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Bone-Targeted Src Kinase Inhibitors: Novel Pyrrolo- and Pyrazolopyrimidine Analogues

Raji Sundaramoorthi,* William C. Shakespeare, Terence P. Keenan, Chester A. Metcalf, III, Yihan Wang, Ukti Mani, Merry Taylor, Shuangying Liu, Regine S. Bohacek, Surinder S. Narula, David C. Dalgarno, Marie Rose van Schravandijk, Sheila M. Violette, Shuenn Liou, Susan Adams, Mary K. Ram, Jeffrey A. Keats, Manfred Weigele and Tomi K. Sawyer

ARIAD Pharmaceuticals, Inc., 26 Landsdowne Street, Cambridge, MA 02139-4234, USA

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Abstract—Src tyrosine kinase is a therapeutic target for bone diseases that has been validated by gene knockout studies. Furthermore, in vitro cellular studies implicate that Src has a positive regulatory role in osteoclasts and a negative regulatory role in osteoblasts. The potential use of Src inhibitors for osteoporosis therapy has been previously shown by novel bone-targeted ligands of the Src SH2 (e.g., AP22408) and non-bone-targeted, ATP-based inhibitors of Src kinase. Significant to this study, compounds **2**–**12** exemplify novel analogues of known pyrrolopyrimidine and pyrazolopyrimidine template-based Src kinase inhibitors that incorporate bone-targeting group modifications designed to provide tissue (bone) selectivity and diminished side effects. Accordingly, we report here the structure-based design, synthetic chemistry and biological testing of these compounds and proof-of-concept studies thereof.

Bone diseases provide significant opportunities for therapeutic intervention with small-molecule inhibitors of varying targets, including farnesyl diphosphate synthase, estrogen steroid receptor, and protein kinases.[1] Most bone pathology is due to an imbalance in bone remodeling which is a dynamic process of bone formation and bone resorption.[1,2–4] While a large number of protein kinases are potential targets for bone disease, attention has been focused on Src kinase because of the discovery that the c-Src −/−mouse had little overt pathology apart from osteopetrosis.[5] The defect in these mice was traced to a cell-autonomous defect in the activity of mature osteoclasts, and not in osteoclastogenesis.[6] Recently, it was shown that the Src activity also has an overall negative regulatory effect on osteoblastic cells.[7] The selectivity of the Src −/−phenotype for bone tissue and the complementary effects on bone resorption and bone formation, implicate Src as an attractive target for the treatment of diseases with excessive bone loss, such as post-menopausal osteoporosis, Paget's disease, and osteolytic metastases.

Recently, we reported a SH2 inhibitor (AP22408) having a bone targeting group incorporated into its structure.[8] By bone-targeting this nonpeptide small molecule, we were able to achieve tissue selectivity by selectively localizing it to the bone surface and achieve higher concentrations for inhibiting osteoclasts. We have extended the same approach to a series of known Src tyrosine inhibitors. By introducing the bone targeting groups to these known inhibitors, we retrieved a wealth of SAR data to advance important 'proof-of-concept' studies. In this paper, we describe the design and synthesis of bone-targeted-pyrrolo[2,3-*d*]pyrimidines, originally reported by the Novartis group[9] as well as bone-targeted-pyrazolo[2,3-*d*]pyrimidines, reported by Pfizer group.[10] A molecular model of Src kinase was developed to help deter-mine the precise regiospecificity of the bone-targeting group that would maximize the molecular interactions of the inhibitor at the enzyme active site.

A 3D model of the catalytically-active conformation of Src kinase was constructed using the crystal structures of inactive Src kinase, Lck kinase and active insulin receptor kinase.[11] The model was tested by docking diverse series of Src kinase inhibitors found in the

*Corresponding author. Fax: +1-617-494-8144; e-mail: raji.sundaramoorthi@ariad.com

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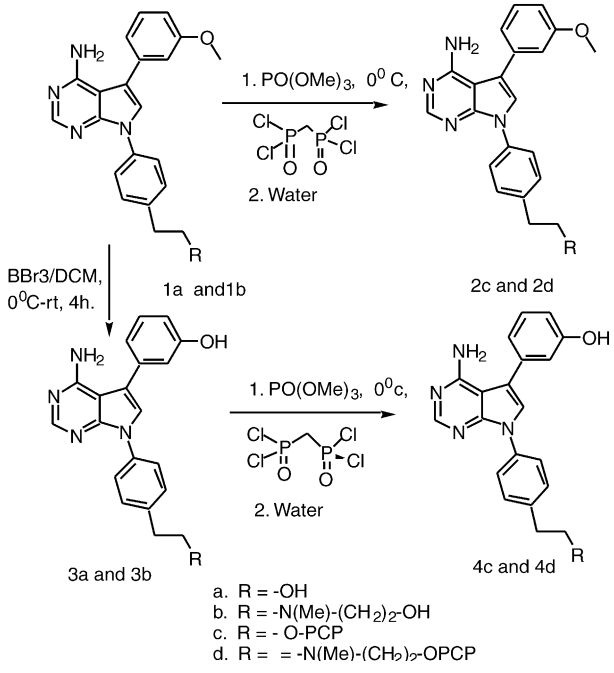
literature. Adjustments were made until the model could rationalize the relative Src kinase inhibitory potencies of such compounds (i.e., both active and inactive). Docking studies using the FLO software were carried out to predict how **3b** would bind to Src kinase active site (Fig. 1a–c). The pyrrolopyrimidine ring of **3b** was determined to overlay with the purine ring of ATP, including hydrogen bonds to Met-319 and Ser-320, and the phenol group of **3b** interacts with a hydrophobic pocket not occupied by ATP. Finally, the phenyl ring of **3b** attached to the N9 nitrogen occupies the region at the solvent interface of the binding site. Modeling studies suggested bone-targeting group incorporation at this position, and similar drug design rationale was suggested for bone-targeted pyrazolopyrimidine analogues.

Figure 2 shows the candidate bone-targeting groups and their abbreviated nomenclature. These groups were prepared according to the procedures described elsewhere[13] for incorporation into either pyrrolopyrimidine or pyrazolopyrimidine templates (examples in Fig. 3). A few different bone-targeting groups, having varying hydroxyapatite affinity properties were examined. Synthesis of compounds **2**–**4** are depicted in Scheme 1 and **7g**–**n** in Scheme 2. The experimental details of the synthesis of **2**–**14** is described elsewhere.[13] Reported compounds **1a** and **1b**[9] were phosphonomethylphosphonylated using methylenebis (phosphonic dichloride) in trimethyl phosphate at 0 °C following a previously reported procedure[14] to give **2c** and **2d**. Compounds **1a** and **1b** were demethylated with boron tribromide in DCM at rt for 5 h to provide compounds **3a** and **3b**. This was then phosphonomethyphosphonylated using methylenebis (phosphonic dichloride) in trimethyl phosphate at 0 °C to give **4c** and **4d**. Compounds **2**–**12** were tested in vitro using a scintillation proximity assay (SPA) developed by Amersham.[15]



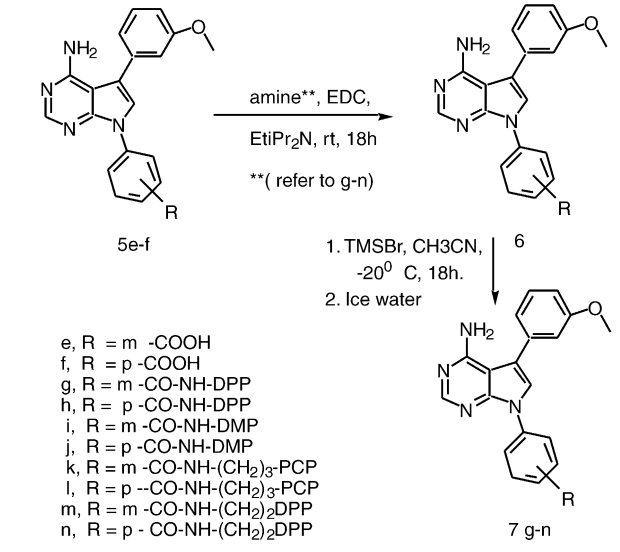
Scheme 1.

Table 1 shows the IC_{50} values of compounds **2**–**12.** The IC_{50} values of compounds **2c** and **2d** were similar to that of their non-bone-targeted parent molecules **1a** and **1b**. Molecular modeling studies suggest that the bone-targeting group interacts with the Mg^{2+} binding pocket of the Src kinase active site in a manner related, but not identical, to that of the triphosphate of the ATP. The Src kinase inhibitory potency of these compounds could be further improved to provide IC_{50} values as low as 4 nM by demethylating the *O*-methyl group (cf. compounds **3a** and **3b** vs **1a** and **1b**, respectively, as well as compounds **4c** and **4d** vs compounds **2c** and **2d**, respectively). Molecular modeling studies suggest that the phenol OH group H-bonds with residues in the hydrophobic specificity pocket of Src kinase. Relative to compounds **5e** and **5f**, a series of systematically modified pyrrolopyrimidines were synthesized (Scheme 2) to explore varying bone-targeting groups. Both aryl and aliphatic modified, bone-targeting groups were explored (cf. compounds **7g**–**n**). Src kinase inhibitory potencies revealed that all were markedly less potent (50- to 100-fold) than the aforementioned lead compound **4d**.

Hydroxyapatite adsorption chromatography assay was developed to experimentally determine the ability of these bone-targeted compounds to bind to hydroxyapatite (mineral component of bone). As expected,

Table 1. Src kinase inhibition of pyrrolopyrimidines and pyrazolopyrimidines using the SPA[15] kinase assay

Pyrrolopyrimidine				Pyrazolopyrimidine	
Compd	IC_{50}, μM	Compd	IC_{50}, μM	Compd	IC_{50}, μM
1a	1.15	**5e**	4.0	PP1	0.12
2c	0.7	**5f**	1.5	**8**	1.6
3a	0.04	**7g**	2.5	**9**	**0.02**
4c	0.06	**7h**	0.87	**10**	0.40
1b	0.16	**7i**	19	**11**	0.32
2d	0.31	**7j**	40	**12**	0.68
3b	0.04	**7k**	0.36		
4d	**0.004**	**7l**	0.25		



Scheme 2.

compound **2d** gave a K value of 2.26 which was slightly higher than that of H_2N-PCP ($K = 1.8$), whereas the non-bone-targeted analogue **1b** gave a K value ~ 0. Functionally, compounds **2c** and **4c** were active in vitro to inhibit rabbit osteoclast mediated resorption of dentine slices,[15,16] showing IC_{50} values in the 1–3 μM range. In terms of proof-of-concept, these data support the expected physical and biological properties of a bone-targeted pyrrolopyrimidine template-based Src kinase inhibitor.

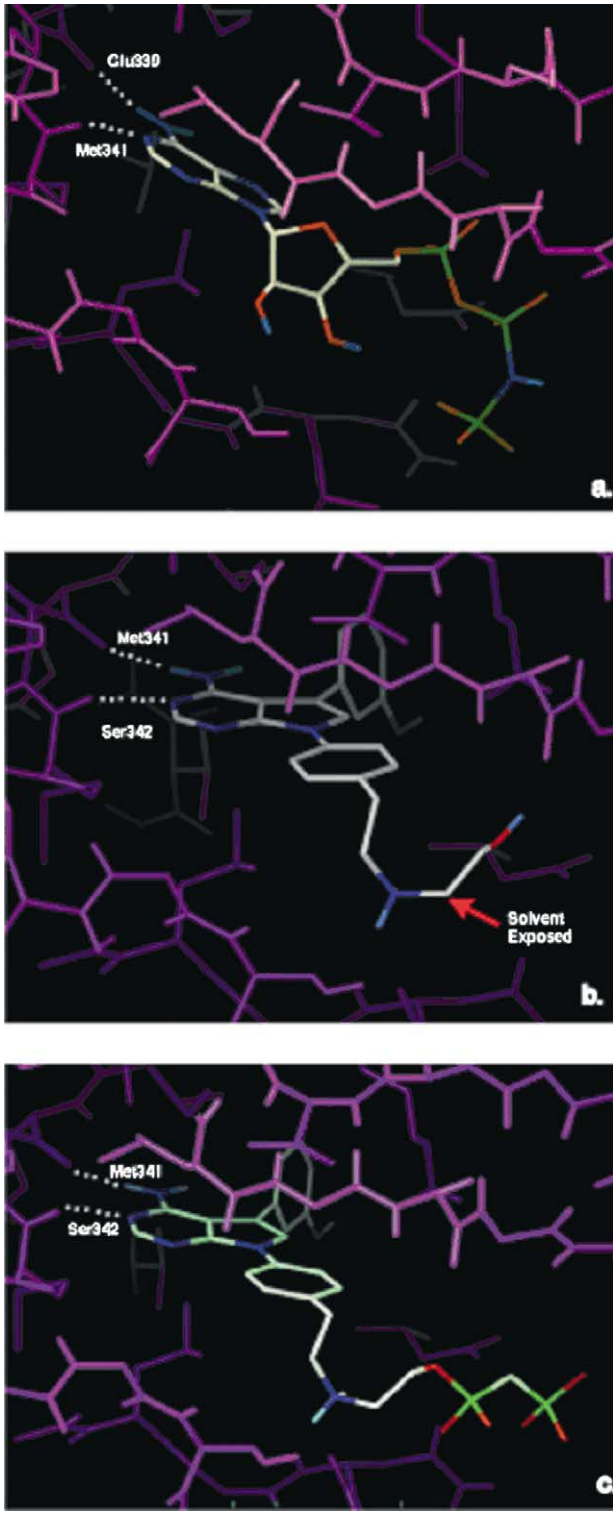


Figure 1. (a) A non-hydrolyzable ATP analogue AMP-PNP used in the crystallographic studies[12] docked into the model of Src kinase illustrating the critical hydrogen bonds; (b) compound **3b** docked in the Src kinase model illustrating the solvent exposed region for placing the bone-targeting group; (c) incorporation of phosphonomethylphosphonic acid (PCP) moiety into **3b**.

Although not as detailed relative to the above studies, a similar effort to test a series of bone-targeted pyrazolopyrimide template-based Src kinase inhibitors was also undertaken. Relative to the known Src kinase inhibitor PP1 (Fig. 4), compounds **8**–**12** were tested for their comparative enzyme inhibitory properties using the SPA kinase assay (Table 1). Noteworthy was compound **9** which showed an Src kinase IC_{50} of 20 nM and supported molecular modeling studies that predicted its bone-targeting group would likely not interfere with binding to the enzyme active site.

In summary, using structure-based drug design we successfully incorporated novel bone-targeting groups into

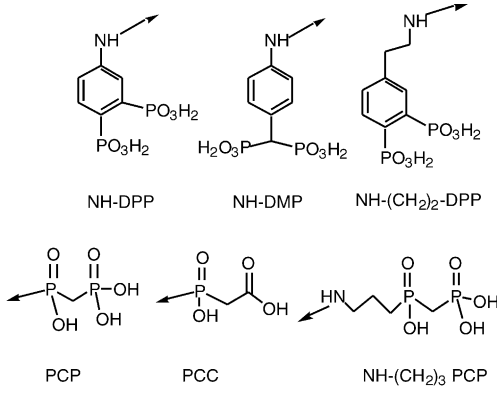


Fig 2. Candidate bone-targeting groups and abbreviated nomenclature.

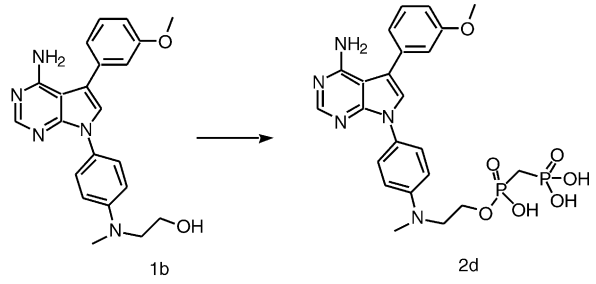


Fig 3. Incorporation of a bone-targeting group onto a pyrrolopyrimidine template-based Src kinase inhibitor **1b** to give **2d**.

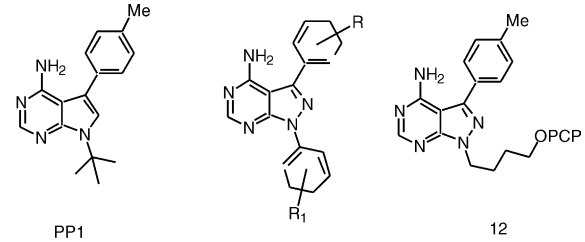
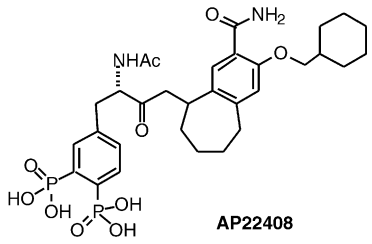


Fig 4. Structures of compounds **8**–**12** and PP1.

both pyrrolopyrimidine- and pyrazolopyrimidine-based templates of known Src kinase inhibitors. Compound **4d** was determined to be more potent than its parent non-bone-targeted Src kinase inhibitor **3b**. The bone-targeted Src kinase inhibitors exhibited high affinities for hydroxyapatite and potent in vitro inhibition of Src-dependent, osteoclast-mediated bone resorption. Collectively, these studies support the proof-of-concept for bone-targeted Src kinase inhibitors relative to heterocyclic templates closely resembling ATP.



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