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Privileged scaffolds for blocking protein–protein interactions: 1,4-disubstituted naphthalene antagonists of transcription factor complex HOX–PBX/DNA

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Abstract—Structure-based-design studies, with the crystal structure of the HOXB1–PBX1/DNA transcription factor complex, were used to identify 1,4-disubstituted naphthalenes as potential antagonists. An initial library of 32 analogs was synthesized, two of which were found to be more potent than the reported activity for a 12 amino acid peptide antagonist. Antagonists were also identified of the related BRN1/DNA and BRN2/DNA transcription factor complexes indicating that a 1,4-disubsituted naphthalene may be a privileged scaffold for preparing screening libraries targeting this family of transcription factor complexes.

The term 'privileged structure' was first introduced by Evans et al.[1] This term has been used to denote a molecular framework (scaffold) that is able to provide ligands for individual multiple targets when suitably decorated with side chains. A variety of privileged scaffolds have been reported since that time.[2] Focused combinatorial libraries, based upon these privileged scaffolds, provide enriched hit rates when screening for receptor agonists/antagonists, ion channels modulators, or enzyme inhibitors. In contrast to these more traditional targets, discovering small molecules capable of disrupting protein–protein interactions has proven to be more challenging, partly because the contact surface between bound proteins typically covers a relative large surface area.[3] Random high-throughput screening for antagonists of protein–protein interactions has generally been less successful than has screening against the traditional categories of drug targets. When the generally accepted MW 500 limit for compounds likely to be orally active is applied to the reported antagonists of protein–protein interactions, then the majority of the remaining antagonists have potencies in the 1–100 μM range. Most of these antagonists were discovered by screening, with only a minority obtained by design.[4]

Transcription factor complexes are responsible for much of the regulation of gene expression[5] and therefore offer many potential protein–protein interaction drug targets,[6] including anticancer targets.[7] The 39 members of the HOX family[8a] and 4 members of the PBX family[8b] are proteins that bind DNA as heterodimers to form transcription factor complexes. The numerous heterodimeric HOX/PBX combinations play critical and complex roles in transcriptional regulation during patterning in early embryonic development[9] and many are utilized again in specific tissues of the adult.[10] A variety of cancers display altered HOX gene regulation.[11] For example, in leukemias and lung cancers HOX genes are overexpressed and consequently antagonists of the HOX/PBX complexes controlling these genes offers the potential for novel anticancer drugs. Also small molecule antagonists of individual (or subsets) HOX/PBX complexes can be used as pharmacological tools to investigation their function.

Herein we report the first small molecule antagonists of HOX–PBX protein–protein interactions.[12] A crystal structure of the minimal HOXB1 and PBX1 fragments necessary for cooperative DNA binding has been reported.[13] This structure showed that HOXB1 and PBX1 bind to overlapping sites on opposite faces of the

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DNA double helix. However, the two proteins also bind to each other, and thereby stabilize the ternary complex, where HOXB1 places a short peptide arm (FDWMK) into a hydrophobic binding pocket (a 'hot spot') on the surface of PBX1. Previous in vitro studies have shown that disruption of the cooperative DNA binding by HOX to PBX1 proteins can be accomplished by point mutations in this HOX peptide domain.[14] These studies also showed that the Trp 182 (W) and Met 183 (M) residues within the highly conserved pentapeptide region of the HOX family of peptide domains (F/Y-P/D-W-M-K/R) were critical for cooperative DNA binding with PBX. The crystal structure shows HOXB1 Trp 182 and Met 183 packing against each other and binding in the hydrophobic pocket on the surface of PBX1.[13] This cooperative binding can be competitively blocked by the 12-residue peptide, QPQIYPWMRKLH ($IC_{50} = 100\,\mu M$), containing the conserved pentapeptide sequence.[14]

The human HOXB1–PBX1/DNA (code 1B72) complex described above was downloaded from the Protein Data Bank (www.rcsb.org). All molecular modeling, ligand docking, and library design studies were carried out using SYBYL 6.8, and the associated modules FlexX, CScore, and LeapFrog, all obtained from Tripos, Inc. (www.tripos.com). The pentapeptide region of HOXB1 that binds in the hydrophobic pocket on the surface of PBX1 as described above was deleted to expose the critical peptide-binding region for designing small molecule antagonists, and the resulting complex was minimized. A variety of potential scaffolds, with representative appended side chains, were docked into the now exposed hydrophobic pocket and the surrounding surface of PBX1. These candidate scaffolds were selected by a combination of visual evaluation of the binding surface, hand docking of candidate ligands, automated docking (FlexX), de novo design experiments (LeapFrog), ease of synthesis, and predictions of binding affinity (FlexX and CScore). An important criteria that was also applied to the candidate scaffold selection process is the ability to produce potential antagonists with MW<500 and that have the ability to meet the additional 'rule of 5' criteria developed by Lipinski et al.[15] for compounds likely to be successful as oral therapeutics. Finally, rigid scaffolds that result in antagonists with a limited number of rotatable bonds were given priority since this rigidity is also predicted to improve the probability of obtaining orally active drugs.[16]

With the above criteria in mind the first scaffold selected for synthesis and testing was the 1,4-disubstituted naphthalene scaffold (Table 1). This rigid scaffold provides two diversity side chains able to interact with the PBX protein at the mouth of the hydrophobic pocket and a phenyl ring to penetrate into the hydrophobic pocket. The library of potential antagonists prepared from this scaffold all have molecular weights between 340 (**7**) and 489 Da (**36**) and have a limited number of freely rotating bonds (Table 1).

A modeled complex (after minimization) of the parent inhibitor based upon this scaffold, **7**, is illustrated in Figure 1. As shown, the naphthalene ring is positioned within the hydrophobic pocket, the C-1 amide side chain NH is forming a hydrogen bond with Leu-252 and the 3-fluoro atom on the phenyl amide side chain is hydrogen bonding with Try-291. The C-4 ether amide side chain is extending across the surface of PBX1 in the direction of the bound DNA. From these modeling studies the C-4 side chain appeared to have multiple potential binding opportunities so a range of side chains at this position was selected for experimental evaluation (Table 1).

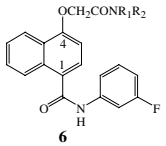
A focused combinatorial library of thirty-two 1,4-disubstituted naphthalene derivatives with the modeled 3-fluorophenyl amide C-1 side chain and various ether amide side chains at C-4 were synthesized (Fig. 1). The synthesis begins from commercially available 4-methoxy-1-naphthaldehyde **1** as outlined in Figure 1. Oxidation of aldehyde **1** provided acid **2**.[17] Amide **3** was obtained by coupling the corresponding acid chloride with 3-fluoroaniline. Demethylation of methyl ether **3** using $AlCl_3$/EtSH produced the corresponding phenol,[18] which was then converted to methyl ester **4**. Acid **5** was obtained by refluxing ester **4** with LiOH in methanol. The final target amides **6** were synthesized using PyBOP as the coupling reagent with a broad range of amines. All final products were purified by silica gel chromatography to greater than 95% purity and gave ^{1}H NMR and MS spectra that are consistent with the expected product.

The selectivity of individual members of the library for the HOXA1–PBX1/DNA target, as well as the ability of some analogs to cross-over to other transcription factor targets was evaluated by testing them in parallel against the BRN1 and BRN2 transcription factors. BRN1 and BRN2 are members of mammalian class III POU transcription factor family and are expressed in the developing embryonic brain.[19] POU domain class transcription factors contain, in addition to the POU domain, a homeodomain helix-turn-helix class DNA binding motif similar to that found in the HOX and PBX class transcription factors.[20] EMSAs were performed with BRN1 and BRN2 exactly as was done with HOXA1–PBX1 except that a sequence recognition element known to bind BRN1/BRN2 was used as the probe.[21]

Of the 32 analogs listed in Table 1, 24 showed measurable inhibition of the formation of the HOX1–PBX1/DNA ternary complex when screened at a 300 μM initial concentration. A relatively high initial screening concentration was chosen for two reasons: (1) the known 12-residue peptide antagonist, QPQIYPWMRKLH, containing the conserved pentapeptide sequence (underlined) that binds to the Pbx1 hydrophobic pocket, has an IC_{50} of only 100 μM, (2) protein–protein interaction targets are significantly more challenging to block with small molecules than the classical drug targets for which lower screening concentrations can be used.

Of these 24 active compounds against the HOXA1–PBX1/DNA target the most potent were **30** and **31** with an IC_{50}'s = 86 and 65 μM, respectively. These com-

Table 1. HOXA1–PBX1/DNA, BRN1/DNA, and BRN2/DNA antagonist activity of 1,4-disubsituted naphthalenes[a]



6

Compound	R_1; R_2	HOXA1–PBX1 IC_{50} (μM)[b]	BRN1 IC_{50} (μM)[c]	BRN2 IC_{50} (μM)[c]
7	H; H	272		
8	NH_2; H	Low activity (300 μM)		
9	Methyl; H	Low activity (300 μM)		
10	Ethyl; H	200		
11	*n*-Propyl; H	278		
12	Isopropyl; H	272		
13	Isobutyl; H	298		
14	*tert*-Butyl; H	No activity (300 μM)		
15	Cyclopropyl; H	173	810	>10,000
16	Propargyl; H	331		
17	2,2,2-Trifluoroethyl; H	111	162	204
18	2-Hydroxyethyl; H	No activity (300 μM)		
19	2-Benzyloxyethyl; H	Low activity (300 μM)		
20	2-Dimethylaminoethyl	No activity (300 μM)		
21	$-CH_2COOCH_2CH_3$; H	No activity (300 μM)		
22	$-CH_2COOH$; H	No activity (300 μM)		
23	Phenyl; H	254		
24	2-Fluorophenyl; H	433		
25	3-Fluorophenyl; H	Low activity (300 μM)		
26	4-Fluorophenyl; H	293		
27	2-Methoxyphenyl; H	718		
28	3-Methoxyphenyl; H	580		
29	4-Methoxyphenyl; H	161		
30	3-Hydroxyphenyl; H	86	142	148
31	4-Hydroxyphenyl; H	65	124	138
32	3,5-Dimethoxyphenyl; H	Low activity (300 μM)		
33	2,5-Dimethoxyphenyl; H	Low activity (300 μM)		
34	3,4-Dimethoxyphenyl; H	464		
35	2,4-Dimethylphenyl; H	Low activity (300 μM)		
36	3,5-Dimethoxybenzyl; H	No activity (300 μM)		
37	Methyl; methyl	No activity (300 μM)		
38	R_1, $R_2 = (CH_2CH_2)_2O$	No activity (300 μM)		

[a] Determined by electrophoretic mobility shift assay (EMSA). IC_{50} data results from an averaged densitometric analysis of multiple EMSA gels.

[b] 2.5 ng each of the mouse HOXA1 192–288 protein and the mouse/human 233–319 PBX1 protein in a final volume of 25 μL binding buffer containing 10 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM DTT, 5% Ficoll-400, 0.1 mg/mL BSA, and 135 mM NaCl was pre-incubated for 15 min at 4 °C with 0.5 μg poly dIdC (to reduce nonspecific DNA binding) and 1 μL of an appropriate dilution of the inhibitory compound in DMSO. Proteins in binding buffer were incubated for 30 min at approximately 22 °C with 1 μL of ^{32}P labeled oligonucleotide probe prepared as described below (1–2×10^4 CPM) prior to electrophoresis on a 4% polyacrylimide gel in 50 mM TBE buffer at 150 V for 2 h 15 min. Two oligonucleotides, HOXPBX-CT Top, 5′CTCTCCTTTTGATTGATTAA-3′, and HOXPBX-CT Bottom 5′-AGAGCTTAATCAATCAAAAGG-3′ were annealed and the ends extended with Klenow in the presence of α32P-dCTP to prepare the oligonucleotide probes. Following electrophoresis, gels were dried and exposed to X-ray autoradiographic film for 18–24 h.

[c] EMSAs were performed with BRN1 and BRN2 exactly as was done with HOXA1–PBX1 when using nuclear extracts from RA treated P19 cells (for BRN induced by RA in P19 cells see Ref. 23) except that a sequence recognition element known to bind BRN1 or BRN2 monomers was used as the probe.[21]

pounds are more potent than that reported for the much larger 12-residue peptide antagonist QPQIYPWMR-KLH containing the conserved pentapeptide sequence[16] and differ from each other only by the position of the hydroxyl group in the C-4 aryl amide moiety.

Figure 2 illustrates that **31** inhibits the formation of the PBX1/DNA binary complex as well the ternary HOX1–PBX1/DNA complex whereas **15** inhibits formation of ternary complex only. Antagonist **31** differs from **15** by replacing the small cyclopropyl R_1 with the larger 4-hydroxyphenyl group. Our modeled complex (Fig. 1) indicated that these substituents are directed toward the bound DNA. Consequently, the larger 4-hydroxyphenyl group may be altering the conformation of the PBX1/DNA binary complex so as to destabilize it whereas the smaller cyclopropyl group does not extend far enough into the DNA binding region to do this. On the other hand both antagonists can bind in the hydrophobic pocket thereby preventing HOXA1 from binding to the binary complex. Upon closer evaluation of the **31** dose–response (data not shown) of the binary PBX/DNA band intensity relative to the ternary HOXA1–PBX/DNA band intensity it was noted that the binary PBX/

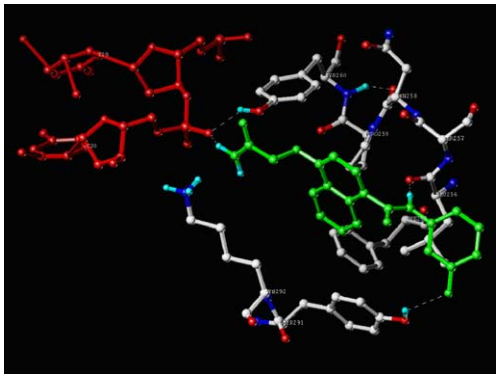
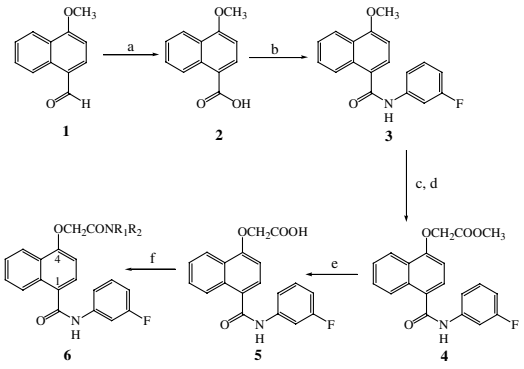


Figure 1. Modeled complex of **7** docked into PBX1/DNA hydrophobic pocket with hydrogen bonds indicated by dash lines. Ligand **7** is in green, DNA is in red, PBX1 is atom color coded.



Scheme 1. (a) $NaClO_2$, NaH_2PO_4, H_2O, *t*-BuOH, 2-methyl-2-butene, rt, 24 h, 95%; (b) (1) $SOCl_2$, CH_2Cl_2, reflux (50 °C), 6 h; (2) 3-fluoroaniline, THF, DIEA, reflux, 0.5 h, 90%; (c) $AlCl_3$, CH_2CH_2SH, CH_2Cl_2, 0 °C to rt, 2 h, 93%; (d) $BrCH_2COOCH_3$, K_2CO_3, THF, reflux, 1 h, 100%; (e) LiOH, MeOH, H_2O, reflux, 2 h, 95%; (f) PyBOP, R_1R_2NH, DMF, 0 °C to rt, 2 h, 90–95%.

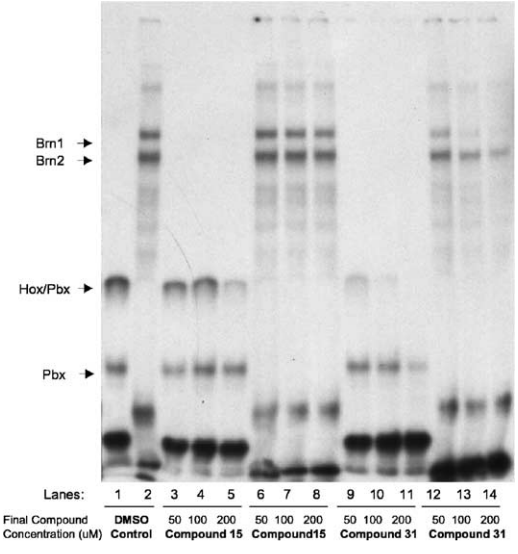


Figure 2. Selectivity of **15** and **31** for HOXA1–PBX1/DNA, PBX1/DNA, BRN1/DNA, and BRN2/DNA.

DNA band initially *increases* in intensity at low **31** concentrations. One potential explanation for this result is that HOXA1 is released from the ternary complex generating a higher initial concentration of the binary complex. As the concentration of **31** is further increased the binary PBX/DNA complex formation is inhibited enough to overcome the release of this binary complex from the ternary complex and therefore the binary band intensity begins to decrease.

Collectively, the above results suggest, but do not prove that compounds based on the 1,4-disubstituted naphthalene scaffold are binding to PBX1 rather than directly binding to DNA. If **15** were directly binding to DNA then it would need to be binding to a region of the DNA not contacted by PBX1 in order to explain the selective inhibition of the formation of the HOXA1–PBX1/DNA ternary complex versus the PBX1/DNA binary complex. Likewise if **31** were directly binding to DNA then it would need to be binding to a region of DNA not contacted by PBX1 since low concentrations of **31** *increase* the concentration of the PBX1/DNA binary complex. Since both results are more readily explained by binding of the compounds in the pentapeptide binding pocket, for which they have complementary topological features by design, it is likely that the mechanism of inhibition is direct binding to PBX1.

Figure 2 illustrates that **15** is also selective toward the HOXA1–PBX1/DNA target ($IC_{50} = 173\,\mu M$) versus BRN1/DNA ($IC_{50} = 810\,\mu M$) or BRN2/DNA ($IC_{50} = >10{,}000\,\mu M$). This result again suggests direct binding to PBX1 rather than to DNA. Extension **15** from the pentapeptide pocket into the DNA binding region provided **31**, which now inhibits BRN1 and BRN2 from binding to their recognition sequences with IC_{50}'s of 124 and 138 μM, respectively, versus 65 μM against HOXA1–PBX1/DNA. Overall, **15** is selective for the blocking the HOXA1–PBX1/DNA ternary complex relative to all of the other complexes tested whereas **31** is an active antagonist of all of the complexes with some selectivity for the HOXA1–PBX1/DNA complex. Since **31** can extend into the DNA binding region of the PBX1/DNA complex, and thereby block DNA from binding, BRN1 and 2 may have similar hydrophobic pockets resulting in the ability of **31** to block DNA from binding to them as well.

The ability of the 1,4-disubstituted naphthalenes to cross-over from PBX to the BRN transcription factors and antagonize their DNA complex, when suitable side chains are present, suggests that this may be a privileged scaffold for related transcription factors. The known tendency[22] of proteins to reuse general binding motifs for a new purpose, after evolving the necessary modifications to attain a new function, suggests that the PBX hydrophobic pocket targeted by these antagonists may be reused in BRN and other transcription factor complexes. Consequently, larger screening libraries based upon this scaffold may produce antagonists, including more potent ones, of various members of this class of transcription factors. A library of such antagonists, including those selective for ternary versus binary

complexes as discovered herein, would serve as valuable bio-probes (or drug leads) for investigating the role of these transcription factors in various biological processes and diseases.

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