograft tumors. Arrowheads identify the location of the tumors. C, growth of DU145 xenograft tumors in NOD/SCID mice treated with saline or pNaKtide. D, inhibition of Src activity in pNaKtide treated xenograft tumors. **, P<0.01.
DISCUSSION

The recent appreciation of the interaction between Na/K-ATPase and Src has greatly expanded our understanding of the ouabain-activated signaling relayed by Na/K-ATPase. On one hand, through the association with Src in the caveolae on the plasma membrane, Na/K-ATPase converts the ouabain binding to activation of Src and then further amplifies the signaling to multiple down-stream pathways through protein phosphorylation (Wang et al. 2004; Tian et al. 2006). On the other hand, through interaction with multiple proteins, i.e., IP3R, PLC-γ, Na/K-ATPase forms a signalosome which has been demonstrated to participate in the regulation of intracellular Ca^{2+} (Yuan et al. 2005; Chen et al. 2008). Most importantly, it has been demonstrated that the direct interaction between Src and Na/K-ATPase is the key component for the formation of ouabain-regulable Na/K-ATPase signalosome (Tian et al. 2006).

Regarding to the significance of Na/K-ATPase-mediated signaling, it is our top priority to further address the molecular mechanism how Na/K-ATPase interacts and regulates Src. So herein, we present evidence to show NaKtide, a peptide of 20 amino acids derived from the N domain of Na/K-ATPase α1 subunit, not only binds but also inhibits Src. We also have developed a permeable NaKtide to efficiently transduce NaKtide across the cell membranes. This procedure has made it possible for us to demonstrate that permeable NaKtide inhibits basal Src and then regulates the basal ERK and FAK activity as well in live cells. More importantly, permeable NaKtide attenuates the formation of Na/K-ATPase/Src complex and further abrogates
ouabain-induced activation of Src and ERKs in neonatal cardiac myocytes. Also the regulation of multiple kinases activities by permeable NaKtide leads us to further check its effect on the viability of various cancer cells.

The formation of Na/K-ATPase/Src receptor complex

The identification of KD1 in the kinase domain of Src and NaKtide region in N domain of Na/K-ATPase as contacting sites leads us to propose a tentative working model of the Na/K-ATPase/Src receptor complex. As depicted in Fig. 19, we believe that both CD2/SH2 and N domain/kinase domain interactions could simultaneously occur because our in vitro studies showed that ouabain did not reduce binding of Src to the Na/K-ATPase (Tian et al. 2006). Second, based on crystal structures of Src, Na/K-ATPase, and SERCA, we believe that formation of the Na/K-ATPase/Src receptor complex would occur (or stabilize the Na/K-ATPase) in an E1-like conformation. We further suggest that the Src kinase domain would dissociate from the Na/K-ATPase in an E2-like conformation due to rotation of the A domain and the inward movement of the N domain. Third, we found that the CD2/SH2 interaction had a higher affinity than that of the N domain/kinase domain interaction (Fig. 3D). Therefore, we contend that the CD2/SH2 interaction would facilitate the interaction between the N domain and the kinase domain, thus helping the formation of the Na/K-ATPase/Src receptor complex. Moreover, when ouabain turned the Na/K-ATPase into an E2-like conformation (Carilli et al. 1982; Suzuki et al. 1985), rotation of the A domain (i.e., CD2) would work in concert with the movement of the N domain,
resulting in the release of the Src kinase domain and thus the activation of Src. Needless to say, this proposal has to be experimentally tested.

Moreover, as mentioned before, Na/K-ATPase functions as a scaffold protein or signal integrator by recruiting Src as well as multiple protein partners, including IP3R, PI3K, caveolin-1, ankyrin and AP-1. It is important to further address the issue how the interaction between Na/K-ATPase and Src affects and regulates the formation and function of the Na/K-ATPase signalosome.
FIGURE 19. Modeling of Na/K-ATPase/Src interaction. Modeling of Na/K-ATPase E1- and E2-like structures was based on SERCA1a structure files 1SU4 and 1IWO as well as the newly published Na/K-ATPase structure, and then generated using SPDBView V3.7 program. The A domain (N-terminus and CD2) in Na/K-ATPase was labeled in blue, P domain in green, N domain in black. The SH2 domain of Src was labeled in orange, kinase domain in light blue. The N-terminus of Src kinase domain and NaKtide region in the ND1 were labeled in pink and red respectively.
Development of pNaKtide as an effective ouabain antagonist

Previously we have shown that the Na/K-ATPase α1 subunit forms functional receptor complex through direct interaction with Src (Tian et al. 2006). The interaction between the CD3 domain of Na/K-ATPase and the kinase domain of Src keeps Src in an inactive state, while ouabain binding to Na/K-ATPase will release the kinase domain and then activate Src. Here we confirm the interaction and further map the contacting sites. Specifically, GST pull-down assay showed that the N-terminus of N domain in the CD3 is sufficient to bind and inhibit Src in the test tube as well as in live cells. With reference to the available 3D structure of Na/K-ATPase N domain (Hilge et al. 2003; Morth et al. 2007), ND1 contains a flexible and less structured loop region (P2 in Fig. 7) which may be involved in interactions with other proteins. To our surprise, this polypeptide does not inhibit Src in vitro (Fig. 8A). Instead, NaKtide has the Na/K-ATPase’s ability to inhibit Src. It is important to note that we cannot exclude the possibility that other binding motifs in ND1 may exist. Also the detailed mechanism by which NaKtide and Na/K-ATPase inhibit Src is still unknown. Systematic mutation analysis or co-crystallization of Na/K-ATPase/Src complex may address this issue eventually.

NaKtide peptide itself is a relatively hydrophilic macromolecule and therefore hard to penetrate through the lipid bilayer membrane. Theoretically, this problem could be resolved by increasing the permeability of cell membrane or peptide. Increasing the permeability of cell membrane with saponin has its own disadvantages which limit us to expand our research to tissues or whole animals. Interestingly, the coupling of biological molecules to varieties of CPPs has been shown to facilitate their uptake into cultured
mammalian cells as well as animal tissues through some pathways still under investigation (Shokolenko et al. 2005; Mae et al. 2006). For example, conjugating of δV1-1 peptide with TAT, a peptide derived from the Tat human immunodeficiency virus, was proved to be able to inhibit δPKC and prevent microcerebrovascular dysfunction after acute ischemia (Bright et al. 2007). Also, coupling of the caveolin-1 scaffolding domain to penetratin peptide was effective in delivery of this peptide to the lung, and the same peptide had been used in the isolated heart preparations (Young et al. 2001; Jasmin et al. 2006). Herein, we first showed that CPP conjugated NaKtide resided in the plasma membrane as well as intracellular compartments in various cells (Fig. 9B and 15A). Secondly, permeable NaKtide was still able to inhibit Src in the test tubes as well as in various cells (Fig. 8A, 9, 10 and Table 5). This is consistent with the observation that expression of either pumping-null Na/K-ATPase α1 (D371E) or ND1 (Fig. 4C) was sufficient to reduce cellular Src activity (Liang et al. 2006). More importantly, basal ERK activity was also inhibited after exposure to pNaKtide in various cells expressing Src, but not in SYF cells where Src, Yes and Fyn are knocked out (Fig. 10B). Clearly, pNaKtide is able to regulate Src effector via inhibiting Src.

The NaKtide sequence is highly conserved among mammalian α1 subunits. However, it is not known whether other isoforms act like α1. Sequence comparison shows 70 ~ 85% identity among different isoforms in this region. Interestingly, a recent study indicates that α3 and α4, but not α2, function like α1 in mediating ouabain-induced ERK activation in SF-9 cells (Pierre et al. 2007). Thus, it is important to further study the effectiveness of NaKtide sequences of other isoforms in interacting with Src. Moreover, at
least nine Src family members have been identified and divided into two groups: tyrosine kinases with a broad expression range (Src, Fyn, Yes) and those with limited expression mainly in hematopoietic cells (Fgr, Lyn, Hck, Lck, Blk, Yrc and Yrk) (Thomas et al. 1997). We have shown NaKtide exerted inhibitory effect on Lyn autophosphorylation with less potency (Fig. 8D). It will be of interest to test whether NaKtide could affect other SFKs. The results from above-mentioned experiments may help us to further address the structural basis required for the NaKtide-induced kinase inhibition.

We and others have shown that ouabain activates Src in cell cultures and in vivo (Haas et al. 2000; Ferrandi et al. 2004; Kotova et al. 2006). This activation leads to stimulation of several protein kinase cascades. Consistently, knock out of Src or addition of Src kinase inhibitor PP2 blocks many ouabain-activated signaling pathways. Therefore, it is not surprising that pNaKtide is effective in blocking ouabain-induced ERK activation in both LLC-PK1 cells and cardiac myocytes (Fig. 12). However, it is important to note the following unique properties of pNaKtide. First, unlike PP2, pNaKtide does not compete with ATP (Fig. 8C). Secondly, pNaKtide targets to the plasma membrane mainly in both LLC-PK1 cells and cardiac myocytes (Fig. 9B). Thirdly, pNaKtide disrupts the formation of Na/K-ATPase/Src complex, thus functions as a relatively specific ouabain antagonist. To this end, we found that pNaKtide appeared to target the Na/K-ATPase-interacting pool of Src. Moreover, unlike PP2, it has less effect on basal Src activity and it does not affect IGF-induced ERK activation in cardiac myocytes (Fig. 10A and 12C). Finally, it is also important to point out that pNaKtide could be further modified so that it can be targeted to different intracellular compartments. In short, we
have identified and developed an effective ouabain antagonist that is potent in abolishing ouabain-induced signal transduction in cultured cells. It remains to be tested whether this peptide is effective in vivo. Moreover, additional experiments are needed to assess its effect on ouabain-induced stimulation of other pathways including the PI3K/Akt and PKC/Ca^{2+} pathways.

Our previous studies have demonstrated that ouabain-induced transcriptional regulation of several cardiac growth-related genes and activation of multiple signaling pathways contributed to the hypertrophic growth of myocytes (Peng et al. 1996; Mohammadi et al. 2003). More recently, studies have shown that PP2, a Src inhibitor, can block ouabain-induced hypertrophy of neonatal cardiac myocytes (Liu et al. 2007). Interestingly, whole animal studies clearly demonstrated that subnanomolar concentrations of ouabain activate Src-dependent signaling pathway and induce cardiac hypertrophy in addition to hypertension (Ferrandi et al. 2004). Since our data clearly showed that permeable NaKtide can abrogate ouabain-induced mitogenic signaling pathway (Fig. 12), it will be interesting to test whether our findings reported here may be relevant to the prevention of ouabain-induced hypertrophy in the heart.
Can Na/K-ATPase Function as a Tumor Suppressor?

Na/K-ATPase has long been viewed as a house-keeping protein because of its essential role in maintaining the ionic gradient across the cell membrane. Of ~1 million Na/K-ATPase molecules in most mammalian cells, less than half are performing the ion pumping which consumes up to 30 to 40% of the cells’ energy production. Interestingly, downregulation of Na/K-ATPase α1 in some tumor tissues has been known for a long time (Verna et al. 1983; Yamanaka et al. 1989; Seligson et al. 2008). Therefore, Na/K-ATPase α1 expression patterns may be a useful prognosticator for certain cancers (Seligson et al. 2008).

With the recent appreciation of the Na/K-ATPase/Src receptor complex, we estimated that ~ 25% of membrane Na/K-ATPase α1 has the potential to interact with Src, which may contribute to the regulation of Src activity (Tian et al. 2006; Liang et al. 2007). In many cancer cells where Na/K-ATPase α1 is downregulated, Src activities as well as proliferation rates are higher than those in non-cancerous cells, suggesting Na/K-ATPase may be directly involved in the regulation of Src and cell growth. This is consistent with what we have reported here. When LLC-PK1 cells with different densities were assessed for Na/K-ATPase and Src activity, Na/K-ATPase α1 amount increased and Src activity decreased with an increase in cell density, while Src pY418 levels decreased (Fig. 13). Interestingly, it has been reported that Na/K-ATPase β1 subunit plays an important role in regulating cell motility through the physical interaction with annexin II (Barwe et al. 2005). Moreover, a recent study showed that reduction of β1 expression caused an activation of ERK1/2 (Inge et al. 2008). Although the role of α1 in these regulations has not
been rigorously tested, it is clear that the Na/K-ATPase could be a functional tumor suppressor.

Permeable NaKtide as a Potential Therapeutic for Cancers

Since the discovery of v-Src, the molecule responsible for the cell-transforming ability of the Rous sarcoma virus (Brugge et al. 1977), extensive studies have revealed the abnormal expressing of SFKs in many human cancers, i.e. lung, breast, prostate, colon, ovarian, gastric, pancreatic, brain, neck, bladder cancer and leukemia etc. (Summy et al. 2003). Moreover, malignant activation of SFKs may lead to at least four of the six hallmarks of cancer and the increases in their activities are proportional to the progressive stages of cancers (Hanahan et al. 2000; Summy et al. 2003). Mechanistically, SFKs relay extracellular stimuli signals through the ubiquitous MAPK signaling pathways. The MAPK signaling pathway regulates cellular proliferation, survival, differentiation, adhesion and motility by relaying extracellular stimuli (i.e., growth factors, hormones, neurotransmitters etc.) signals to multiple down-stream cytosolic and nuclear effectors (Seger et al. 1995; McKay et al. 2007). Besides of SFKs and MAPK kinases, FAK, as another Src effector, has also been implicated in many biological processes including cell motility, invasion, metastasis and angiogenesis (McLean et al. 2005). FAK is also able to enter the nucleus and bind p53 through its FERM (FAK N-terminal band 4.1/ezrin/radixin/moesin homology) domain, thus promotes cell proliferation and survival (Lim et al. 2008). It is not surprising that FAK kinase inhibitors, including PF-562271 and TAE226, have anti-tumor activity (Halder et al. 2007; Roberts et al. 2008).
As discussed above, there is a clear correlation between $\alpha_1$ amount, Src activity and cell proliferation in cancer cells. These findings suggest the possibility of using exogenous $\alpha_1$ or $\alpha_1$ equivalent as an anti-cancer agent. Since ND1 has been identified to be sufficient to bind and inhibit Src as the full-length $\alpha_1$ does, we tested whether expression of ND1 may lead to cell death. In both MCF-7 and DU145 cancer cells, Na/K-ATPase $\alpha_1$ subunit expression level is lower and Src activity is higher than that in LLC-PK1 cells. We found that overexpression of YFP-ND1 reduced cell growth in DU145 cells (Fig. 14A). Essentially, the same observation was made in breast cancer MCF-7 cells (Fig. 14B). Moreover, expression of YFP-ND1 inhibited the ability of MCF-7 cells to attach to culture dishes. Taken together, our new findings indicate that supplement of ND1 to cancer cells could be an effective mean to inhibit the growth of these cells.

With the cell permeable NaKtide peptide derived from ND1, we were able to evaluate its effect on the viability of various cancer cells. First, consistent with the cell death induced by ND1 overexpression in DU145 cells, permeable NaKtide also caused significant cell death at the concentration of 10 ~ 20 $\mu$M. This cell death is also observed in many types of cancer cells, including breast cancer cells and neuroblastoma cells, but not in colon cancer cells (HT-29 and DLD-1), LLC-PK1 or TCN23-19 cells (Fig. 15B). The differences of sensitivity between these cancer cells may be related to the SFKs activity as well as the Na/K-ATPase expression level.

Evidence suggests that apoptosis is not involved in permeable NaKtide-induced rapid cell death in DU145 cells nor in BE(2)-C cells. Distinct from apoptosis, necroptosis has been a recently identified cell death, which
does not involve key apoptosis regulators. Morphologically, necroptotic cells are similar to necrotic cells, such as loss of plasma membrane integrity, lack of nuclear fragmentation etc. Notably, small molecule necrostatin-1 has been shown to inhibit RIP1 kinase and then specifically block necroptosis (Degterev et al. 2005; Degterev et al. 2008). We show that necrostatin-1 can partially rescue permeable NaKtide-induced DU145 cell death (Fig. 16B), which suggests that permeable NaKtide may trigger mixed types of cell death.

Although permeable NaKtide inhibits Src and causes cell death in multiple prostate cancer cells in the cell-based systems, the anticancer effect has to be verified in animals, because the process of tumor development is complicated and involves both the tumor cells and the host environment. NOD/SCID mice lacking both B- and T-cell-mediated immune responses have been widely used as a preclinical system to investigate whether candidate compounds have a major role in the control of cancer xenograft growth in vivo. Administration of permeable NaKtide significantly inhibits the growth of the xenograft tumors and decreases the tumor incidence (Fig. 17). Further histochemical analysis of xenografts suggests permeable NaKtide may inhibit tumor cell proliferation (Fig. 18). Meanwhile, administration of permeable NaKtide does not cause significant changes in whole body weight, as well as plasma cholesterol and lipoprotein profiles. No dramatic liver or kidney toxicity was observed in permeable NaKtide treated NOD/SCID mice. These results suggest the clinical potential of the permeable NaKtide as an anti-prostate cancer agent. Obviously, chronic toxicity studies have yet to be performed to further evaluate the safety of permeable NaKtide. Considering the essential role of SFKs in the immune system, additional studies are needed to identify
more subtle effects of permeable NaKtide in immunocompetent animals before human studies are contemplated.

Overall, this study suggests that Na/K-ATPase-derived permeable NaKtide may serve as a potential therapeutic intervention for many types of cancers. Moreover, our findings point out the possibility of targeting Na/K-ATPase for developing novel therapeutics against cancer. Specifically, we contend that induction of $\alpha_1$ expression may be sufficient to control tumor cell growth.
CONCLUSIONS

1. Development of permeable NaKtide as a potential ouabain antagonist.
   - The ND1 region in the N domain of Na/K-ATPase is sufficient to function as a Src binding and regulatory domain.
   - 20-mer polypeptide NaKtide derived from the ND1 inhibits Src \textit{in vitro} as well as \textit{in vivo}.
   - NaKtide specifically inhibits Src family kinases with no apparent effect on PKC.
   - Conjugating of CPPs makes NaKtide highly permeable to various cells. Permeable NaKtide displays different distribution patterns in a cell-specific manner.
   - Permeable NaKtide disrupts the formation of functional Na/K-ATPase/Src receptor complex. Consequently, ouabain-provoked activation of Src as well as ERK kinase is significantly blocked by permeable NaKtide.
   - Permeable NaKtide has no apparent effect on IGF-1-induced ERK activation in cardiac myocytes. Thus, permeable NaKtide could be relatively more specific to the Na/K-ATPase/Src receptor complex than general Src inhibitor PP2.

2. Potential function of permeable NaKtide as an anticancer agent.
   - Reduction of Na/K-ATPase $\alpha_1$ is accompanied with increase of Src pY418 in many cancer cells.
   - Transient transfection of YFP-ND1 may induce cell death in DU145 prostate cancer cells.
• Permeable NaKtide induces rapid cell death in various cancer cells, including prostate cancer cells (DU145, LNCaP), neuroblastoma cells (BE(2)-C, SK-N-DZ, IMR32, SMS-SAN), breast cancer cells (BT-20, MCF-7), but not LLC-PK1 cell, MEFs (SYF, SYF + Src), colon cancer cells (DLD-1, HT-29).

• Permeable NaKtide may trigger non-apoptotic and non-caspase dependent cell death.

• Permeable NaKtide-induced rapid cell death in DU145 cell is partially rescued by necorstatin-1, a specific inhibitor for necroptosis.

• Permeable NaKtide significantly inhibits the growth of human DU145 xenograft tumor in NOD/SCID mice.


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ABSTRACT

The Na/K-ATPase interacts with Src to form a functional receptor complex. Bind of ouabain to this receptor complex stimulates Src and subsequently activates multiple protein kinase cascades. To understand the molecular mechanism of Na/K-ATPase/Src interaction, we have further mapped the contacting sites between these two proteins. These studies have produced the following new findings. First, GST pulldown assays identified the N-termini of the α1 N-domain (ND1) and the Src kinase domain (KD1) as the contacting sites that are responsible for Src inhibition. Second, while ND1 works as the Na/K-ATPase and inhibits Src, the KD1 is capable of blocking Na/K-ATPase- or ND1-induced Src inhibition. In addition, expression of YFP-ND1 targets the Na/K-ATPase/Src receptor complex, suggesting that ND1 may function as an ouabain antagonist. Third, further mapping analyses lead to the identification and development of 20 amino acid NaKtide as a potent Src inhibitor. Moreover, coupling of NaKtide with a cell-penetrating peptide GRKKRRQRRRPPQ makes NaKtide cell permeable and allows targeting of this conjugated peptide (pNaKtide) to the plasma membrane. Functionally, pNaKtide is a potent Src inhibitor with an IC_{50} of 5 nM. Significantly, it has no effect on PKC family of kinases and inhibits Lyn kinase, a closely related Src family kinase, with a much higher IC_{50}. When applied to cell cultures, pNaKtide disrupts the formation of Na/K-ATPase/Src receptor complex and blocks ouabain-induced activation of ERKs in both LLC-PK1 cells and cardiac myocytes. In comparison to PP2, a widely used Src inhibitor, the effects of pNaKtide appear to be more specific to the Na/K-ATPase-interacting Src. While PP2 inhibits IGF-induced Src activation, pNaKtide shows no effect.
Taken together, pNaKtide represents a novel ouabain antagonist and can be utilized for probing the physiological function of receptor Na/K-ATPase/Src complex in isolated organs or in vivo.

Because Src and Src family kinases are highly expressed in many cancer cells, we have determined whether downregulation of Na/K-ATPase contributes to the elevated Src activity and thus proliferation of these cells. It appears that Na/K-ATPase expression is low in some prostate (DU145) and breast (MCF-7) cancer cells that exhibit high Src activity. Moreover, expression of YFP-ND1 is sufficient to inhibit Src activity in these cells. Significantly, expression of this mini-gene reduces cell growth in DU145 cells. It also inhibits the cell attachment. These findings suggest that the Na/K-ATPase may be a tumor suppressor by regulating the Src activity.

Consistently, addition of pNaKtide to cancer cells that express less Na/K-ATPase has proven to be effective in inhibiting cell growth and attachment. Furthermore, in vivo studies have demonstrated the effectiveness of pNaKtide in blocking the formation and growth of xenografted prostate tumors in NOD/SCID mice. Taken together, these findings suggest pNaKtide as a potential therapeutics against tumors that the Na/K-ATPase expression is decreased.