



Pergamon

Bone-Targeted 2,6,9-Trisubstituted Purines: Novel Inhibitors of Src Tyrosine Kinase for the Treatment of Bone Diseases

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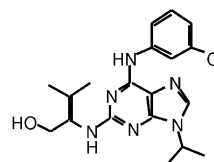
Received 31 March 2000; accepted 6 May 2003

Abstract—Novel bone-targeted 2,6,9-trisubstituted purine template-based inhibitors of Src tyrosine kinase are described. Drug design studies of known purine compounds revealed that both positions-2 and -6 were suitable for incorporating bone-seeking moieties. A variety of bone-targeting groups with different affinity to hydroxyapatite were utilized in the study. Compound **3d** was determined to be a potent Src inhibitor and was quite selective against a panel of other protein kinases.

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Src is a non-receptor protein tyrosine kinase which functions as an early upstream signal transduction protein activated by specific cellular receptors.¹ A broad spectrum of functional properties exists for Src, including cell growth, differentiation, survival, cytoskeletal alterations, adhesions and migration.² These process are critically involved in the progression of cancer by impacting tumor growth, metastasis and angiogenesis.^{3–5} Src also plays multifunctional roles in osteoclast and osteoblast activities, thus impacting bone remodeling (resorption and formation) and bone related diseases, including osteoporosis, rheumatoid arthritis, periodontal disease, Paget's disease, hypercalcaemia of malignancy and metastasis of certain cancers to bone.^{6,7} In osteoclasts, Src is important for osteoclast activation (ruffled border formation), survival, motility and adhesion through various signal transduction pathways.^{8–12} In osteoblasts, Src recently has been implicated as a negative regulator of osteoblast functional activity and, conversely, as a mediator of sex steroid-induced antiapoptotic signalling in osteoblasts.^{13,14} Thus, Src inhibitor drug discovery holds high potential to treat bone related diseases and cancer-induced bone metastasis. Recent reports exemplify Src inhibitors that illustrate progress in such efforts.^{15,16}

There exists a plethora of templates available to advance Src kinase inhibitors.^{17,18} Since Src is widely expressed in mammalian cells, strategies to selectively deliver the drug to bone are desirable. In this paper we describe a strategy to incorporate a bone-targeting moiety to Src kinase inhibitors. It is anticipated that such compounds would localize to bone when administered in vivo, thus achieving tissue selectivity. Guided by structure-based drug design, we sought the optimal sites on a series of ATP-related inhibitors of Src kinase to incorporate such bone-targeting moieties. Bisphosphonates exhibit exceptional affinity to bone, which have led to exploration of their utility for targeting pharmacological agents to bone.¹⁹ Encouraged by such studies, we describe the design and synthesis of a series of novel bone-targeted 2,6,9-trisubstituted purines as well as their molecular and cellular properties related to the inhibition of Src.²⁰



Purvalanol A

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The 2,6,9-trisubstituted purines were first identified as potent inhibitors for cyclin-dependent kinases.²¹ For example, Purvalanol A has an IC_{50} of 4 nM against CDK2.²² A model of the catalytically active conformation of Src kinase was constructed using the crystal

structures of inactive Src kinase,²³ Lck kinase²⁴ and active insulin receptor kinase.²⁵ The model was tested by docking a diverse series of Src kinase inhibitors found in the literature. Adjustments were made until the model could rationalize differences between active and inactive compounds. Docking studies using the FLO software were performed to predict the binding of Purvalanol A to Src kinase. These studies predicted that the purine moiety of Purvalanol A would occupy a position slightly different to that of adenosine of ATP and form hydrogen bonds to Met-341 and Ser-342. The isopropyl group interacted with a hydrophobic pocket not accessed by ATP (Fig. 1a and b). The 3D Src kinase model also indicated that bone-targeting groups incorporated at the *para*-position of the aniline ring, would project into solvent (Fig. 1b) and would have minimal effect on the binding affinity. The C2 hydroxyethylamino group of Purvalanol A was predicted to project toward the Mg^{+2} binding pocket, hence incorporation of bone-targeting groups were conceptualized to possibly mimic some interactions between ATP triphosphate moiety and the Mg^{+2} binding site.

Synthesis of the compounds **3a–f** (Scheme 1 and Table 1) was initiated with the preparation of 2-fluoro-6-chloro-9-cyclopentylpurine **1**. The syntheses of respective bone-targeting aniline or amine phosphonic esters were previously reported.²⁶ The free phosphonic acids (**A** to **D**) were obtained after treatment of corresponding phosphonic esters with concentrated HCl. These free phosphonic acids (**A** to **D**) and alendronate **E** were then used in the subsequent steps to replace C6 chlorine or C2 fluorine. Three anilino bisphosphonates (**A**, **B**, **C**) were used as R_1-NH_2 to replace C6 chlorine, and two amino bisphosphonates (**D** and **E**) were used as R_2-NH_2 to replace the C2 fluorine. The final compounds **3a–f** were obtained in good yields after purification by reverse-phase HPLC chromatography.

Compounds obtained were screened for their Src kinase inhibitory effect using ELISA methods.²⁷ Compound **3a** is a potent Src kinase inhibitor, with an IC_{50} of 141 nM. Incorporation of a gem-diphosphonomethyl group at the *para*-position, or two phosphonic acids at the *para*- and *meta*-positions, resulted in about 100-fold loss of potency (**3b** IC_{50} = 22.0 μ M and **3c** 15.7 μ M). Molecular modeling predicted negative binding interactions for such *meta*-phosphate or gem-diphosphate groups which was consistent with the biological data. Compound **3d** with a linear phosphinomethylphosphonic acid moiety, showed a strong inhibitory effect towards

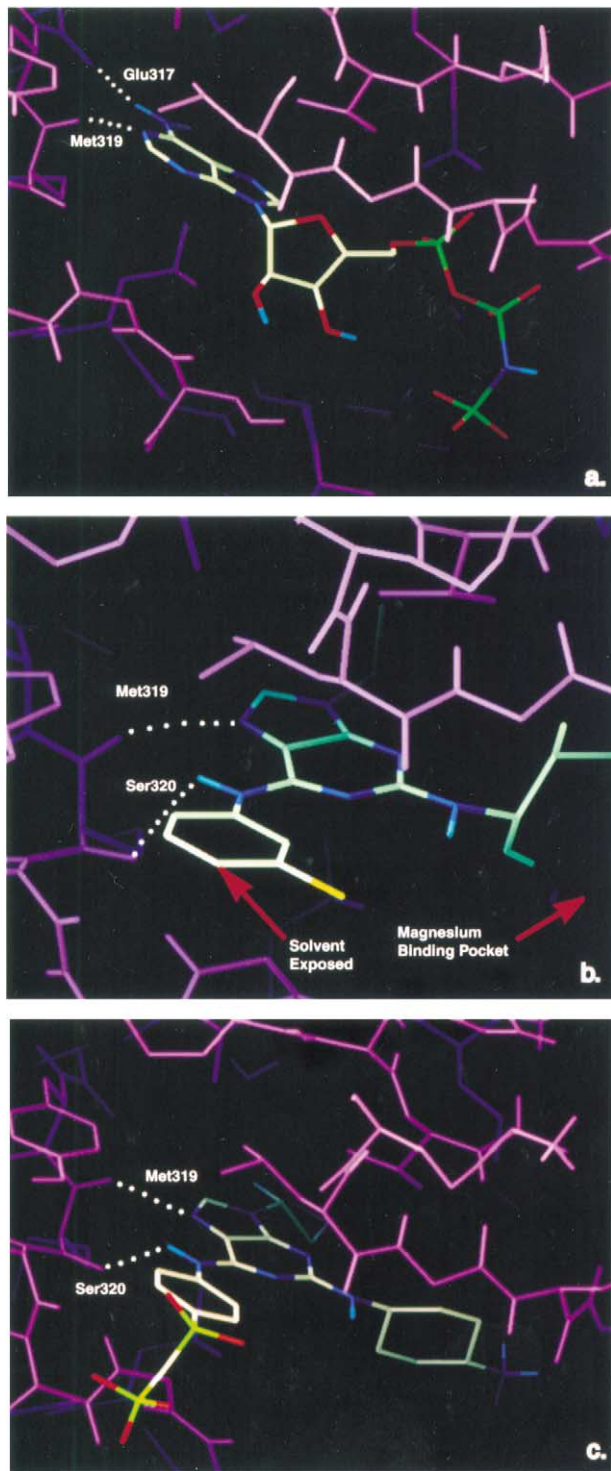
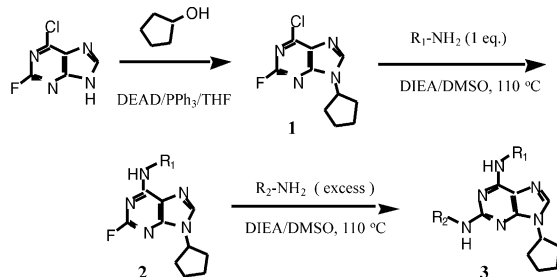


Figure 1. (a) AMP-PNP binding in the 3D model of Src tyrosine kinase; (b) Purvalanol A binding in the 3D model illustrating the solvent exposed *para* position on the aniline ring and the extension of hydroxyethylamino group towards the Mg^{+2} binding pocket; (c) compound **3d** binding in the 3D model.



Scheme 1. Synthesis of novel bone-targeted purines.

Table 1. Bone-targeted 2,6,9-trisubstituted purines and their comparative Src kinase inhibitory and hydroxyapatite affinity properties

Analogue	R ₁ -NH-	R ₂ -NH-	Src kinase IC ₅₀ (μM)	Hydroxyapatite affinity (K') ¹⁶
3a			0.141	0
3b			22.0	3.3
3c			15.7	1.8
3d			0.041	2.5
3e			9.81	0
3f			5.23	2.3
3g			48.2	3.6

Src. Its more than 3-fold increased potency may be due to the favorable interactions of the phosphinic acid group with the protein, whereas the second phosphonic acid extends into the solvent (Fig. 1c).²⁸ We also evaluated the attachment of bone-targeting moieties at the R₂ position. Compound **3e**, with an *N,N*-dimethylethylene-diamine group at R₂, is a much weaker inhibitor than **3a**. Replacement of the R₂-NH group of **3e** with linear bisphosphonate **D**, resulted in slightly more potent compound **3f**. Yet detrimental effects were found with alendronate (**E**), compound **3g** has about 5-fold loss of potency compared to **3e**.

Compounds **3a–f** were tested for their relative bone-targeting affinities using a hydroxyapatite column.¹⁶ Whereas non-bone-targeted compound **3a** showed a $K' = 0$, the highest propensity bone-targeted compound **3f** showed a $K' = 3.6$. The relative affinities of bone-targeting groups for hydroxyapatite were as follows, **3g** > **3b** > **3d** ≥ **3f** > **3c**, consistent with that of their respective bone-targeting moieties (Fig. 2).

Because Src has been implicated in the regulation of osteoclast functional activity, some of the bone-targeted

compounds were assayed for their ability to inhibit rabbit-osteoclast mediated resorption of dentine slices.²⁹ We tested the most potent compound from each category (compound **3d** for R₁ replacement, and compound **3f** for R₂ replacement). Compound **3d** inhibited resorption of dentine slices with an IC₅₀ of ~2.0 μM, whereas compound **3f** was not active up to 100 μM. These results are consistent with the 127-fold difference in Src kinase inhibitory potencies of compounds **3d** and **3f**. Neither compound showed sign of toxicity as monitored by the presence of tartrate resistant acid phosphatase (TRAP)-positive cells and surrounding fibroblasts. Interestingly, the non-bone-targeted parent compound **3a** was found to be a potent inhibitor in the osteoclast assay (IC₅₀ < 0.16 μM), however it was also determined to be more toxic than the bone-targeted analogues **3d** and **3f** (TRAP staining decreased by >50% at ~1.0 μM). Therefore, compound **3d** is an exceptional lead compound relative to its Src kinase inhibitory potency, cellular activity and non-toxicity.

Compound **3d** was further tested against a panel of other protein kinases. It exhibited mixed selectivity for Src

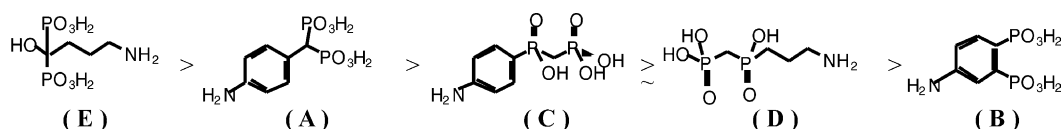
**Figure 2.** Relative hydroxyapatite binding affinities of bone-targeting intermediates.

Table 2. Protein kinase inhibition selectivity of compound **3d**

Selectivity	Protein kinases
1–10	Fyn
10–100	CDK1, CDK2, CDK5
100–500	Lck, PAK3, CSK
> 500	CK2, DNAPK, ERK1, GSK3b, ILK1, MST2, PIM1, PKA, PKB, PKC, WEE1, LAR, MEG2, EST-PT1, PTP1b, TC-PTP

family kinases Fyn and Lck, but showed consistently increasing selectivities toward CDKs (10- to 100-fold range), PAK3 and CSK (100- to 500-fold range), and 16 other protein kinases (> 500-fold selectivity) (Table 2).

Collectively, these results provide initial proof-of-concept data for a potent bone-targeted Src kinase inhibitor which may be advanced by simply modifying a 2,6,9-trisubstituted purine template. Further optimization work to identify in vivo lead compounds for treating bone diseases is ongoing and will be reported in due course.

Acknowledgements

We would like to thank our collaborators Drs. Danny Leung and Jasbinder Sanghera at Kinetek Pharmaceuticals, Inc. for selectivity data on compound **3d**, and Mr. James Biggie and Mr. Richard Brawley at ARIAD for their help in manuscript preparation.

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