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Structure-based de novo design and biochemical evaluation of novel Cdc25 phosphatase inhibitors

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ABSTRACT

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 32 novel Cdc25 phosphatase inhibitors with micromolar activity by means of a structure-based de novo design method with the two known inhibitor scaffolds. 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.

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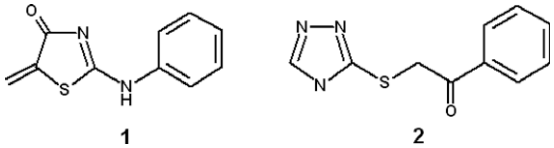
Cdc25 phosphatases are able to dephosphorylate both threonine and tyrosine side chains of a protein substrate, and therefore belong to a class of dual-specificity phosphatase. Of the three Cdc25 homologues (Cdc25A, Cdc25B, and Cdc25C) encoded in human genome, Cdc25A and Cdc25B are shown to have oncogenic 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 evidence 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 B 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]

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] Most of these new inhibitors proved to be competitive in their respective Lineweaver–Burk plots and could be categorized into the two scaffolds, **1** (5-methylene-2-phenylamino-thiazol-4-one) and **2** (1-phenyl-2-(4*H*-[1,2,4]triazole-3-ylsulfanyl)-ethanone) as shown in Figure 1. In present study, we apply a de novo design approach to find the derivatives of the two inhibitor scaffolds 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 implemen-



1 2

Figure 1. Chemical structures of the two inhibitor scaffolds under investigation.

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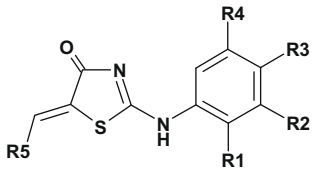
Table 1
Structures and inhibitory activities of the derivatives of **1**



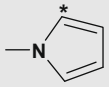
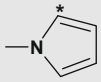
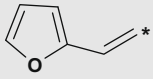
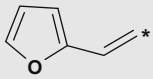
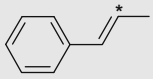
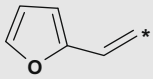
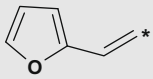
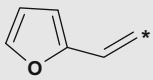
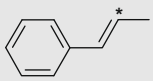
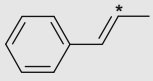
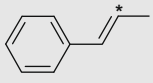
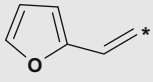
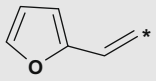
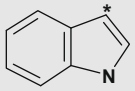
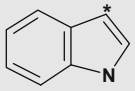
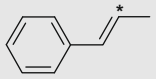
	R1	R2	R3	R4	R5[a]	IC_{50} (μM)	
						Cdc25A	Cdc25B
1a	H	H	Cl	H		15.4	3.1
1b	H	H	F	H		14.3	3.7
1c	H	Br	H	H		7.7	2.7
1d	H	Cl	H	H		9.1	3.2
1e	H	Cl	H	Cl		5.1	1.2
1f	H	Me	Me	H		7.1	1.8
1g	Cl	H	H	H		10.1	2.1
1h	Cl	Cl	H	H		6.2	2.1
1i	H	H	F	H		30.7	8.9
1j	H	Cl	H	H		12.8	3.3
1k	H	Me	Me	H		8.1	2.3
1l	H	Cl	H	H		11.1	4.4
1m	H	H	Br	H		17.3	5.1
1n	H	Cl	H	Cl		4.1	2.3
1o	H	OH	H	H		7.8	2.8

Table 1 (*continued*)

	R1	R2	R3	R4	R5[a]	IC_{50} (μM)	
						Cdc25A	Cdc25B
1p	Cl	H	Cl	H		6.7	4.5
1q	H	Cl	H	Cl		12.7	2.3
1r	Cl	H	Cl	H		12.6	2.6
1s	Cl	H	H	H		10.3	3.9

[a] Asterisk indicates the atom attached to the position of substitution.

tation of an accurate solvation model in calculating the binding free energy between Cdc25 phosphatases and the putative inhibitors, which would have an effect of increasing 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.

LigBuilder program[20] was used in the structure-based de novo design of Cdc25 phosphatase inhibitors 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 two inhibitor scaffolds identified in the virtual screening (**1** and **2** in Fig. 1) were used as the starting structures 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 structures of Cdc25B in complex with **1** and **2** obtained from docking simulations with the AUTODOCK program[24] were used as input for POCKET to find the key interaction residues in the active site. The next step involves the generation of the derivatives of the two inhibitor scaffolds 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.[25]

The catalytic domains of the two Cdc25 phosphatases (Cdc25A: residues 336–523; Cdc25B: residues 378–566)[26] were overexpressed in *Escherichia 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

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