Discovery of Novel and Potent Cdc25 Phosphatase Inhibitors Based on the Structure-Based De Novo Design

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Cdc25 phosphatases have been considered as attractive drug targets for anticancer therapy due to the correlation of their overexpression with a wide variety of cancers. We have been able to identify five novel Cdc25 phosphatase inhibitors with micromolar activity by means of a structure-based de novo design method with a known inhibitor scaffold. Because the newly discovered inhibitors are structurally diverse and have desirable physicochemical properties as a drug candidate, they deserve further investigation as anticancer drugs. The differences in binding modes of the identified inhibitors in the active sites of Cdc25A and B are addressed in detail.

Key Words: Cdc25 phosphatase, De novo design, Inhibitor, Anticancer agents

Introduction

Cdc25 phosphatases are able to dephosphorylate both a threonine and a tyrosine side chain of a protein substrate, and therefore belong to a class of dual-specificity phosphatase. Of the three Cdc25 homologues (Cdc25A, Cdc25B, and Cdc25C) encoded in human genome, Cdc25A and Cdc25B are shown to have oncogenic properties.[1] Cdc25A acts on the control of G_1-to-S and G_2-to-S transitions in cell cycle whereas Cdc25B is mainly responsible for regulating the progression at the G_2-to-M transition.[2] Cdc25 phosphatases can thus serve as the central regulators of the cell cycle with the role of driving each state of cell division. So far, several lines of experimental evidences have been provided for the involvement of Cdc25 phosphatases in oncogenic transformations and various human cancers.[3-6] The inhibition of Cdc25 phosphatases may thus represent a novel approach for the development of anticancer therapeutics.

Structural studies of Cdc25 phosphatases have lagged behind the mechanistic and pharmacological studies. The X-ray crystal structures of the catalytic domains of Cdc25A and Cdc25B have been reported so far in their ligand-free forms only.[7,8] The lack of structural information about the nature of the interactions between Cdc25 phosphatases and small molecule inhibitors has made it a difficult task to discover a good lead compound for anticancer drugs. Nonetheless, a number of effective inhibitors of Cdc25 phosphatases have been discovered with structural diversity as recently reviewed in a comprehensive manner.[9,10] Most of the Cdc25 inhibitors reported in the literature have stemmed from either the isolation of new scaffolds by high throughput screening[11] or the generation of the improved derivatives of pre-existing inhibitor scaffolds.[12-14] Binding modes of the newly found Cdc25 inhibitors have also been addressed with docking simulations in the active site to gain structural insight into their inhibitory mechanisms.[15-17]

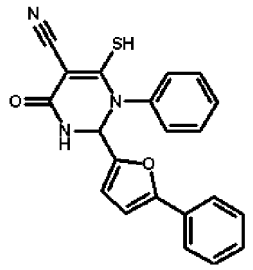

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Figure 1. Chemical structures of the inhibitor scaffold under investigation.

Recently, we have identified several novel classes of Cdc25 phosphatase inhibitors with micromolar activity based on the structure-based virtual screening with docking simulations.[18] All of these inhibitors proved to be competitive in the Lineweaver-Burk plots and include 9-mercapto-4-oxo-1-phenyl-2-(5-phenyl-furan-2-yl)-1,2,3-tetrahydro-pyrimidine-5-carbonitrile **1** that is shown in Figure 1. In present study, we apply a de novo design approach to find the derivatives of this inhibitor scaffold that have an improved inhibitory activity with good physicochemical properties as a drug candidate. The characteristic feature that discriminates our de novo design approach from the others lies in the implementation of an accurate solvation model in calculating the binding free energy between Cdc25 phosphatases and the putative inhibitors, which would have an effect of increaseing the accuracy in predicting the binding affinity.[19] On the basis of docking simulations, we will also address the interactions of the newly identified inhibitors with the active site residues of Cdc25 phosphatases.

[a]These two authors contribute equally to this work.

Computational and Experimental Methods

LigBuilder program[20] was used in the structure-based de novo design of Cdc25 phosphatase using the crystal structure of Cdc25B (PDB entry: 1cwr).[8] In order to score the derivatives according to the relative binding affinity, the program employs the empirical binding free energy function including van der Waals, hydrogen bond, electrostatic, and entropic terms.[21] Gasteiger-Marsilli atomic charges[22] were used for both proteins and ligands in the calculation of the electrostatic interaction term. The current scoring function of LigBuilder lacks a solvation term although the effects of ligand solvation have been shown to be critically important in protein-ligand interactions.[19] Therefore, the solvation free energy function developed by Kang *et al.*[23] was added to improve the original scoring function. The inhibitor scaffold identified in the previous virtual screening (**1** in Figure 1) was used as the starting structure of de novo design. The first step to design the new derivatives was to analyze the binding pocket of the active site using the POCKET module. The structure of Cdc25B in complex with **1** obtained from docking simulations with the AutoDock program[24] were used as the input for POCKET to find the key interaction residues in the active site. In this docking simulation, we employed the improved scoring function with the new solvation model that had been shown to enhance the accuracies of predicting the binding mode and the free energy of binding in protein-ligand association.[25,26] The next step involved the generation of the derivatives of **1** by applying the genetic algorithm as implemented in the GROW module. The bioavailability rules were also applied to screen the derivatives with good physicochemical properties as a drug.[27]

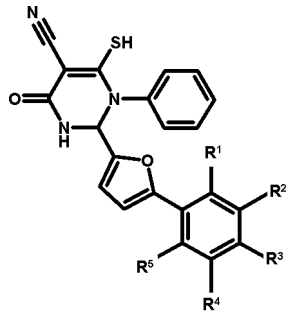
The catalytic domains of the two Cdc25 phosphatases (Cdc25A: residues 336-523; Cdc25B: residues 378-566)[28] were overexpressed in *E. coli* by using pET28a (Novagen) with 6xHis tag in the N-terminus. The overexpressed Cdc25 phosphatases were purified by Ni-NTA affinity resin (Qiagen) and frozen (-75 °C) in a buffer of pH 8.0 containing 20 mM Tris-HCl, 0.2 M NaCl, and 5 mM DTT until enzyme assay. In the phosphatase assay using 96 well plates, the reaction mixture included 180 μL of reaction buffer (20 mM Tris-HCl, 0.01% Triton X-100, and 5 mM DTT) with 10 μM 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP, molecular probe), 10 μL of enzyme (30 nM Cdc25A or 20 nM Cdc25B), and 10 μL of a designed putative inhibitor dissolved in DMSO. The reaction was performed for 20 min at room temperature and stopped by adding 1 mM sodium orthovanadate (final concentration). The fluorescence was measured (355 nm excitation and 460 nm emission) by a plate reader. IC_{50} values were then estimated at least two times and the averaged values were selected. As the positive controls for the enzyme inhibition assay, we used the Cdc25 phosphatase inhibitor known as Cpd 5. This compound has micromolar inhibitory activity against Cdc25 phosphatases and are known to be one of the most potent growth inhibitors of various tumor cell lines.[29,30] The IC_{50} values of Cpd 5 were found to be 4.6 and 23.6 μM for Cdc25A and Cdc25B, respectively, in the present enzyme assay.

Results and Discussion

In the present structure-based de novo design study, we explore the substituent space on the promising core at five positions to identify the substituents that can enhance the inhibitory activity against Cdc25 phosphatases with the desirable physicochemical properties as a drug candidate. This de novo design was operated by varying the substituents to optimize the binding free energy with the core structure being kept fixed. Structures were evolved from the substitution points of the hydrogen atoms attached to the molecular core. 10000 derivatives were generated and scored according to the calculated binding affinities. The top-scored 500 molecules were then selected and checked for commercial availability. Finally, 96 derivatives of **1** were purchased from InterBioScreen Ltd. (http://www.ibscreen.com) and tested for inhibitory activities against Cdc25A and B.

Of the 96 derivatives of **1** tested for the inhibitory activities against Cdc25A and B, five were found to have IC_{50} values less than 10 μM for at least one of the two Cdc25 phosphatases. Table 1 lists the structures and IC_{50} values of the newly found inhibitors. We note that most of the derivatives exhibit a higher inhibitory activity for Cdc25B than for Cdc25A. This is not surprising because the structure-based de novo design was carried out using the 3-D structure of Cdc25B. It is also noteworthy that the chloride atom should be a suitable substituent at R^2 and R^5 positions on the terminal phenyl ring of **1**. A nitro group is also allowed at R^3 position for the micromolar inhibitory activities. Judging from the absence of a substituent at R^1 and R^4 positions, the vacancies in one of the ortho and meta positions with respect to the adjacent furan ring seem to be indispensable for the micromolar inhibitory activities. All of the inhibitors shown in Table 1 satisfy the Lipinski's "Rule of Five" for physicochemical properties as a drug candidate.[27] indicating that they deserve further investigation as an anticancer drug.

Table 1. Structures and inhibitory activities of the derivatives of 1.



	R^1	R^2	R^3	R^4	R^5	IC_{50} (μM)	
						Cdc25A	Cdc25B
1a	H	Cl	H	H	H	6.7	5.0
1b	H	Cl	H	H	Cl	2.9	2.7
1c	H	H	NO_2	H	Cl	4.8	2.3
1d	H	H	H	H	Cl	7.4	5.8
1e	H	Cl	Cl	H	H	2.3	1.7

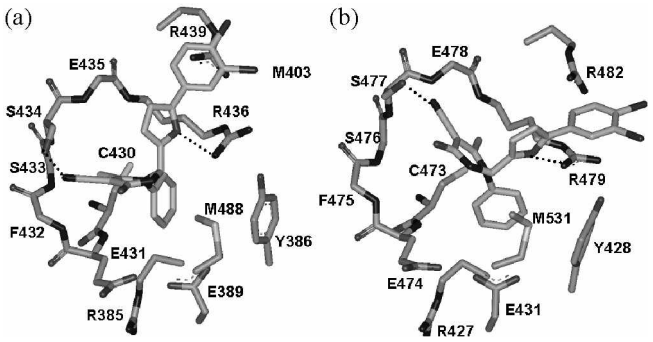


Figure 2. Calculated binding modes of **1e** in the active sites of (a) Cdc25A and (b) Cdc25B. Each dotted line indicates a hydrogen bond.

To obtain some energetic and structural insight into the inhibitory mechanisms by the newly identified inhibitors of Cdc25 phosphatases, their binding modes in the active sites of Cdc25A and B were investigated using the AutoDock program.[24] The calculated binding modes of the most potent inhibitor **1e** in the active sites of the two Cdc25 phosphatases are compared in Figure 2. Some substantial differences are observed in the binding modes in the active sites of the two Cdc25 phosphatases: the terminal phenyl ring of **1e** resides at the entrance of active site in the Cdc25A-**1e** complex whereas it is situated deep in the active site of Cdc25B, establishing a hydrophobic contact with the side chains of Tyr428, Arg479, and Met531. The involvement of stronger hydrophobic interactions in the Cdc25B-**1e** complex than in the Cdc25A-**1e** one can be invoked to explain the differences in IC_{50} values of the derivatives of **1** for the two Cdc25 phosphatases. The dichlorophenyl group of **1e** points toward the side chains of Arg and Tyr residues in Cdc25A-**1e** and Cdc25B-**1e** complexes, respectively. Despite such differences in the overall binding configurations, the patterns for the formation of hydrogen bonds appear to be similar in the two complexes. As can be seen in Figure 2, the cyano and furan moieties of **1e** receive the hydrogen bonds from the side chains of Ser (Ser434 in Cdc25A and Ser477 in Cdc25B) and Arg residues (Arg436 in Cdc25A and Arg479 in Cdc25B) in the active sites of Cdc25A and B, respectively. It is also common to the two binding configurations that the thiol moiety of the inhibitor attached to 6-mercapto-2,3-dihydro-1*H*-pyrimidin-4-one ring interacts with the catalytic Cys residues (Cys430 in Cdc25A and Cys479 in Cdc25B) at a distance within 4.5 Å. Judging from the proximity to the Cys residues in the active site, 6-mercapto-2,3-dihydro-1*H*-pyrimidin-4-one ring seems to serve as a surrogate for the phosphate group of the substrate of Cdc25 phosphatases.

The difference in binding modes of the inhibitor in the active sites of the two Cdc25 phosphatases can be attributed to the difference in the active-site geometry. In this regard, the volume of the active site of Cdc25A is known to be smaller than that of Cdc25B due to the differences in the positioning of the side chains of Leu403 and Met488 in Cdc25A and those of the corresponding Leu445 and Met531 in Cdc25B.[18] This is consistent with the results of the current docking simulations in which the terminal phenyl group of **1e** is situated deeper in the active site of Cdc25B than in that of Cdc25A. Despite the differences in the active site geometry and in the binding modes, the inhibitor appears to be stabilized in a similar way through the establishment of multiple hydrogen bonds and hydrophobic contacts with active-site residues of the two Cdc25 phosphatases.

Conclusions

We have identified five novel Cdc25 phosphatase inhibitors with micromolar activities based on the structure-based de novo design approach with the inhibitor scaffold including 6-mercapto-2,3-dihydro-1*H*-pyrimidin-4-one moiety. The modified scoring function with a suitable solvation energy term was used in this de novo design. Although the inhibitor is found to be bound in different ways in the active sites of the two Cdc25 phosphatases, it is a common feature of the binding modes that the inhibitor should be stabilized through the simultaneous establishments of multiple hydrogen bonds and hydrophobic interactions. The newly found inhibitors deserve further investigation as anticancer drugs because they have desirable physicochemical properties as a drug candidate.

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References and Notes

1. Kristjansdottir, K.; Rudolph, J. *Chem. Biol.* **2004**, *11*, 1043.
2. Rudolph, J. *Biochemistry* **2007**, *46*, 3595.
3. Galaktionov, K.; Lee, A. K.; Eckstein, J.; Draetta, G.; Meckler, J.; Loda, M.; Beach, D. *Science* **1995**, *269*, 1575.
4. Takemara, I.; Yamamoto, H.; Sekimoto, M.; Ohue, M.; Noura, S.; Miyake, Y.; Matsumoto, T.; Aihara, T.; Tomita, N.; Tamaki, Y.; Sakita, I.; Kikkawa, N.; Matsuura, N.; Shiozaki, H.; Monden, M. *Cancer Res.* **2000**, *60*, 3043.
5. Ngan, E. S. W.; Hashimoto, Y.; Ma, Z.-Q.; Tsai, M. J.; Tsai, S. Y. *Oncogene* **2003**, *22*, 734.
6. Boutros, R.; Dozier, C.; Ducommun, B. *Curr. Opin. Cell Biol.* **2006**, *18*, 185.
7. Fauman, E. B.; Cogswell, J. P.; Lovejoy, B.; Rocque, W. J.; Holmes, W.; Montana, V. G.; Piwnica-Worms, H.; Rink, M. J.; Saper, M. A. *Cell* **1998**, *93*, 617.
8. Reynolds, R. A.; Yem, A. W.; Wolfe, C. L.; Deibel, M. R.; Chidester, C. G.; Watenpaugh, K. D. *J. Mol. Biol.* **1999**, *293*, 559.
9. Contour-Galcéra, M.-O.; Sidhu, A.; Prevost, G.; Bigg, D.; Ducommun, B. *Pharmcol. Ther.* **2007**, *115*, 1.
10. Ham, S. W.; Carr, B. I. *Drug Des. Rev.* **2004**, *1*, 123.
11. Lazo, J. S.; Aslan, D. C.; Southwick, E. C.; Cooley, K. A.; Ducruet, A. P.; Joo, B.; Vogt, A.; Wipf, P. *J. Med. Chem.* **2001**, *44*, 4042.
12. Sohn, J.; Kiburz, B.; Li, Z.; Deng, L.; Safi, A.; Pirrung, M. C.; Rudolph, J. *J. Med. Chem.* **2003**, *46*, 2580.
13. Brault, L.; Denancé, M.; Banaszak, E.; Maadidi, S. E.; Battaglia, E.; Bagrel, D.; Samadi, M. *Eur. J. Med. Chem.* **2007**, *42*, 243.
14. Huang, W.; Li, J.; Zhang, W.; Zhou, Y.; Xie, C.; Luo, Y.; Li, Y.; Wang, J.; Li, J.; Lu, W. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1905.
15. Lazo, J. S.; Nemoto, K.; Pestell, K. E.; Cooley, K.; Southwick, E. C.; Mitchell, D. A.; Furey, W.; Gussio, R.; Zaharevitz, D.W.; Joo, B.; Wipf, P. *Mol. Pharmacol.* **2002**, *61*, 720.
16. Lavecchia, A.; Cosconati, S.; Limongelli, V.; Novellino, E. *Chem. Med. Chem* **2006**, *1*, 540.
17. Park, H.; Carr, B. I.; Li, M.; Ham, S. W. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2351.

18. Park, H.; Bahn, Y. J.; Jung, S.-K.; Jeong, D. G.; Lee, S.-H.; Seo, I.; Yoon, T.-S.; Kim, S. J.; Ryu, S. E. *J. Med. Chem.* **2008**, *51*, 5533.
19. Shoichet, B. K.; Leach, A. R.; Kuntz, I. D. *Proteins* **1999**, *34*, 4.
20. Wang, R.; Gao, Y.; Lai, L. *J. Mol. Model.* **2000**, *6*, 498.
21. Wang, R.; Liu, L.; Lai, L. *J. Mol. Model.* **1998**, *6*, 379.
22. Gasteiger, J.; Marsili, M. *Tetrahedron* **1980**, *36*, 3219.
23. Kang, H.; Choi, H.; Park, H. *J. Chem. Inf. Model.* **2007**, *47*, 509.
24. Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. *J. Comput. Chem.* **1998**, *19*, 1639.
25. Lee, W.; Park, H.; Lee, S. *Bull. Korean Chem. Soc.* **2008**, *29*, 363.
26. Park, J.-H.; Ko, S.; Park, H. *Bull. Korean Chem. Soc.* **2008**, *29*, 921.
27. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug. Delivery. Rev.* **1997**, *23*, 3.
28. With respect to the numbering scheme for the amino acids, we followed those used in the X-ray crystallographic studies of Cdc25A (Ref. 7) and Cdc25B (Ref. 8).
29. Tamura, K.; Southwick, E. C.; Kerns, J.; Rosi, K.; Carr, B. I.; Wilcox, C.; Lazo, J. S. *Cancer Res.* **2000** *60*, 1317.
30. Osada, S.; Osada, K.; Carr, B. I. *J. Mol. Biol.* **2001**, *314*, 765.