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p16^{INK4a} Peptide mimetics identified via virtual screening

Mark A. Klein [a,b,*], Kevin H. Mayo [c], Robert A. Kratzke [a,b,d,*]

[a] Research Service, Minneapolis Veterans Affairs Medical Center, Minneapolis, MN 55417, USA
[b] Department of Medicine, University of Minnesota, Minneapolis, MN 55455, USA
[c] Departments of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA
[d] Department of Surgery, University of Minnesota, Minneapolis, MN 55455, USA

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ABSTRACT

The transition from G1 to S phase in the cell cycle is highly regulated by Cdk4 and Cdk6, which in turn is inhibited by the tumor suppressor p16^{INK4a}. Replacement of lost p16^{INK4a} activity in cancer cells via gene therapy has worked in vivo to decrease tumor progression; however, practical issues limit gene therapy applications at this time. Here, we report the discovery of compounds that inhibit Cdk4 and Cdk6 activity. The NMR structure of a peptide that exhibits p16^{INK4a} activity was solved and combined with known functional data to generate a pharmacophore that was used to mine the NCI chemical database. The hits were filtered utilizing the program Qikprop. Four compounds were subsequently shown to inhibit Cdk4 and/or Cdk6 with IC_{50} in the μM range. These compounds form lead compounds upon which further cell cycle inhibitors can be developed.

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The cell cycle plays a critical role in cancer progression. Among the regulators of the cell cycle is p16^{INK4a}, a member of a family of protein inhibitors of Cdk4 and Cdk6 (Cdk4/6).[1–3] Lack of expression of p16^{INK4a} has been identified in a number of cancers, including lung cancer.[1,4] It has been demonstrated that re-expression of p16^{INK4a} activity in lung cancer cell lines results in cell proliferation inhibition and cell cycle arrest.[5,6] In addition, tumor suppression using viral vectors recapitulating this activity has been demonstrated in vivo in mice.[7]

It has been shown that a small 20 amino acid peptide from the third ankyrin-like repeat of p16^{INK4a} can significantly inhibit Cdk4/6 activity.[8] It has also been demonstrated that a 10 amino acid subset from that same peptide could also significantly inhibit Cdk4 kinase activity. Frizelle et al. demonstrated that a 32 amino acid peptide consisting of the HIV TAT protein transduction domain, a glycine linker, and the 20 amino acid sequence described above showed decreased mesothelioma cell proliferation, cell cycle arrest, hypophosphorylation of pRb, and activity against mouse mesothelioma xenografts.[9]

The goal of this project was to see if small molecule inhibitors could be found that mimic the pharmacophore site of a 10 amino acid peptide similar in sequence to that described above.[10] Here, we describe the discovery of four lead agents by the utilization of structure-function relationships in screening a small molecule database.

NMR spectra were obtained from peptides dissolved in water or SDS micelles via a homonuclear approach (TOCSY and NOESY) described previously.[11–14] Initial NMR spectra for p16_10 in water failed to yield significant internuclear NOEs consistent with structure. Specifically, intermediate range NOEs were not observed. This is consistent with random coil. However, it is well known that co-solvents or dissolution of peptides in a micellar environment can help to stabilize certain structural elements. When p16_10 was dissolved in 100 mM SDS/H_2O, a significant number of intermediate NOEs and temperature factors consistent with helix were found; therefore, it follows that even though peptides interconvert between structures, that p16_10 likely is in a helical conformation a significant part of the time.

Analysis of NOEs revealed a total of 130 internuclear distance constraints, consisting of 70 intraresidue constraints, 37 sequential constraints, and 23 medium range ($|i-j|<5$) constraints. Backbone NOEs are summarized in Figure 1 and are consistent with the presence of helix from L2 to V7. Chemical shift temperature dependence was determined to identify evidence for hydrogen bonds (Fig. 1). A total of five hydrogen bonds were inferred from chemical shift temperature differences ($\Delta\delta\Delta T > -5$ ppb/K), and included the amide protons of L5, V6, V7, L8, and R10; therefore, 10 hydrogen bond constraints were included in the structural modeling.

One hundred structures were calculated via the X-PLOR routine. The 23 lowest energy structures were selected and are displayed in Figure 1. NOEs did not deviate more than 0.5 Å. Structural statistics are summarized in Table 1. The calculated structures are consistent with helix from residues 2–10 with less structurally defined areas

* Corresponding authors.
E-mail address: klein062@umn.edu (M.A. Klein).

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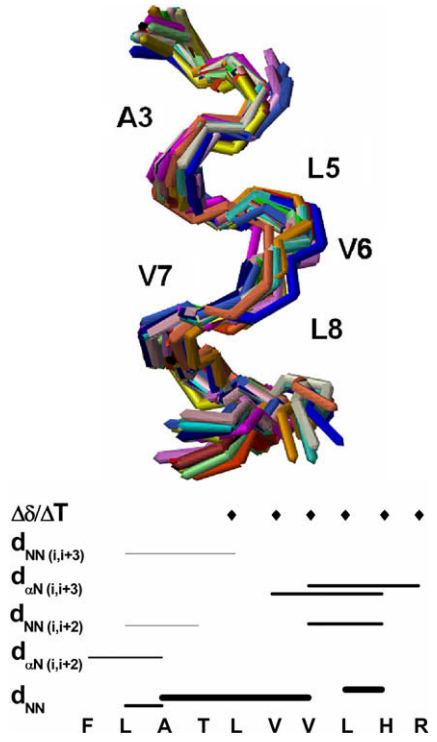


Figure 1. (A) NOE (nuclear Overhauser effect) and temperature factor-derived structures (overlay of 23 structures). (B) Backbone–backbone NOEs are shown with relative thickness proportional to the strength of the interaction. Temperature factors ($\Delta\delta/\Delta T$) consistent with hydrogen bonds are displayed via diamonds.

Table 1
Structural statistics for the lowest energy structures

	p16_10[21,a]
RMS deviations (Å)[b]	
NOE (130)	0.082 ± 0.02
H-bonds	10
Deviations from idealized geometry	
Bonds (Å)	0.0066 ± 0.0005
Angles (°)	0.9 ± 0.04
Energies (kcal mol^{-1})	
E_{NOE}[c]	53 ± 8
E_{BOND}	78 ± 1
E_{ANGLE}	40 ± 3
E_{TOTAL}	130 ± 27

[a] The number of structures over which values have been averaged.
[b] RMS Deviations from experimental distance restraints (Å). None of the final structures exhibited distance restraint violations greater than 0.5 Å or dihedral angle violations greater than 5°. RMSD values represent the mean and standard deviations for the structures.
[c] The final values of the NOE (ENOE), torsion angle (ECDIH) and NCS (ENCS) potentials have been calculated with force constants of 50 kcal mol^{-1} Å^{-2}, 200 kcal mol^{-1} rad^{-2}, and 300 kcal mol^{-1} Å^{-2}, respectively.

at the amino and carboxy terminals. RMSD of the backbone was 1.1 Å in comparison with the native structure of p16^{INK4a}.

Information from mutational studies conducted previously and our NMR studies was used to model a proposed pharmacophore site.[8] Previous research has shown that amino acid residues corresponding to L94A, V95A, V96A, and L97A decreased the peptide affinity to Cdk4 and Cdk6 while D92A increased affinity.[8] Three potential pharmacophore sites were modeled based on this synthesis of data corresponding to the representative residues from peptide p16_10 ($F_1L_2A_3T_4L_5V_6V_7L_8H_9R_{10}$): $A_3V_6V_7$, $A_3L_5V_6V_7$, $A_3V_6V_7L_8$, (AVV demonstrated in Fig. 2). Initial screening of $A_3V_6V_7$ yielded 555 compounds out of 234,054 as hits. Lipinski's rules were applied in conjunction with the program Qikprop to narrow 'hits' to the compounds predicted to have the greatest drug-like qualities (oral absorption and solubility).[15–17] This resulted in 164 compounds out of an original 234,054. The other two pharmacophores yielded <100 compounds each and were not used further.

To triage for those compounds that might have the highest affinity for the p16^{INK4a}-binding site on Cdk6, these 164 compounds were docked along the p16^{INK4a}-binding face of Cdk6 (no X-ray structure of Cdk4 existed at the time of the docking) using the program Glide.[18–20] The majority of these compounds dock to the same binding surface as p16^{INK4a}, albeit encompassing a smaller surface area. The top 20 compounds with the highest scores (indicating tightest predicted binding) were then ordered from the Developmental Therapeutics Program at the NCI.

Fourteen compounds were evaluated for kinase inhibitory activity via an Adapta Kinase assay (Invitrogen) utilizing time-resolved fluorescence resonance energy transfer.

Of the 14 compounds that were evaluated for the ability to inhibit Cdk4/cyclin D1 and Cdk6/cyclin D1 in vitro, four showed inhibition against Cdk4 (NCI 29992, 14893, 13719, and 29997), while two showed activity against Cdk6 (29992, 14893) (see Table 2).

In this Letter, we describe a multifaceted approach to replacing p16^{INK4a} Cdk4/Cdk6 inhibitory activity utilizing known functional contributions of individual residues to peptide activity, structural studies, virtual screening, computational docking, and kinase activity assays. Previously, it has been demonstrated that the 20 amino acid peptide (DAAREGFLDTLVVHRAGAR) and the 10 amino acid peptide (FLDTLVVLHR) could significantly replace the activity of full length p16^{INK4a}.[8,10,21] It has also been established that replacement of L_{11}, V_{12}, V_{13}, and L_{14} with alanine greatly decreased the activity of the 20 amino acid peptide, while replacement of D_9 with alanine improves its activity. Here, we applied that information to the evidence (as derived from NMR structural studies) for helix structure of the peptide p16_10 (FLATLVVLHR) to generate a possible pharmacophore that may be responsible for the peptides' activities.

The pharmacophore $A_3V_6V_7$ as derived from $F_1L_2A_3T_4L_5V_6V_7L_8H_9R_{10}$ forms 3 points on the face of one turn of the helix and yielded 555 hits compared to few for the pharmacophores $A_3L_5V_6V_7$ and $A_3V_6V_7L_8$. These 3 pharmacophores were chosen due to the results obtained previously.[8,10] Five of six residues significantly change the activity of the original 20 amino acid peptide. Even though the p16_10 micellar peptide structures statistically correlate well with that found in the X-ray structure of p16^{INK4a} bound to Cdk6, it is unclear how the peptide fragment actually binds to Cdk4 or Cdk6.[22] In the Letter by Russo et al., the corresponding helix makes contact with Cdk6 via D92 and F90, but not via the residues $L_{94}V_{95}V_{96}L_{97}$. Therefore, we postulated that while a 3 or 4 point pharmacophore may be involved in binding to Cdk4/6, it is unlikely that a 5 point pharmacophore is essential to binding. Lack of hits via the 4 point pharmacophores may be due to the restrictive nature of a 4 point query.

Qikprop and Lipinski rule filtering for drug-like qualities and solubility was chosen to make the list of hit compounds more manageable for docking and to allow for a hierarchy to triage which compounds to test first. The NCI database was chosen in part due to its unique structural diversity.[23] Computational docking to Cdk6 was chosen to further rank compounds that would most likely bind to the p16^{INK4a}-binding face of Cdk6 and due to the fact that no Cdk4 crystal structure existed at the time of the study. Cyclin-dependent kinases rely on ATP binding for their activity. The

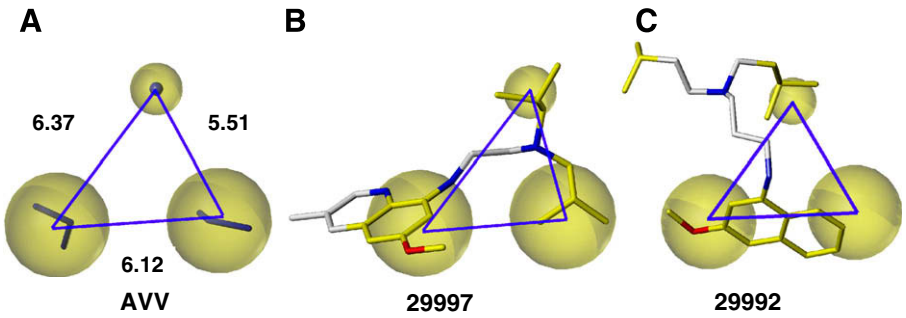
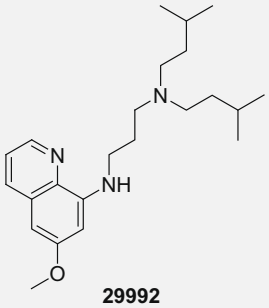
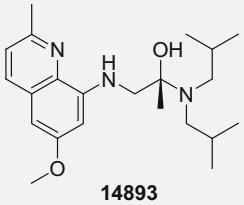
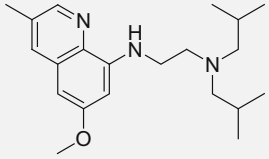
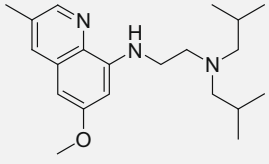


Figure 2. (A) Representation of the pharmacophore AVV derived from the lowest energy structure depicted in Figure 2. Spheres represent the hydrophobic space constraints with the centers of the terminal ends of the residues as the center. Distance constraints (in angstroms) between centers of pharmacophore points are labeled next to the lines between such points. (B) Compound 29997 is superimposed on the AVV pharmacophore depicted in A. (C) Compound 29992 is superimposed on the AVV pharmacophore in depicted in A.

Cdk6/p16^{INK4a} structure shows that the ATP-binding site is at least partially distorted and likely contributes to the inhibition of Cdk6 activity by p16^{INK4a}. Docking studies demonstrated that many of the compounds, namely, 29992 and 29997, docked to the opposite side of the ATP binding site in Cdk6. While not evidence for mechanism, it is possible that such binding could underlie some of the activity exhibited by these compounds.

Table 2

NCI Compound	CDK4 IC_{50} (μM)	CDK6 IC_{50} (μM)
 29992	162	325
 14893	179	691
 29997	128	N.A.
 13719	147	N.A.

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References and notes

1. Liggett, W. H., Jr.; Sidransky, D. *J. Clin. Oncol.* **1998**, *16*, 1197.
2. Sausville, E. A.; Elsayed, Y.; Monga, M.; Kim, G. *Annu. Rev. Pharmacol. Toxicol.* **2003**, *43*, 199.
3. Swanton, C. *Lancet Oncol.* **2004**, *5*, 27.
4. Benson, C.; Kaye, S.; Workman, P.; Garrett, M.; Walton, M., et al *Br. J. Cancer* **2005**, *92*, 7.
5. Craig, C.; Kim, M.; Ohri, E.; Wersto, R.; Katayose, D., et al *Oncogene* **1998**, *16*, 265.
6. Lee, J. H.; Lee, C. T.; Yoo, C. G.; Hong, Y. K.; Kim, C. M., et al *Anticancer Res.* **1998**, *18*, 3257.
7. Frizelle, S. P.; Rubins, J. B.; Zhou, J. X.; Curiel, D. T.; Kratzke, R. A. *Cancer Gene Ther.* **2000**, *7*, 1421.
8. Fahraeus, R.; Paramio, J. M.; Ball, K. L.; Lain, S.; Lane, D. P. *Curr. Biol.* **1996**, *6*, 84.
9. Frizelle, S. P.; Kratzke, M. G.; Carreon, R. R.; Engel, S. C.; Youngquist, L., et al *Anticancer Res.* **2008**, *28*, 1.
10. Fahraeus, R.; Lain, S.; Ball, K. L.; Lane, D. P. *Oncogene* **1998**, *16*, 587.
11. Piotto, M.; Saudek, V.; Sklenar, V. *J. Biomol. NMR* **1992**, *2*, 661.
12. Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355.
13. Wider, G.; Macura, S.; Kumar, A.; Ernst, R. R.; Wüthrich, K. *J. Magn. Reson.* **1984**, *56*, 207.
14. Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley-Interscience: New York, 1986.
15. Jorgensen, W. L.; Duffy, E. M. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1155.
16. Jorgensen, W. L.; Duffy, E. M. *Adv. Drug Deliv. Rev.* **2002**, *54*, 355.
17. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Deliv. Rev.* **1997**, *23*, 3.
18. Halgren, T. A.; Murphy, R. B.; Friesner, R. A.; Beard, H. S.; Frye, L. L., et al *J. Med. Chem.* **2004**, *47*, 1750.
19. Friesner, R. A.; Banks, J. L.; Murphy, R. B.; Halgren, T. A.; Klicic, J. J., et al *J. Med. Chem.* **2004**, *47*, 1739.
20. *Glide*; Schrodinger, LLC: New York.
21. Orsini, M. J.; Klein, M. A.; Beavers, M. P.; Connolly, P. J.; Middleton, S. A., et al *J. Med. Chem.* **2007**, *50*, 462.
22. Russo, A. A.; Tong, L.; Lee, J. O.; Jeffrey, P. D.; Pavletich, N. P. *Nature* **1998**, *395*, 237.
23. Voigt, J. H.; Bienfait, B.; Wang, S.; Nicklaus, M. C. *J. Chem. Inf. Comput. Sci.* **2001**, *41*, 702.