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Development of a Chimeric c-Src Kinase and HDAC Inhibitor

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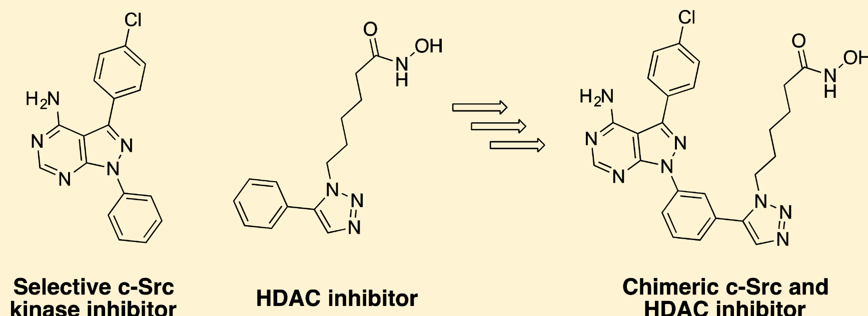
Supporting Information



ABSTRACT: On the basis of synergism observed between a selective c-Src kinase inhibitor with an HDAC inhibitor, the development of the first chimeric c-Src kinase and HDAC inhibitor is described. The optimized chimeric inhibitor is shown to be a potent c-Src and HDAC inhibitor. Chimeric inhibitor **4** is further shown to be highly efficacious in cancer cell lines and significantly more efficacious than a dual-targeting strategy using discrete c-Src and HDAC inhibitors.

KEYWORDS: *c-Src, kinase, chimera, HDAC, combination therapy*

The nonreceptor tyrosine kinase c-Src plays an important role in many aspects of cell physiology, regulating diverse cellular processes including division, motility, adhesion, angiogenesis, and survival.[1,2] c-Src was the first proto-oncogene identified and is frequently overexpressed in cancer, and the extent of overexpression of c-Src correlates with malignant potential.[1,2] Furthermore, c-Src expression levels inversely correlate with patient survival.[1,2] Recently, c-Src activity was shown to be a main mode of resistance to Herceptin, a first line therapy for Her2+ breast cancer.[3] Therefore, c-Src kinase is an attractive therapeutic target in cancer.

We recently reported the first highly selective inhibitor of c-Src (Figure 1).[4] Despite potent biochemical activity against c-Src, our selective c-Src inhibitor (**1**) is only modestly potent in cellular proliferation assays using breast cancer cell lines.[4] Following the success of combinatorial drug therapies in the treatment of HIV,[5] tuberculosis,[6] and other microbial infections,[7] the use of multiple targeted drugs for cancer chemotherapy is increasingly being pursued.[8] We reasoned that multitarget inhibition using our selective c-Src inhibitor might lead to improved cellular efficacy.

To identify drug combinations that would be synergistic with c-Src inhibition, we examined a small library of targeted inhibitors in combination with our selective c-Src inhibitor **1**. These studies were performed in SK-BR-3 cells, a Her2+ breast cancer cell line previously shown to be growth dependent upon c-Src kinase activity.[4,9] From these experiments, we identified that panobinostat, a histone deacetylase (HDAC) inhibitor in clinical trials,[10] was highly synergistic with c-Src inhibitor **1** (Figure 2). HDAC inhibitors have been shown to promote the growth arrest and apoptosis of cancer cells with minimal toxicity.[11] We believe that the observed synergy is due to previously reported mechanisms whereby HDAC inhibitors can

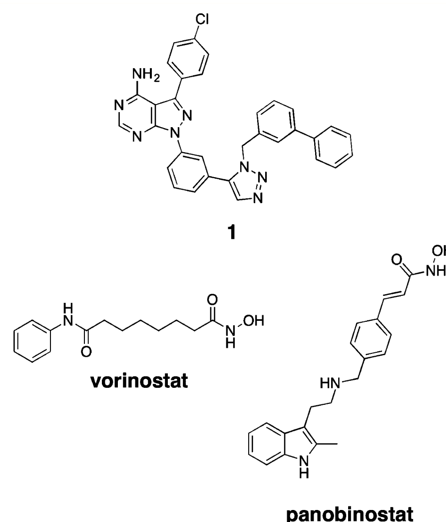


Figure 1. Structures of highly selective c-Src inhibitor **1**, vorinostat, and panobinostat.

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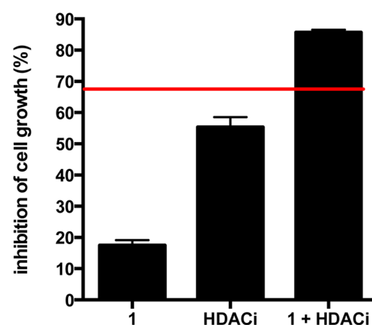


Figure 2. Synergy studies of selective c-Src inhibitor **1** (2 μM), panobinostat (HDACi, 10 nM), and combination (**1** + HDACi, 2 μM **1**, 10 nM panobinostat) in SK-BR-3 cell line. Red line denotes predicted additivity [(eA+eB)-(eA*eB)] of **1** + panobinostat. The higher level of inhibition than the predicted additivity indicated synergism between **1** and panobinostat.

down-regulate c-Src levels through repression of SRC transcription.[12]

To determine whether the synergy observed with c-Src inhibition and panobinostat was general for any HDAC inhibitor, we performed combination experiments with vorinostat,[13] an FDA approved HDAC inhibitor, and c-Src inhibitor **1** (Table 1). c-Src inhibitor **1** and vorinostat have a

Table 1. Cellular Efficacy of Selective c-Src Inhibitor 1, Vorinostat, 1/Vorinostat (1:1), and Chimera 4

	GI_{50} (μM), SK-BR-3	GI_{50} (μM), HMEC	therapeutic index
compound **1**	4.8 ± 0.2	4.3 ± 0.4	0.9
vorinostat	1.2 ± 0.1	5.8 ± 0.2	4.8
1 + vorinostat	0.80 ± 0.03	5.4 ± 0.2	6.8
chimera **4**	0.20 ± 0.03	4.7 ± 0.3	23.5

GI_{50} of 4.8 and 1.2 μM, respectively, for SK-BR-3 proliferation. In combination, c-Src inhibitor **1** + vorinostat (1:1) has a GI_{50} for SK-BR-3 proliferation of 0.8 μM, which is an improvement over either inhibitor dosed alone.[14] Next, as a measure of cellular toxicity, we examined each compound's ability to inhibit proliferation of primary human mammary epithelial cells (HMEC). c-Src inhibitor **1** and vorinostat have a GI_{50} of 4.3 and 5.8 μM, respectively, for HMEC proliferation. The combination of **1** + vorinostat (1:1) has a GI_{50} of 5.4 μM against primary mammary epithelial cells.

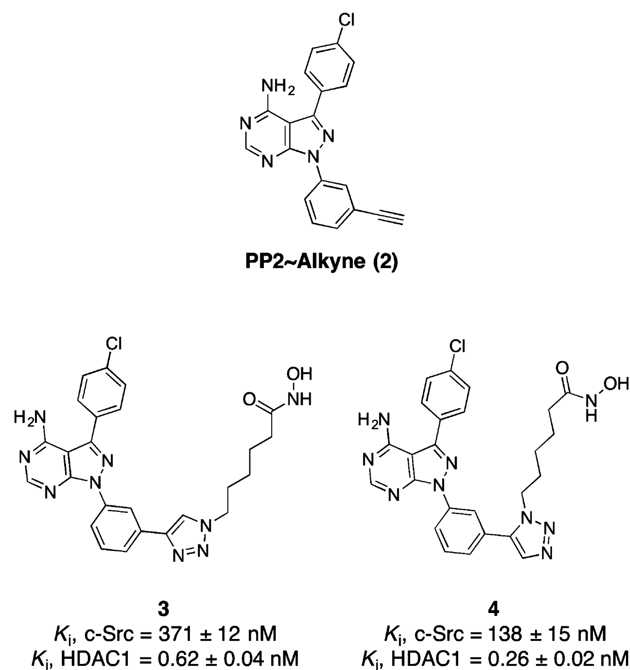
Using the SK-BR-3 and HMEC data, we calculated a therapeutic index (GI_{50} HMEC/GI_{50} SK-BR-3) for c-Src inhibitor **1**, vorinostat, and the combination of **1** + vorinostat (Table 1).[15] c-Src inhibitor **1** has a poor therapeutic index of 0.9, while vorinostat's therapeutic index is 4.8. Disappointingly, the combination of **1** + vorinostat has an insignificant improvement in therapeutic index (6.8) relative to vorinostat alone (4.8).

We wondered whether there would be any advantage for a chimeric inhibitor, where a single molecule could serve as both a c-Src kinase and HDAC inhibitor, rather than using two separate agents in combination. For example, we thought that we might obtain improved cellular efficacy. In addition, using a single agent to inhibit both c-Src and HDAC does not lead to the additive toxicity that is often observed with combination therapy.[14] Chimeric kinase-HDAC inhibitors have previously been developed; however, no Src-HDAC chimeric compounds have been reported.[16−18] In addition, previously reported studies of kinase-HDAC chimeras lack a comparison of therapeutic indices between combination therapy and chimeric inhibition.[16−18]

We previously reported PP2~alkyne (**2**), a modular and selective c-Src inhibitor scaffold.[4] We envisioned using this kinase inhibitor scaffold to append HDAC pharmacophores. The classic pharmacophore for HDAC inhibitors consists of a zinc-binding motif, a hydrophobic linker, and a recognition cap.[19] Using PP2~alkyne, HDAC elements can readily be appended using "click" chemistry.[20] Importantly, the use of a triazole ring as the recognition cap in HDAC inhibitors has previously been reported and shown to be highly efficacious both in vitro and in cellulo.[21] Previous reports with triazole-based HDAC inhibitors have demonstrated that a 6-carbon hydrophobic linker will provide potent HDAC inhibition.[21] While only 1,4-[1,2,3]-triazoles have been reported as HDAC inhibitors,[21] we reasoned that because our selective c-Src inhibitor **1** contains a 1,5-[1,2,3]-triazole,[4] we would synthesize and evaluate both regioisomers.

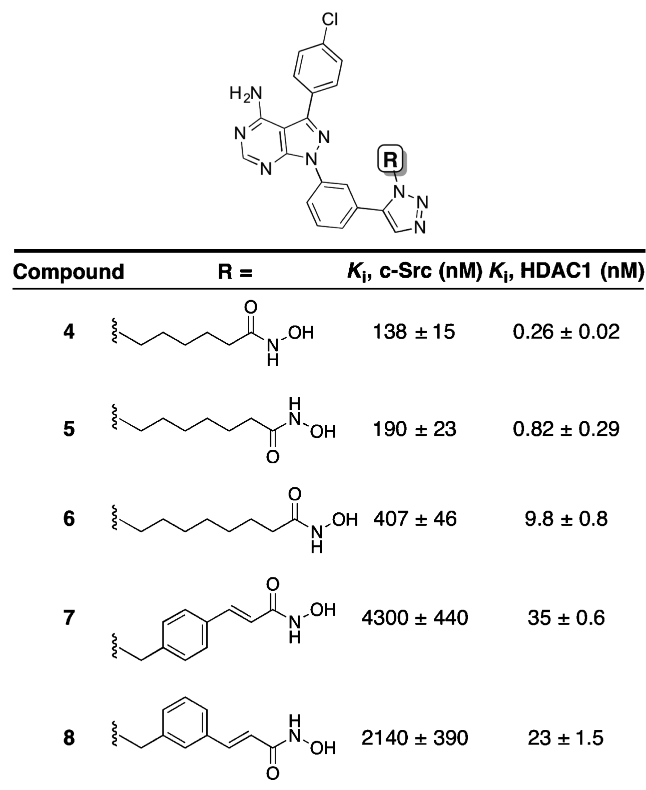
We synthesized compounds **3** and **4** as chimeric Src/HDAC inhibitors. Compound **3** has a 1,4-triazole and was synthesized using a copper-mediated cycloaddition reaction,[20] while compound **4** has a 1,5-triazole synthesized using a ruthenium-mediated cycloaddition reaction (Chart 1, see Supporting Information for synthetic details).[22] Using a previously reported fluorescence assay for c-Src kinase activity,[23] we found that **3** and **4** were competent c-Src kinase inhibitors (K_i = 371 and 138 nM, respectively). We next examined the ability of **3** and **4** to inhibit HDAC1 using a Fluor de Lys based-assay[24] and found both compounds were potent inhibitors of HDAC1 (K_i = 0.62 and 0.26 nM, respectively). In our assays, compound **4** was a better inhibitor of both c-Src and HDAC1. Thus, the 1,5-triazole regiochemistry was used exclusively for subsequent linker optimization.

Chart 1. Structure of PP2~Alkyne (2) and Chimeric Inhibitors 3 and 4



In an effort to optimize potency for both c-Src and HDAC1, we synthesized a series of chimeric HDAC-Src inhibitors containing varied hydrophobic linkers (Table 2). This series included alkyl linkers of varied length as well as styrene-containing linkers that are found in panobinostat.[10] The six-carbon alkyl linker (compound **4**) was found to be optimal for inhibition of both c-Src kinase and HDAC1. Of note, we found the styrene linkers (compounds **7** and **8**) were ineffective as c-Src inhibitors and only modest inhibitors of HDAC1 compared to the *n*-alkyl linkers.

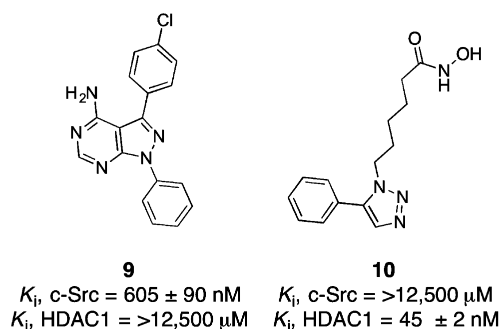
Table 2. SAR of Linker



Compound	R =	K_i, c-Src (nM)	K_i, HDAC1 (nM)
4		138 ± 15	0.26 ± 0.02
5		190 ± 23	0.82 ± 0.29
6		407 ± 46	9.8 ± 0.8
7		4300 ± 440	35 ± 0.6
8		2140 ± 390	23 ± 1.5

Chimeric inhibitor **4** is one of the most potent HDAC1 inhibitors reported to date (K_i = 260 pM) and is also a potent c-Src inhibitor (K_i = 138 nM). To decipher the binding contributions for each half of the chimera, two fragments of inhibitor **4** were synthesized (Chart 2). Compound **10** contains only the HDAC inhibitor pharmacophore, while compound **9** includes the c-Src kinase binding elements. Interestingly, we observe a marked decrease in affinity for both c-Src and HDAC1 when both elements are not present. Specifically, compound **10**, which retains all of the HDAC inhibitor pharmacophore elements, has a K_i for HDAC1 that is >170-times higher than found with chimeric inhibitor **4**. These data imply that the c-Src binding elements enhance HDAC1 inhibition observed with compound **4**. Likewise, the c-Src inhibitor fragment **9** has nearly 10× less affinity for c-Src than chimera **4**, suggesting that the addition of the HDAC fragment is important for c-Src inhibition. Together, these data demonstrate that chimera **4** is not simply two inhibitors linked together, but rather represents a merged inhibitor where both elements are required for affinity against each target.

Chart 2. c-Src Inhibitor 9 and HDAC Inhibitor 10



Our chimeric inhibitor was initially optimized for HDAC inhibition using HDAC1; however, we assumed it could be a promiscuous inhibitor of HDACs. Profiling of compound **4** against a panel of 11 HDACs was performed by Reaction Biology (Malvern, PA). The HDAC profiling revealed that our chimera is a potent and nonselective inhibitor against class I, IIa, and IV HDACs (Table 3). Consistent with vorinostat's selectivity, chimera **4** is not an effective inhibitor of class IIa HDACs (Table 3). Relative to vorinostat, chimera **4** has improved affinity to all HDACs except HDAC8 and HDAC11.

Table 3. HDAC Profiling of Chimera 4 and Vorinostat

	HDAC class	IC_{50} (nM), Chimera 4	IC_{50} (nM), vorinostat[a]
HDAC1	I	86 ± 11	306
HDAC2	I	231 ± 39	232
HDAC3	I	19 ± 1	132
HDAC4	IIa	3982 ± 920	76000
HDAC5	IIa	3891 ± 681	27200
HDAC6	IIb	2.7 ± 0.5	20
HDAC7	IIa	13220 ± 860	105000
HDAC8	I	2311 ± 251	306
HDAC9	IIa	28020 ± 1910	141000
HDAC10	IIb	51 ± 3	432
HDAC11	IV	224 ± 26	200

[a]Data from Reaction Biology (Malvern, PA).

In previously published work, we found that c-Src inhibitors that are selective for c-Src over c-Abl are more efficacious in cell culture with nonhematopoietic cancers.[4] Thus, we wanted to determine whether chimera **4** has selectivity for c-Src over c-Abl. Gratifyingly, in our biochemical assay, chimera **4** was selective for c-Src over c-Abl (K_i for c-Src = 138 nM; K_i for c-Abl = 2350 nM). We next tested the ability of **4** to inhibit Hck, a SRC-family kinase with 85% similarity across the kinase domain to c-Src, and found it has a K_i = 504 nM. Together, these data suggest that chimera **4** is selective for c-Src over homologous kinases. Given that our compound shares many features with our highly selective c-Src inhibitor **1**,[4] it is likely that chimera **4** is also selective for c-Src.

In an effort to compare chimeric inhibition to dual-targeting c-Src and HDAC1, we examined the efficacy of chimera **4** in cellulo. Combination dosing of selective c-Src inhibitor **1** + vorinostat (1:1) was found to have a GI_{50} = 0.78 μM for SK-BR-3 cells and a GI_{50} = 5.4 μM for noncancer HME cells. This resulted in a therapeutic index of 6.8 (vide supra). In comparison, chimeric inhibitor **4** was more efficacious at inhibiting the growth of SK-BR-3 cells (GI_{50} = 0.2 μM) and has similar noncaner cellular toxicity (GI_{50} = 4.7 μM for HME cells), resulting in a cellular therapeutic index of 23.5 (Table 1). This corresponds to chimeric inhibitor **4** having an improve-

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ment in therapeutic index significantly higher than dual targeting c-Src and HDACs with two distinct compounds (23.5 versus 6.8, respectively). These results highlight an important advantage for chimeric inhibition over dual-agent targeting: we observe synergistic activity against cancer cells while not increasing the cellular toxicity relative to the single agent counterparts.

To better characterize the cellular efficacy of our chimeric c-Src/HDAC inhibitor, compound **4** was submitted to the National Cancer Institute for screening in the NCI-60 panel (see Supporting Information for full NCI-60 data).[25] From this panel, chimera **4** has an average GI_{50} = 0.26 μM. Significantly, the efficacy of chimera **4** across the NCI-60 is better than vorinostat (NCI-60 average GI_{50} = 0.53 μM) and an FDA-approved c-Src inhibitor (dasatinib, NCI-60 average GI_{50} = 5.7 μM). In addition to the improved efficacy across the NCI-60 panel, chimera **4** does not have increased toxicity relative to primary human mammary cells (chimera **4**, HMEC GI_{50} = 4.7 μM; vorinostat, HMEC GI_{50} = 5.8 μM; dasatinib, HMEC GI_{50} = 1.8 μM).

Analysis of the NCI-60 data demonstrates that chimera **4** is a highly efficacious agent in cell lines where vorinostat and dasatinib are ineffective alone (Table 4a). Furthermore, chimera **4** is more efficacious than vorinostat when c-Src inhibition is shown to be efficacious (Table 4b). For example, chimera **4** is an efficacious inhibitor of Hs 578T, a triple negative breast cancer cell line, cell growth (GI_{50} = 0.17 μM), while vorinostat is not (GI_{50} = 4.83 μM), due to c-Src inhibition having an important role in Hs 578T cell proliferation (dasatinib GI_{50} = 0.03 μM). This dramatic increase in efficacy demonstrates that chimera **4** is acting as more than a HDAC inhibitor alone in cellulo. Finally, chimera **4** is observed to be more effective than dasatinib in cell lines where cellular proliferation is dependent upon HDAC1 activity (Table 4c). Together, these data demonstrate that compound **4**'s impressive cellular efficacy in the NCI-60 panel is inherent in its chimeric nature, and the ability to inhibit both c-Src kinase and HDAC1 is required for the cellular potency observed.

Table 4. NCI-60 Panel Data for Chimera 4, Vorinostat, and Dasatinib against Select Cell Lines

	cell line	GI_{50} (μM), chimera 4	GI_{50} (μM), vorinostat	GI_{50} (μM), dasatinib
a	KM12	0.47	1.88	7.44
	MCF7	0.35	2.19	8.32
	U251	0.28	1.53	2.81
b	DU-145	0.39	1.36	0.16
	HS 578T	0.17	4.83	0.03
	MDA-MB-231	0.39	2.32	0.02
c	HCT-116	0.22	0.37	3.70
	MALME-3M	0.07	0.37	6.61
	SW-620	0.25	0.54	8.43

In summary, we have reported the first chimeric c-Src kinase and HDAC inhibitor. Furthermore, we have performed detailed studies that demonstrate that chimera **4** is a potent and selective c-Src kinase inhibitor as well as a potent and nonselective HDAC inhibitor. We demonstrated that our chimeric inhibitor has improved efficacy in cellular experiments compared to dosing two individual inhibitors targeting c-Src and HDACs. Chimera **4** has significant efficacy in the NCI-60 panel, while not possessing significant toxicity to primary human cells, and represents a novel small molecule probe that can provide simultaneous inhibition of c-Src and HDACs. Our approach to constructing kinase-HDAC inhibitor hybrids using a triazole linkage should be general and readily adapted to any kinase and/or HDAC pair of interest.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures and characterization data, assay conditions, and cell data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors, and all authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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