A Src SH2 selective binding compound inhibits osteoclast-mediated resorption

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Background: The observations that $Src^{-/-}$ mice develop osteopetrosis and Src family tyrosine kinase inhibitors decrease osteoclast-mediated resorption of bone have implicated Src in the regulation of osteoclast-resorptive activity. We have designed and synthesized a compound, AP22161, that binds selectively to the Src SH2 domain and demonstrated that it inhibits Src-dependent cellular activity and inhibits osteoclast-mediated resorption.

Results: AP22161 was designed to bind selectively to the Src SH2 domain by targeting a cysteine residue within the highly conserved phosphotyrosine-binding pocket. AP22161 was tested *in vitro* for binding to SH2 domains and was found to bind selectively and with high affinity to the Src SH2 domain. AP22161 was further tested in mechanism-based cellular assays and found to block Src SH2 binding to peptide ligands, inhibit Src-dependent cellular activity and diminish osteoclast resorptive activity.

Conclusions: These results indicate that a compound that selectively inhibits Src SH2 binding can be used to inhibit osteoclast resorption. Furthermore, AP22161 has the potential to be further developed for treating osteoporosis.

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Introduction

The Src family of tyrosine kinases (Src, Fyn, Yes, Yrk, Lyn, Hck, Fgr, Blk, Lck, Frk/Rak and Iyk/Bsk) plays an important role in regulating intracellular signaling events. Their physiological importance is relative to the cell type in which they reside, their level of expression, and the upstream signaling events that lead to their activation (for reviews see [1,2]). The Src tyrosine kinases contain several conserved domains including a unique myristoylated amino-terminal domain, an SH2 domain, an SH3 domain, a catalytic kinase domain and a negative regulatory carboxyl tail [1–3]. Although the catalytic kinase domain is important for autophosphorylation and downstream phosphorylation of protein substrates, the SH2 domain has been postulated to play an important role in regulating intracellular signaling events by acting as an adaptor that brings specific protein substrates into the signaling complex and as a determinant of Src tyrosine kinase subcellular localization [4,5]. The physiological function(s) of many Src tyrosine kinases have been elucidated from mice in which a specific tyrosine kinase gene has been disrupted or eliminated using gene targeting technology [6]. These studies have provided information regarding the potential effect(s) of compounds that block the activity of a particular tyrosine kinase protein. Studies with $Src^{-/-}$ mice, in which the major phenotype is osteopetrosis (overly thick bones), have indicated that Src plays an important function in regulating osteoclast-mediated resorption of bone [7–9]. Src is normally expressed at high levels as a membrane-associated protein in osteoclasts [10,11], and $Src^{-/-}$ osteoclasts do not form an organized actin cytoskeleton or a membrane-associated ruffled border, and do not produce the lacunae that are necessary for proper resorption of bone [7–9,12]. The defect in bone homeostasis in $Src^{-/-}$ mice therefore appears to be linked to the regulation of osteoclast adhesion and subsequent signaling events that are integrated into the resorption process. A compound that blocks specifically the function of Src should inhibit osteoclast-mediated resorption of bone and have a potential role in the treatment of osteoporosis. Src family kinase inhibitors, both *in vitro* and *in vivo*, have been found to inhibit osteoclast-mediated resorption of bone [13–16]. Because these compounds are not specific for the Src kinase domain (they also inhibit other Src kinase family members), studies with these compounds do not demonstrate that the effects are the result of inhibiting any one Src family tyrosine kinase protein.

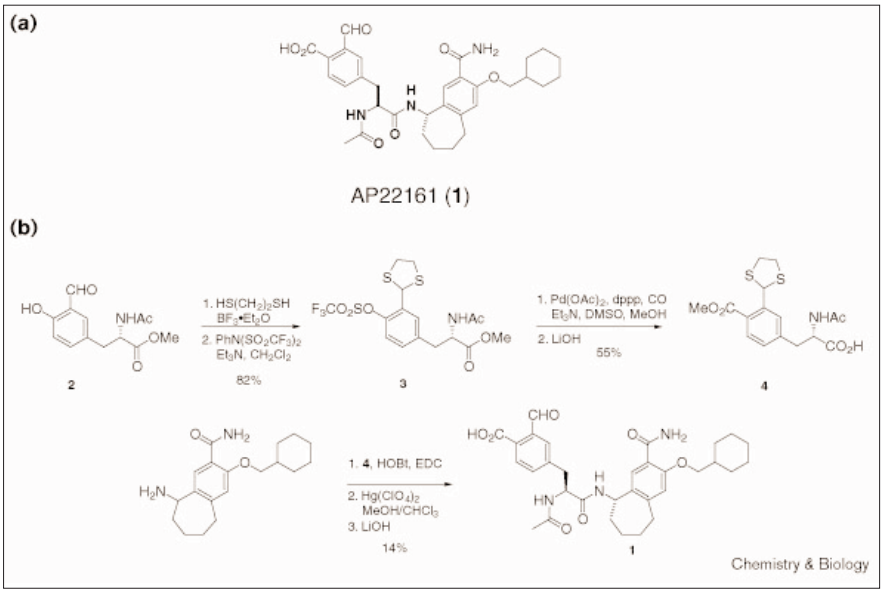
A major challenge in developing small-molecule inhibitors specific for the Src tyrosine kinase is designing a compound that selectively binds to one member of the family. This is particularly true when targeting the SH2 domain, which is highly conserved within this family of proteins [17–19]. In a separate report we will describe bone binding as a means to target selectively Src family SH2 inhibitors to osteoclasts (S.M.V., *et al.*, unpublished observations). In this paper we describe using a biochemical approach to target the Src SH2 domain. Unique to Src among this family of protein tyrosine kinases is the presence of a cysteine residue (Cys188) in the phosphotyrosine-binding pocket of its SH2 domain. We and others [20–22] have investigated the importance of this cysteine residue in the design of pTyr mimics. Modeling predicted the thiol moiety of the cysteine residue of Src SH2 might impart biochemical selectivity. Src is the only member of the family to contain this residue (all others possess serine or threonine). Precedent for this type of interaction comes from the previously reported design and synthesis of reversible, high-affinity ligands for cysteine proteases in which the active site contains a reactive thiol [23–26]. Although it is unlikely that the thiol of Cys188 is fully ionized, the concentration of positively charged residues in the pocket is likely to decrease the p*K*a and increase its reactivity. In support of this, it has been determined that the p*K*a of His57 and pTyr, in a PLC-γ_1 SH2 domain–pY1021 complex, are significantly reduced because of the proximity of these groups to the highly charged pTyr binding pocket [27]. The cysteine residue in the Src SH2 domain may therefore be more reactive than cysteine residues in general and in the context of the SH2-binding pocket provides a potential site for designing a compound that binds with selectivity. As an important note, an elegant biochemical approach has been previously described for the design and synthesis of selective and potent inhibitors of the Grb2 SH2 domain [28]. Here we describe the synthesis of a cysteine-targeted compound that selectively binds to the Src SH2 domain with nanomolar binding affinity. We also demonstrate that this compound blocks Src SH2 binding to peptide ligands in cells, decreases Src-dependent cellular activity and inhibits osteoclast-mediated resorption.

Results

Inhibition of Src SH2-ligand binding

AP22161 was designed and synthesized as a compound that would selectively bind to the Src SH2 domain and inhibit binding to its peptide ligand (Figure 1). AP22161 was designed to target binding to the cysteine residue in the phosphotyrosine-binding pocket of the Src SH2 domain. A fluorescence-polarization-based competitive binding assay was utilized to determine the IC_{50} of AP22161 and control peptides binding to the Src SH2 domain, the Yes SH2 domain and the tandem ZAP SH2 domains [29]. The binding assay was therefore used to monitor both relative binding affinity and selectivity of binding. AP22161 was assayed for its ability to inhibit Src, Yes and ZAP SH2 domains binding to fluorescein-conjugated peptide ligands. The Yes SH2 domain (Figure 2) was chosen from the Src family of tyrosine kinase proteins for comparison with the Src SH2 domain because it is the most structurally related to the Src SH2 domain [18,30]. The ZAP tyrosine kinase protein was included in the binding assay because it contains structurally distant SH2 domains and confirms binding specificity. pYEEI, a known Src SH2

Figure 1



Chemical synthesis scheme and structure of AP22161. **(a)** Chemical structure of AP22161. **(b)** Synthesis scheme.

Figure 2

Sequence comparison of human Src, Yes and ZAP SH2 domains. Residues that correspond to Cys188 in the Src SH2 domain are bold and underlined.

ligand [1,31], which served as a positive control for comparison with AP22161, inhibited Src SH2 binding to the fluorescein-conjugated ligand with an IC_{50} of 5.5 μM (Table 1). The native ZAP SH2 domain ligand, ζ-1-ITAM (NQLpYNELNLGRREEpYDVLD, using single-letter amino-acid code where pY is phosphotyrosine), found in the cytoplasmic domain of the CD3 receptor, also served as a positive control and inhibited Src SH2 binding with an IC_{50} of 11.07 μM. By comparison, AP22161 had a significantly lower IC_{50} of 0.24 μM in the Src SH2 binding assay (Table 1 and Figure 3). In addition, AP22161 had selectivity for binding to the Src SH2 domain because it had a 100-fold higher IC_{50} for blocking the binding of the same fluorescein-conjugated peptide to the structurally related Yes SH2 domain. The positive control peptide, pYEEI, had a similar IC_{50} value in the Src SH2 and Yes SH2 binding assays. As expected, AP22161 did not inhibit the binding of the fluorescein-conjugated peptide ligand to the tandem ZAP SH2 domains. AP22161 was therefore identified as a compound that demonstrated high affinity and selective binding to the Src SH2 domain.

Mammalian two-hybrid assay

Given that AP22161 showed high-affinity binding to the Src SH2 domain *in vitro* we wanted to demonstrate further that it inhibited Src SH2 binding to peptide ligands in cells. The mammalian-cell-based two-hybrid assay provided a model to evaluate whether compounds permeate cells, demonstrate inhibition of Src SH2 binding to peptide ligands, and elicit any signs of cellular toxicity [32]. Cells were engineered to contain an SH2 domain transcriptional activation domain fusion protein and an SH2 peptide ligand–GAL4 DNA binding domain–estrogen receptor ligand binding domain–Src kinase fusion protein (Figure 4). Compounds were screened in two different Src SH2 cell lines (Src-SH2/IgE-β and Src-SH2/ζ-ITAM), a negative control GerVP cell line and a ZAP SH2 cell line. Details of these two-hybrid cell lines have been described previously [32]. The Src SH2 cell lines express the secreted alkaline phosphatase (SEAP) reporter gene under the control of a Src SH2 ligand-binding interaction. Both of the Src SH2 cell lines contain the same Src SH2 domain construct, but each express different ligand-binding peptides. Compounds that inhibit binding to the Src SH2 domain would be expected to inhibit SEAP expression in both of these cell lines. The ZAP SH2 cell line expresses SEAP under the control of a ZAP SH2 ligand-binding interaction, whereas the GerVP negative control cell line expresses the SEAP reporter gene independent of an SH2–ligand interaction. Both the GerVP and the ZAP two-hybrid cells serve as negative controls to determine whether compounds selectively inhibit Src SH2-ligand binding. The ZAP cell line, containing a structurally

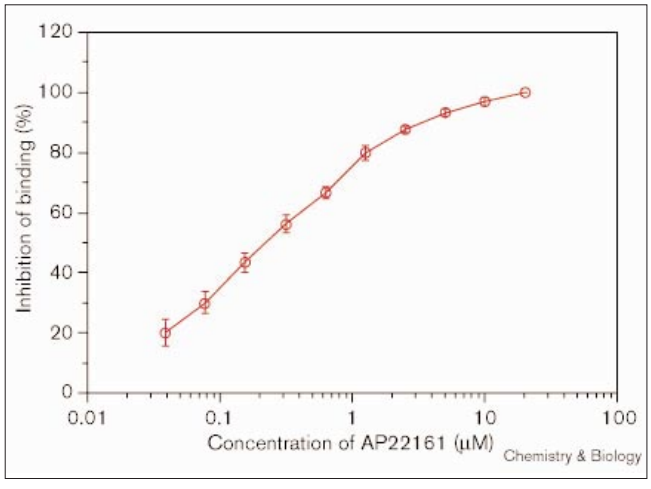
Table 1

Inhibition of Src, Yes and ZAP SH2 domains binding to peptide ligands *in vitro*.

	Src SH2 IC_{50} (μM)	Yes SH2 IC_{50} (μM)	ZAP SH2 IC_{50} (μM)
AP22161	0.24 ± 0.04	29.38 ± 3.60	421.86 ± 47.69
pYEEI	5.56 ± 0.21	4.26 ± 0.25	530.12 ± 49.02
ζ-ITAM peptide*	11.07 ± 0.09	23.63 ± 1.19	0.05 ± 0.01

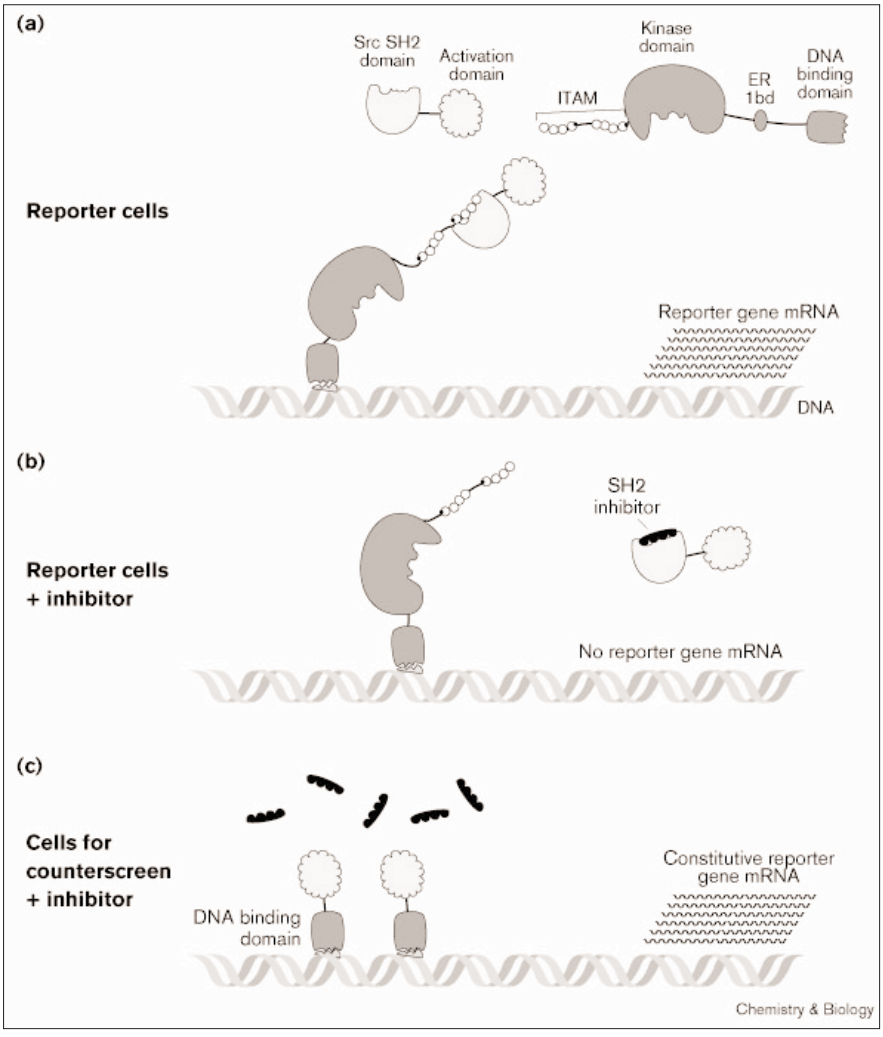
*ζ-1-ITAM peptide = Ac-NQLpYNELNLGRREEpYDVLD-NH_2 and pY = (H_2O_3PO)-Tyr. AP22161 and control peptides were assayed for their ability to inhibit Src, Yes and tandem ZAP SH2 domains binding to a fluorescein-conjugated peptide ligand. Results are shown as mean IC_{50} values (μM) ± S.E.M.

Figure 3



Inhibition of Src SH2 binding to peptide ligand by AP22161. Increasing concentrations of AP22161 were assayed for inhibition of Src SH2 domain binding to a fluorescein-conjugated peptide. Data are shown as the mean % inhibition of binding ± S.E.M.

Figure 4



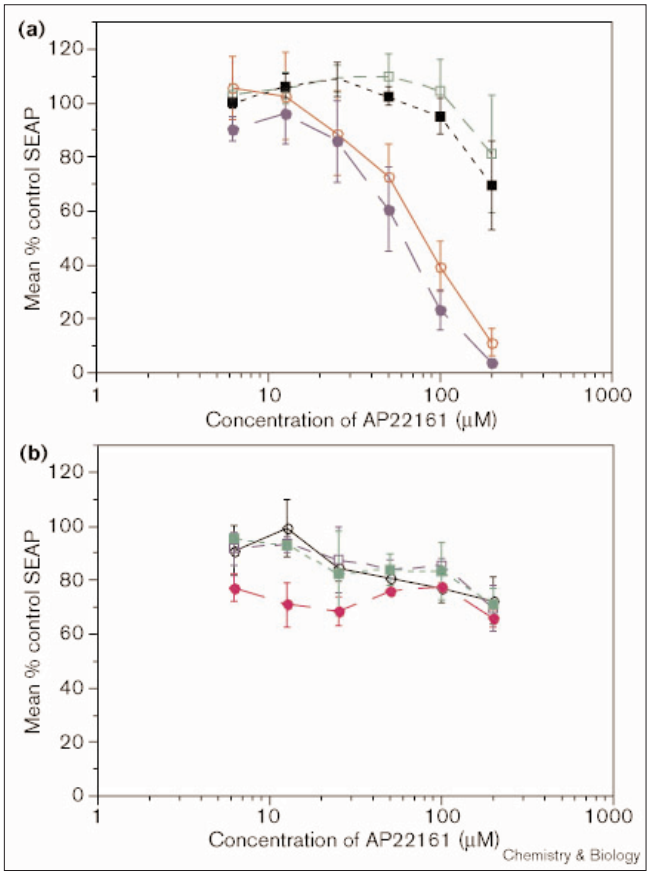
The mammalian two-hybrid cell assay. Cells contain an integrated secreted alkaline phosphatase (SEAP) reporter gene under the control of a GAL4-responsive promoter. Cells used to monitor SH2 domain-dependent binding were engineered to constitutively express a GAL4 DNA binding domain–estrogen receptor (ER) ligand-binding domain–Src kinase domain–SH2 domain ligand fusion protein; and a transcriptional activation domain–SH2 domain fusion protein. SH2 domain–peptide ligand interactions are observed when cells are exposed to estrogen. **(a)** Binding of the two-hybrid constructs within cells resulting in reporter gene expression. **(b)** Binding of compounds to the Src SH2 domain preventing activation domain binding and subsequent reporter gene expression. **(c)** The negative control GerVP cell line (containing a constitutively expressed GAL4 DNA binding domain–estrogen receptor ligand-binding domain–VP16 transcriptional activation domain fusion protein) where reporter gene expression is independent of an SH2-ligand binding interaction. Details of two-hybrid cell lines have been previously described [32].

distant related SH2 domain, monitors for nonspecific SH2-binding interactions. The GerVP and ZAP cell lines are also used to monitor nonspecific inhibition of SEAP production as a result of toxicity. All the cell lines described express SEAP in an estrogen-inducible manner. Cells were incubated for 16 hours with AP22161 and estrogen, and SEAP activity was examined. Cellular toxicity was also monitored by alamar activity and [^{3}H]-uridine incorporation. AP22161 selectively inhibited SEAP production from both of the Src SH2 two-hybrid cell lines (Figure 5a) with an IC_{50} of 60.0 μM in the Src SH2 ζ cell line and an IC_{50} of 80.1 μM the Src SH2 IgE-β cell line. In the negative control cell lines, AP22161 had minimal effects on estrogen-induced SEAP production. AP22161 did not induce any significant toxicity at any concentration as monitored by alamar reactivity (Figure 5b) or [^{3}H]-uridine incorporation (data not shown). The inhibition curve observed for AP22161 in the Src SH2 two-hybrid cell assay was steeper than that in the fluorescence polarization binding assay. In the two-hybrid assay there was ~80% inhibition across one log concentration of AP22161, whereas in the fluorescence polarization binding assay the same extent of inhibition was observed across a two log concencentration of AP22161. The fluorescence polarization inhibition curve fits the expected model that given one binding site, there should be approximately a two log difference in the inhibitor concentration going from 10 to 90% occupancy. In contrast, in the two-hybrid assay, the expression of the reporter gene, under the control of multiple DNA-binding events and five tandem Gal4-binding sites upstream of the reporter gene [32], is expected to introduce cooperative binding and thus a steeper curve of inhibition.

Effect of compounds on the morphology and growth of cSrcY527F transformed cells

Activated Src is a potent inducer of cellular transfomation, resulting in altered growth and morphology of cells [3].

Figure 5



Inhibition of Src SH2 domain binding to ligands in mammalian two-hybrid cells. AP22161 was tested at increasing concentrations for **(a)** its effects on the production of SEAP and **(b)** alamar reactivity in Src-SH2/IgE-β (open circles), Src-SH2/ζ ITAM (closed circles), GerVP (open squares) and ZAP (closed squares) two-hybrid cells. Data are shown as mean % of control ± S.E.M. SEAP production and alamar reactivity relative to vehicle alone treated cells.

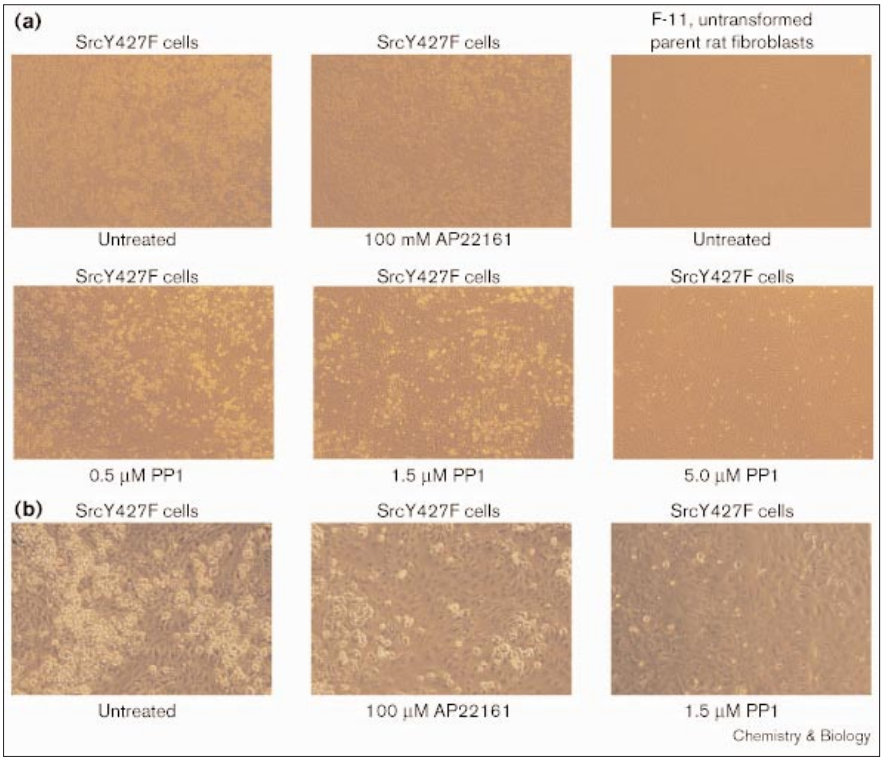
Thus, cells transformed with an activated form of Src could serve as a cellular system to assay compounds for inhibition of Src activity. AP22161 was examined for its ability to revert the morphology and inhibit the growth of rat fibroblasts transformed with mutant cSrcY527F as a means to monitor repression of Src cellular activity. In this mutant version of Src, the tyrosine kinase remains in an open conformation, and the kinase is constitutively active. cSrcY527F transformed cells appear rounded as they reach confluence and show loss of contact inhibition of growth [33], whereas the parent fibroblasts grow to form a confluent monolayer (Figure 6). The Src family kinase inhibitor PP1 was also tested as a known blocker of Src activity [34]. PP1 induced a concentration-dependent reversion of the morphological phenotype with complete reversion observed at 5 μM (Figure 6). PP1 also inhibited the growth of cSrcY527F transformed cells 74.5% after 5 days of treatment with 1.5 μM compound (Figure 7). The Src SH2 domain inhibitor AP22161 showed partial morphological reversion of the transformed phenotype at 100 μM. The effect of AP22161 on the morphology of the cSrcY527F cells was similar to that observed with 0.5 μM PP1. 100 μM AP22161 inhibited the growth of the cSrcY527F cSrc cells 26.3 % after 5 days of exposure (Figure 7). Given the IC_{50} of AP22161 in the Src two-hybrid assay of 60–80 μM, we would expect an IC_{50} in the reversion assay to be in a similar range or higher. Although not as potent as the kinase inhibitor PP1, AP22161 demonstrated the ability to inhibit Src functional activity in cSrcY527F transformed cells. The difference in potency could be related to a number of factors including mechanism of action, cellular permeability and compound stability.

Effect of AP22161 on rabbit osteoclast resorption

Because Src has been implicated in the regulation of osteoclast functional activity, AP22161 was examined for its ability to inhibit osteoclast-mediated resorption of dentine. AP22161 was tested at concentrations in the range of 1.2–100 μM. AP22161 inhibited resorption of dentine with an IC_{50} of 42.92 μM (Table 2). AP22161 did not demonstrate signs of toxicity at any of the concentrations tested as monitored by the presence of tartrate resistant acid phosphatase (TRAP)-positive cells and surrounding fibroblasts. These findings indicate that a compound that binds selectively to the Src SH2 domain, and inhibits Src binding to ligand in cells, can inhibit osteoclast-mediated resorptive activity. PP1 was also tested as a control compound and found to have an IC_{50} of 1.18 μM. This is consistent with its activity on the reversion of morphology and inhibition of growth of cSrcY527F transformed cells.

On the basis of its IC_{50} in the two-hybrid assay, we expected AP22161 to have an IC_{50} in the high micromolar range in the SrcY527F transformed cell assay and in the osteoclast-resorption assay. Although AP22161 has an IC_{50} of 0.24 μM in the fluorescence polarization binding assay, the aldehyde moiety of the pTyr mimic or AP2216 may not be chemically or metabolically stable. We have also determined that structurally similar compounds without the aldehyde have a 100-fold higher IC_{50} in the fluorescence polarization binding assay (W.C.S., unpublished observations). If the intracellular concentration of the active species is in fact in the high micromolar range we may expect inhibition of other Src SH2 family members such as Yes. If AP22161 were nonspecifically inhibiting other Src SH2 family members we would expect to see nonspecific signs of toxicity, such as decreased transcription and alamar reactivity in the two-hybrid cells or decreased numbers of fibroblasts and TRAP staining osteoclasts in the resorption assay. These types of effects have been observed with compounds that nonspecifically inhibit Src family kinases, as also seen with concentrations of PP1 > 2 μM. Rather, we believe that AP22161 may be

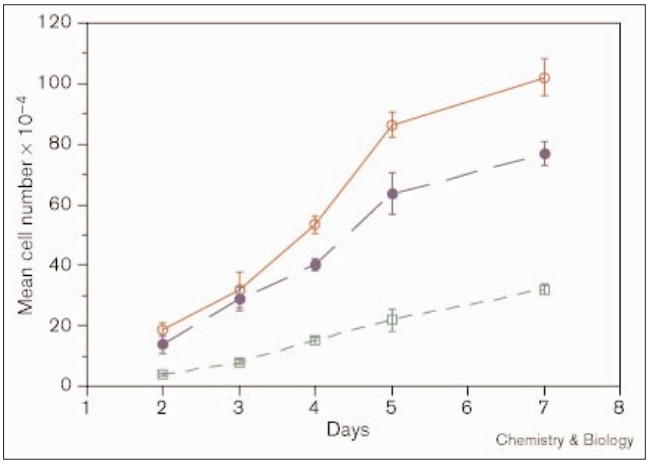
Figure 6



Morphological reversion of cSrcY527F transformed rat fibroblasts. cSrcY527F transformed rat fibroblasts were incubated with vehicle alone, 0.5 μM PP1, 1.5 μM PP1, 5 μM PP1 or 100 μM AP22161 for 4 days. Parent F1-11 rat fibroblasts are shown as the untransformed matched control cells. Cells were examined using light microscopy and compared with vehicle alone treated cSrcY527F and F1-11 rat fibroblasts. **(a)** Cells are shown at 10× magnification. **(b)** Cells are shown at 20× magnification.

less stable intracellularly and that the extracellular concentration is not a true indication of the intracellular concentration. It is also noted that there was a structure–activity relationship for the 3-formyl-4-carboxy-substituted-phenyalanine-containing compounds when comparing *in vitro* binding data with two-hybrid assay data. Compounds ranked similarly comparing the two assays. In addition, only compounds that had an IC_{50} less than 1 μM in the binding assay showed significant inhibition of osteoclast-mediated resorption at concentrations ≤ 200 μM.

Figure 7



Growth inhibition of cSrcY527F transformed rat fibroblasts. cSrcY527F rat fibroblasts were cultured for 2, 3, 4, 5 and 7 days with vehicle alone (open circles), 100 μM AP22161 (closed circles), or 1.5 μM PP1 (open squares). Data are shown as the mean number of cells counted ± S.D.

Table 2

Inhibition of rabbit osteoclast-mediated resorption of dentine.

AP22161 concentration (μM)	% Inhibition
1.23	26.86 ± 8.05
3.7	26.05 ± 7.68
11.1	34.66 ± 3.25
33.3	42.53 ± 12.14
100	67.65 ± 12.09
IC_{50} = 42.92 μM	
PP1 concentration (μM)	**% Inhibition**
25	99.37 ± 0.61
5	96.93 ± 2.85
1	44.63 ± 27.56
0.2	17.14 ± 28.61
0.04	0 ± 18.92
IC_{50} = 1.18 μM	

AP22161 and PP1 were assayed at increasing concentrations. Percent inhibition of resorption and IC_{50} values were calculated on the basis of three separate experiments containing triplicate samples for each concentration tested.

Discussion

Although Src has been implicated in regulating osteoclast-mediated resorption of bone, there has been little evidence that selective inhibition of Src activity using pharmacological approaches would result in inhibition of osteoclast-resorptive activity. In addition, Src contains many different regulatory domains. One of these domains, the SH2-ligand-binding domain, is known to regulate the activation state of Src and its ability to mediate intracellular signaling. There has been little information to indicate that inhibition of Src SH2 binding to its cognate ligand will diminish its cellular activity, however. Most information regarding the effects of inhibiting Src protein tyrosine kinases in osteoclasts has come from studying the effects of tyrosine kinase inhibitors on osteoclast resorption and genetic targeting of the Src gene in mice. Src family tyrosine kinase inhibitors have been found to inhibit osteoclast function in both *in vitro* and *in vivo* models of bone resorption [13–16]. Because the compounds tested do not selectively inhibit Src, however, it is not clear whether the effects observed are a result of inhibiting more than one member of the Src kinase family. They also do not address whether inhibition could be attained by inhibiting SH2 domain binding to ligand. The observation that osteoclasts in $Src^{-/-}$ mice are not able to resorb bone indicates that selective inhibition of Src function should also lead to inhibition of osteoclast resorption. Transgenic mouse approaches to rescue Src function in $Src^{-/-}$ mice have provided some information regarding the role that individual domains may play in osteoclast-mediated resorption of bone. Genetic rescue experiments in which physiological levels of a kinase-dead mutant were expressed in osteoclasts of $Src^{-/-}$ mice have suggested that the kinase domain may not be a key regulator of Src function in osteoclasts, but rather that the SH2 domain plays an essential role, perhaps as an adaptor protein, in contributing to the resorptive process [4,12]. Rescue experiments with a truncated Src protein construct in which the entire kinase domain was removed led to more severe osteopetrosis, suggesting that the presentation of the SH2 domain as an adaptor requires the conformation of the entire protein.

Studies carried out with fibroblasts isolated from both $Src^{-/-}$ mice and $Src^{-/-}$ mice expressing the kinase-dead mutant Src construct indicate that Src SH2 domain function is particularly important for integrin receptor signaling [35,36]. It is well established that the $\alpha_v\beta_3$ integrin receptor is intimately involved in osteoclast adhesion, intracellular signaling and resorption activity. Blocking $\alpha_v\beta_3$ integrin receptor binding inhibits osteoclast resorption both *in vitro* and *in vivo* [37–39]. These findings linked with the observations that Src is involved in regulating $\alpha_v\beta_3$ integrin receptor signaling [40,41] lend further insight into the process of how Src might regulate osteoclast-mediated resorption. They further suggest that blocking $\alpha_v\beta_3$ integrin receptor mediated intracellular signaling, by interfering with the association of an important regulatory protein, such as the SH2 domain of Src, could have the potential to block osteoclast resorption.

That $Src^{-/-}$ mice develop a tissue-specific phenotype restricted primarily to osteoclasts when the Src tyrosine kinase protein is normally expressed ubiquitously suggests that there is compensation of function by other tyrosine kinase proteins in unaffected cell types, because of overlapping patterns of expression and function with other Src tyrosine kinase members [1,2]. Such a possibility is further supported by reports that co-disrupted expression of Src with another member of the Src family of tyrosine kinase proteins in mice results in more profound phenotypes and in some cases lethality [42,43]. We suggest that selective inhibition of osteoclast function would require either a compound that only blocks Src activity or a nonselective Src family inhibitor that is targeted to osteoclasts. In this paper, we describe a compound that inhibits the binding of the Src SH2 domain to peptide ligands using biochemical selectivity and demonstrate its utility in inhibiting Src cellular activity and osteoclast resorption. We are working on a bone targeting approach to provide cellular selectivity to osteoclasts (S.M.V., *et al.*, unpublished observations).

The structural conservation of SH2 domains in the Src family of tyrosine kinases makes it difficult to design compounds that are selective for the Src SH2 domain. The observation that Src is the only member of the family to contain a cysteine residue in the pTyr-binding pocket of its SH2 domain led to the development of compounds designed to interact specifically with this residue. The effectiveness of 3′-formyl-pTyr-containing SH2 inhibitors has been described [21]; these compounds have no cellular activity, however, presumably because of their phosphatase sensitivity and inability to effectively penetrate cells. Similarly, we have reported [44] the synthesis of 3′-formyl-4′-phoshonodifluoromethyl-phenylalanine-containing SH2 inhibitors; these compounds appear not to engage the cysteine residue, however. A 3-formyl-4-carboxy-substituted phenyalanine has been described as an effective pTyr replacement, which targets Cys188 in the phosphotyrosine-binding pocket of the Src SH2 domain [22,45]. In an abstract, it was reported that the same molecule, in the context of a peptidic tail (EEIE-NH_2), irreversibly inhibited phosphopeptide binding to Src SH2 domain and was effective as an antiresorptive agent in a rat long-bone resorption assay [45]. There was no report of selectivity of binding to the Src SH2 domain for this compound, however. With an interest in designing molecules that specifically target Cys188, we incorporated 3′-formyl-4′-carboxy-phenylalanine into a novel nonpeptide template designed to bind to the Src SH2 domain. BIAcore binding studies were carried out with other compounds from this series, preceding the synthesis of AP22161, to determine whether they target Cys188

(W.C.S., unpublished observations). Compounds were tested for their ability to inhibit native Src SH2 and mutant Cys188 Src SH2 binding to peptide ligands. These compounds had a 100-fold lower IC_{50} for binding to the native form of Src SH2 compared with the mutant Cys188 Src SH2 protein. In addition, nuclear magnetic resonance (NMR) analyses with lead compounds demonstrated the formation of a hemithioacetal that is dependent on the interaction of the formyl group with Cys188 of Src SH2 (W.C.S., unpublished observations). These earlier experiments clearly demonstrated that compounds with the 3-formyl-4-carboxy-substituted phenyalanine head group target Cys188 within the Src SH2 domain. The time course of binding was also tested with the lead series to determine whether binding was irreversible. There was no significant difference in binding when incubated with Src SH2 for 0 or 36 hours, indicating that binding was reversible. Fluorescence polarization binding assays were carried out in parallel with the BIAcore binding studies comparing the binding of compounds to both Src and Yes SH2 domains. These studies confirmed that only the cysteine-targeted compounds selectively bound to Src SH2.

Subsequent studies with the lead series indicated that more potent binding compounds were necessary to achieve cellular activity. During our investigations into the design of selective Src SH2 inhibitors, we discovered a very effective bicyclic replacement (**5**) for the EEI peptide which confers increased binding affinity (20-fold) in the context of phosphotyrosine relative to pYEEI (W.C.S., unpublished observations). Moreover, this bicyclic compound is nonpeptidic and, therefore, should be less susceptible to cleavage by proteolytic enzymes and potentially more orally bioavailable. In this paper, we have described a compound, AP22161, that contains both the cysteine-targeted 3-formyl-4-carboxy-substituted phenylalanine and the bicyclic template. We demonstrate that AP22161 binds selectively and with high affinity to the Src SH2 domain. We also demonstrate that this compound inhibits Src cellular activity and osteoclast-mediated resorption. The effects of a Src SH2 inhibitor in osteoclasts highlight the role of the adaptor region of Src.

Significance

There is considerable interest in developing compounds that inhibit osteoclast function as an approach to treating osteoporosis. Because the Src tyrosine kinase protein has been implicated as an important regulator of osteoclast resorptive activity it is believed that inhibition of its activity could block osteoclast resorption of bone. The highly conserved nature of Src's SH2 and kinase domains, which are important in regulating its cellular functions, presents a major challenge for developing a compound that selectively inhibits Src. Gene targeting studies in mice suggest that selective inhibition of Src, within the family of Src tyrosine kinases, is desirable for developing an inhibitory compound that is not toxic to nonosteoclast cell types. To date, most full reports of the pharmacological approaches to inhibit Src function in osteoclasts have been carried out with compounds that inhibit the kinase domain of many members of the Src family of tyrosine kinases. Although the Src SH2 domain is believed to act as an adaptor protein — important in regulating Src interactions with downstream targets during signal transduction — there has been no previously reported evidence that selective inhibition of the Src domain will inhibit Src cellular activity and osteoclast-resorptive processes. We now describe the synthesis and cellular effects of a compound, AP22161, that selectively binds with high affinity to the Src SH2 domain. AP22161 was designed to be selective for the Src SH2 domain by targeting a cysteine residue in the highly conserved phosphotyrosine-binding pocket. This compound was tested in various *in vitro* and cellular assays and found to selectively block Src binding to its peptide ligands, inhibit Src cellular activity and diminish osteoclast-mediated resorption. This is the first full description of a Src selective SH2 binding compounding and its effects in both mechanism-based cellular assays and osteoclast resorption studies. Such a compound provides a prototype lead for further development in the treatment of osteoporosis.

Materials and methods

Materials

PP1 was purchased from Calbiochem (San Diego, CA). cSrcY527F transformed rat fibroblasts were developed by Adele Filson in the laboratory of Joan Brugge (Harvard Medical School, Boston, MA). Two-hybrid cell lines were constructed as previously described [32].

AP22161 synthesis

3-(1,3-Dithiolan-2-yl)-N-acetyl-L-tyrosine methyl ester. Boron trifluoride diethyl etherate (0.12 ml, 0.96 mmol) was added to L-3-formyl-N-acetyltyrosine methyl ester (**2**; 0.13 g, 0.48 mmol) [46] in methylene chloride (5 ml) at 0°C followed by the addition of ethanedithiol (0.044 ml, 0.53 mmol). The mixture was then warmed to room temperature and stirred for 1 h. The solution was mixed with water and the layers separated. The aqueous layer was extracted with methylene chloride and the combined extracts were washed with water, dried over magnesium sulfate, filtered and concentrated to a solid. The solid was recrystallized from ethyl acetate/hexane (0.14 g, 82%); melting point 97–99°C.

*3-(1,3-Dithiolan-2-yl)-4-(trifluoromethansulfonyloxy)-N-acetyl-L-phenylalanine methyl ester (**3**).* N-phenyltrifluoromethanesulfonimide (0.58 g, 1.61 mmol) was added to 3-(1,3-dithiolan-2-yl)-N-acetyltyrosine methyl ester (0.50 g, 1.46 mmol) and triethylamine (0.22 ml, 1.61 mmol) in methylene chloride (10 ml) at 0°C. The mixture was allowed to stir for 5 days then washed sequentially with 1 N NaOH, 1 N HCl and brine. The organic layer was dried over magnesium sulfate, filtered and concentrated to a solid. The solid was recrystallized from ethyl acetate/hexane (0.61 g, 87%); melting point 116–118°C.

3-(1,3-Dithiolan-2-yl)-4-(carboxymethyl)-N-acetyl-L-phenylalanine methyl ester. Triethylamine (0.33 ml, 2.36 mmol) was added to 3-(1,3-dithiolan-2-yl)-4-(trifluoromethansulfonyloxy)-N-acetyl-L-phenylalanine methyl ester (**3**) (0.51 g, 1.08 mmol) in dimethylsulfoxide (DMSO)/methanol (MeOH) (3:2, 5 ml) followed by the addition of palladium acetate (0.0073 g, 0.033 mmol) and 1,3-bis(diphenylphosphino)propane (0.013 g, 0.034 mmol). Carbon monoxide was bubbled

through for 3 min and the mixture was heated to 80°C for 24 h. After cooling, the solution was diluted with water and extracted with ethyl acetate. The combined extracts were washed with water, dried over magnesium sulfate, filtered and concentrated to a solid. The solid was recrystallized from ethyl acetate/hexane (0.30 g, 72%); melting point 113–119°C.

*3-(1,3-Dithiolan-2-yl)-4-(carboxymethly)-N-acetyl-L-phenylalanine (**4**).* Lithium hydroxide monohydrate (0.025 g, 0.60 mmol, 1 ml water) was added to 3-(1,3-dithiolan-2-yl)-4-(carboxymethyl)-N-acetyl-L-phenylalanine methyl ester (0.22 g, 0.57 mmol) in tetrahydrofurane (THF, 10 ml) at 0°C. The mixture was stirred for 45 min, diluted with water, made acidic with 1 N HCl, and extracted with ethyl acetate. The combined extracts were washed with water, dried over magnesium sulfate, filtered and concentrated to a glassy solid, which was homogeneous by reverse-phase high-pressure liquid chromatography (HPLC, 0.18 g, 86%). Mass spectrometry $[M-H]^-$ 368.

4-[(S)-2-Acetylamino-2-(3-carbamoyl-2-cyclohexylmethoxy-6,7,8,9-tetrahydro-5H-benzocyclohepten-(R,S)-5-ylcarbamoyl)-ethyl]-2-[1,3]dithiolan-2-yl-benzoic acid methyl ester. 1-Hydroxybenzotriazole (0.051 g, 0.35 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.072 g, 0.35 mmol) was added to 3-(1,3-dithiolan-2-yl)-4-(carboxymethyl)-N-acetyl-L-phenylalanine (**4**) (0.13 g, 0.35 mmol) in CH_2Cl_2/dimethylformamide (5:1, 5 ml) at 0°C. The mixture was stirred for 10 min and then (*R*,*S*)-9-amino-3-cyclohexylmethoxy-6,7,8,9-tetrahydro-5*H*-benzocycloheptene-2-carboxylic acid amide (**5**) (0.10 g, 0.32 mmol) (W.C.S., unpublished observations) was added and stirring was continued for 1 h. The solution was added to water and the layers separated. The aqueous layer was extracted with methylene chloride and the combined extracts were washed with water, 1 N HCl, dried over magnesium sulfate, filtered and concentrated to a glassy solid (0.20 g, 99%), which was homogeneous by reverse-phase HPLC. Mass spectrometry $[M-H]^-$ 636.

4-[(S)-2-Acetylamino-2-(3-carbamoyl-2-cyclohexylmethoxy-6,7,8,9-tetrahydro-5H-benzocyclohepten-(R,S)-5-ylcarbamoyl)-ethyl]-2-formyl-benzoic acid methyl ester. Mercury (II) perchlorate hydrate (0.38 g, 0.94 mmol) was added to 4-[(S)-2-acetylamino-2-(3-carbamoyl-2-cyclohexylmethoxy-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-(R,S)-5-ylcarbamoyl)-ethyl]-2-[1,3]dithiolan-2-yl-benzoic acid methyl ester (0.20 g, 0.31 mmol) in $CHCl_3$/MeOH (1:1, 6 ml). The mixture was stirred for 5 min and then filtered though Celite ($CHCl_3$ wash). The organic layer was washed with 1 N HCl, saturated $NaHCO_3$, dried over magnesium sulfate, filtered and concentrated to a glassy solid (0.18 g), which was used immediately in the next reaction.

*4-[(S)-2-Acetylamino-2-(3-carbamoyl-2-cyclohexylmethoxy-6,7,8,9-tetrahydro-5H-benzocyclohepten-(S)-5-ylcarbamoyl)-ethyl]-2-formyl-benzoic acid (**1**).* Lithium hydroxide monohydrate (0.018 g, 0.42 mmol, 1 ml water) was added to 4-[(S)-2-acetylamino-2-(3-carbamoyl-2-cyclohexylmethoxy-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-(R,S)-5-ylcarbamoyl)-ethyl]-2-formyl-benzoic acid methyl ester (0.18 g, 0.32 mmol) in THF (5 ml). The mixture was stirred for 1 h, acidified with trifluoroacetic acid and evaporated. The residue was diluted with DMSO (2 ml) and purified by reverse-phase HPLC (CH_3CN/H_2O). Lyophilization left a white solid (0.040 mg, 21%). ^1H NMR (300MHz, DMSO) indicates a 4:1 mixture of hemiacetal (HA)/aldehyde (Ald.) δ 0.96–1.38 (m, 7H), 1.60–1.80 (m, 7H), 2.81 (dd, $J = 12.2$, 10.1 Hz, 1H), 2.96 (m, 1H), 2.56 (d, $J = 5.7$ Hz, 2H), 4.52 (m, 1H), 4.79 (m, 1H), 6.41 (s, 40% HA), 6.50 (s, 40% HA), 6.89 (dd, $J = 8.6$, 4.0 Hz, 1H), 7.13 (br d, $J = 9.1$ Hz, 1H), 7.39 (m, 4H), 7.59 (m, 1H), 7.69 (s, 1H), 7.96 (br s, 1H), 8.41 (br s, 1 H), 10.38 (s, 20% Ald.). Mass spectrometry $[M-H]^-$ 576.

Proteins for binding assay

Human Src SH2 domain protein (residues Ser145–Ser251) and human tandem ZAP SH2 domain protein (residues Met1–Ala259) were produced and purified as described previously [29,47]. Murine Yes SH2 domain protein (residues Ser150–Val256) is identical to human Yes SH2 in sequence except that Val202 in the murine protein is Ile204 in the human protein. This residue is in the CD loop far removed from the binding site and the sequence difference is therefore not expected to affect binding. Murine Yes SH2 was expressed from vector pT7.7 in *E. coli* BL21 (DE3) cells grown in BHI medium containing 200 mg/l ampicillin and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h at 30°C. The cells were resuspended in two volumes of 75 mM Tris/Tris–HCl pH 7.0, containing 300 mM NaCl, 7.5 mM dithiothreitol (DTT), 7.5 mM ethylenediaminetetraacetic acid (EDTA), 1.5 mM phenylmethylsulfonyl fluoride (PMSF), and lysed using a French pressure cell at 16,000 psi. The lysate was diluted twofold with 50 mM Tris/Tris-HCl pH 7.0, 5 mM DTT, 2 mM EDTA, 1 mM PMSF and clarified by filtration and centrifugation at 20,000 g for 30 min. The clarified lysate was diluted 3.5-fold with H_2O, adjusted to pH 7.0, and purified over carboxy-sulfon (J.T. Baker, Phillipsburg, NJ) a weak-strong cation exchanger, equilibrated with 20 mM Tris/Tris-HCl, 5 mM DTT, 0.02% NaN_3, pH 7.0. The Yes SH2 protein was eluted with a 2M NaCl gradient. The protein was then purified by gel filtration on a 2.6×94 cm Sephacryl S-100 (Pharmacia, Uppsala, Sweden) column equilibrated with 20 mM BES, 200 mM NaCl, 5 mM DTT, 0.02% NaN_3, pH 7.0. Gel filtration was essential for removal of a variable fraction of domain-swapped dimers. Purity of the final protein, as measured by sodium dodecylsulfate (SDS) gel electrophoresis was > 95% and the protein was 97% monomer. Once the monomer was isolated, little or no dimer was formed on storage in the S100 buffer at 1.3 mg/ml for up to 1 week at 4°C and several hours at room temperature. The monomer was also stable to multiple freeze-thaws.

Fluorescence polarization binding assay

Details of the fluorescence polarization method to monitor ligand binding to Src SH2 and tandem ZAP SH2 have been described previously [29,47]. The fluorescein-conjugated peptide used to monitor Src SH2 and Yes SH2 binding was fluorescein-pYpYpYIE-NH_2 and the probe used to monitor ZAP tandem SH2 binding was fluorescein-GpYNELNLGRREEpYDVL-NH_2 [47]. The Yes SH2 binding pocket is very similar to Src SH2 except that Cys188 of human Src is Ser193 of murine Yes. Yes SH2 was therefore assayed with the same probe as Src SH2. By performing a complete saturation binding curve with a fixed concentration of peptide probe and increasing concentrations of SH2 protein, the K_d of the peptide probe–SH2 protein interaction was determined. Yes SH2 was found to have an ~twofold higher affinity than Src SH2 for the probe, which required use of a somewhat lower protein concentration in the compound inhibition assays (90 nM instead of 150 nM as for Src SH2). For compound inhibition assays, serial twofold dilutions of compounds in buffer solution (20 mM phosphate buffer pH 7.4, containing 100 mM NaCl, 10 mM DTT, 2% DMSO, 1 mM EDTA, and 0.1% gamma globulin) were tested. Compounds (0.04–500 μM) were then added to a constant concentration of SH2 protein premixed with 20 nM of the corresponding fluorescein-conjugated peptide. The protein concentrations used were 25 nM for tandem ZAP SH2, 90 nM for Yes SH2, and 150 nM for Src SH2. Binding reactions were carried out for 5 min at room temperature. Fluorescence polarization of the samples was measured on a Jolly FPM2 96-well plate reader with standard cutoff filters (excitation $\lambda = 485$ nm, BP = 22; emission $\lambda = 530$ nm, BP = 30). The 50% inhibitory concentration of each compound (IC_{50}) was calculated based on the % binding of the fluorescein-conjugated peptide to SH2 protein in the presence of compound relative to vehicle alone control samples. Results are shown from three or more experiments each containing triplicate samples.

Mammalian two-hybrid assay

Cells were cultured in minimal essential medium eagle (Cellgro, Herndon, VA) supplemented with 10% heat inactivated fetal bovine serum (HIFBS, Hyclone, Logan, UT), 0.5 mg/ml active G418 (Gibco-BRL, Grand Island, NY), 50 units/ml penicillin, 0.05 mg/ml streptomycin, 2 mM glutamine, 50 μg/ml hygromycin, and 0.1 mM MEM nonessential amino acids (Gibco-BRL). Cells were plated into 96-well plates at 20,000 cells per well and incubated for 4 h at 37°C and 5% CO_2. Fresh culture medium containing serially diluted test compounds

was added to cells and incubated for 3 h at 37°C in 5% CO_2, and then estrogen was added to a final concentration of 1 μM. Cells were incubated with test compounds and estrogen for 16 h at 37°C and 5% CO_2. After incubation, supernatant was collected and transferred into 96-well plates to examine SEAP activity and the remaining cells were monitored for alamar reactivity or [^{3}H]-uridine incorporation to assess non-specific toxic effects of compounds. Results are shown as the mean percent of control ± S.E.M. from at least three independent experiments each containing triplicate samples.

SEAP activity

Supernatant transferred to 96-well plates was incubated for 90 min at 65°C to inactivate nonspecific phosphatase activity. 100 μl of heat inactivated supernatant was transferred into 96-well fluorometer plates (Perkin Elmer, Norwalk, CT) followed by the addition of 100 μl 2 M diethanolamine, and 1.32 mM 4-methylumbelliferylphosphate. Plates were covered and incubated in a humidified incubator at 37°C. Fluorescence was measured on an LS 50 B Perkin Elmer Luminescence Spectrometer using 350 nM excitation and 445 nM emission.

Alamar reactivity

After culture media was removed for SEAP analysis 200 μl of fresh culture media containing 10% alamar Blue (Alamar Biosciences Inc., Sacramento, CA) was added to each well and incubated for 2–3 h at 37°C and 5% CO_2. Absorbance was measured at 570 nM.

Treatment of cSrc Δ 527 transformed cells

Cells were plated into 12-well plates at 5×10^4 cells/well in 1.0 ml dulbecco's modified eagle medium (Gibco-BRL) supplemented with 10% HIFBS (Hyclone), 50 units/ml penicillin, 0.05 mg/ml streptomycin, and 2 mM glutamine and incubated overnight at 37°C in 5% CO_2. The next day medium was replaced with fresh culture medium supplemented with test compounds. Cells were refed with fresh medium containing compounds every other day. Cells were counted on days 2, 3, 4, 5 and 7. The effects of compounds on morphological reversion was determined after 4 days of exposure. Data are shown as the mean number of cells counted ± S.E.M. from four separate samples.

Resorption assay

Femurs, tibias and scapulas were isolated from 3–4 day old New Zealand white rabbits. Bones were chopped and minced in minium essential medium alpha (Gibco-BRL) containing 0.55 g/l $NaHCO_3$, 10 mM HEPES (Gibco-BRL), 50 units/ml penicillin, and 0.05 mg/ml streptomycin, pH 7.1. Bone fragments were allowed to settle, supernatant was collected and centrifuged at 400 RPM (Beckman GS-6KR) for two minutes, and the cell pellet was resuspended in the same medium supplemented with 10% HIFBS (Hyclone). 0.75 ml of cell suspension was added on top of dentine slices in 24-well plates that were preincubated for 4 h at 37°C with 0.75 ml culture medium. Osteoclasts were allowed to adhere for 30 min at 37°C and 5% CO_2 and then the medium and nonadherent cells and debris were removed by aspiration. Fresh culture medium containing serially diluted test compounds was added. Cells were incubated on dentine with or without compound for 24 h at 37°C and 5% CO_2. After the resorption phase, dentine slices were soaked for 30 s in 0.5% sodium hypochlorite, wiped clean of adherent cells, and then stained for 30–45 s with 1% toluidine blue. Sperm whale dentine was cut into 1 mm × 6 mm circular discs. Resorption was measured using reflective light microscopy and automated image analysis. The resorbed area was measured on the entire 6 mm disc. Remaining cells in the 24-well plates were stained for TRAP and also assessed visually for the presence of fibroblasts. Experiments were carried out containing triplicate samples for each concentration of compound with five untreated control samples per plate. Results are reported as mean percent of control resorption ± the S.E.M. from three independent experiments each containing triplicate samples.

TRAP staining

Cell culture wells were washed one time with phosphate buffered saline (PBS), fixed with 3.7% formaldehyde for 5 minutes at room temperature, and then incubated with acetone:ethanol (1:1) for 30 s. Cells were stained with 0.5 ml per well of TRAP staining solution [0.24 mM Napthol-AS-MX phosphate sodium salt (Sigma, St. Louis, MO), 0.14 mM N,N-dimethyl formamide (Sigma), 1.6 mM fast red violet LB salt (Sigma), 40 mM anhydrous sodium acetate (Sigma), and 10 mM sodium tartrate (Sigma)] for 5 min. After staining the solution was aspirated and replaced with 0.5 ml PBS.

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