Dual-Specificity Phosphatase CDC25A/B Inhibitor Identified from a Focused Library with Nonelectrophilic Core Structure

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ABSTRACT: Focused libraries of enamine derivatives with a nonacidic, nonelectrophilic core structure were screened for inhibitors of dual-specificity protein phosphatases, and an o-hydroxybenzyl derivative RE44 (10d) was identified as a selective inhibitor of CDC25A/B. This inhibitor induced cell-cycle arrest of tsFT210 cells at the G2/M phase and inhibited dephosphorylation of the CDC25B substrate CDK1. Unlike most quinone-based inhibitors, 10d does not generate reactive oxygen species.

KEYWORDS: dual-specificity protein phosphatases, CDC25, inhibitor, reactive oxygen species, cell cycle, focused library

Cell cycle progression and transition between phases of the cell cycle are controlled by the activation of cyclin-dependent kinases (CDKs), and alterations in CDKs activities are involved in tumor-associated cell-cycle aberration. † Dual-specificity protein phosphatase (DSP) CDC25s (CDC25A, CDC25B, and CDC25C) have the unique function of activating CDKs by dephosphorylating specific phosphotyrosine and phospho-threonine residues on CDKs. ‡, § CDC25s are important checkpoint regulators for handling DNA damage caused by UV light, ionizing irradiation, or chemicals in eukaryotic cells. Thus, misregulation of CDC25s can trigger genomic instability. Furthermore, CDC25A and CDC25B have been suggested to be oncogenic and are overexpressed in various cancer cells. †, § Therefore, inhibitors of CDC25s would be promising candidates for cancer therapy. As has been reviewed recently, ‡, § powerful CDC25s inhibitors with antitumor activity have been found. On the other hand, although all three CDC25 isoforms appear to participate in the regulation of G1-S and G2-M transitions and mitosis, the precise functions of individual CDC25 isoforms still remain to be fully established. This is at least partly due to the lack of inhibitors with high enzyme specificity as well as isoform specificity. ‡

Many of the reported inhibitors of CDC25s have highly acidic, electrophilic, or quinone-based structures and were discovered through screening of natural products or synthetic libraries. † Among the p-quinoid derivatives, NSC663284 (1), † NSC95397 (2), † adociaquinone B, † and IRC-083864 † are representative potent inhibitors, and this class of inhibitors generally inhibits all three isoforms of CDC25 in an unselective manner. These compounds cause remarkable inhibition of tumor cell proliferation via irreversible inhibition of CDC25s by electrophilic modification †, † or oxidation of the thiolate of the cysteine residue in the catalytic P-loop structure. †, § It has been shown that reactive oxygen species (ROS) are generated in cultured cells treated with p- and p-quinone derivatives. †, § The activity of CDC25B is modulated by reversible or irreversible redox reaction, † and the cysteine residue on the P-loop of CDC25B was suggested to be more reactive to oxidant than usual cysteine residues. However, nonselective oxidation of relatively reactive cysteine residues of proteins involved in cellular signaling, including other DSPs and protein tyrosine phosphatases (PTPs), as well as many other redox sensor proteins, by ROS would be likely to seriously disrupt cell function. †, §, ‡ Therefore, the potential toxicity to normal cells and tissues caused by ROS derived from usual quinoid inhibitors would be problematic in biological studies. Although inhibitors such as a H3217 that do not oxidize cysteine residues or generate ROS have been reported, it is expected that nonquinoid inhibitors with high enzyme selectivity would have great potential for the development of pharmaceutical agents and useful biological tools. Herein, we report a novel class of inhibitor, RE44 (10d), which has potent and selective inhibitory activity toward CDC25A/B without generation of ROS.

We have previously reported the development of inhibitors for DSPs/PTPs by screening of a 3-acyltetronic acid library (3) based on RK-682, ‡ in which the 3-acyltrimetronate 4 acts as a phosphate-mimicking core structure (Figure 1). However, the anion-delocalized core 4 resulted in poor cell permeability and enzyme selectivity of this class of inhibitors. Recently, we addressed these problems by designing and synthesizing novel
Selective inhibitor of VHR, RE12 (Figure 1), was used as a representative of PTPs and DSPs (CDC25s, VHR, MKP-1, and MKP-3) were evaluated in vitro by utilizing purified phosphatase proteins as previously described.19 Only a few compounds (8c, 9a, 9d, and 10c) weakly inhibited PTP1B and CDC25C, but generally, the benzyl-substituted RE derivative showed a strong preference for the inhibition of VHR and CDC25A/B. As we had expected, the nature of the substituent on the aromatic ring affects the selectivity between VHR and CDC25A/B. Introduction of a m-substituent, such as methyl or chloro, decreased the inhibitory activity for CDC25A/B and increased VHR-inhibitory potency, and thus, we were able to obtain the selective VHR inhibitors 8a (RE12) and 8b. In contrast, p- or o-methyl or chloro substituent (9a, 10a, 9b, and 10b) did not result in similar changes of inhibitory potency toward VHR and CDC25A/B. Fluoro derivatives 8c, 9c, and 10c showed a slight increase of CDC25A inhibition as compared to the nonsubstituted 7 but still inhibited VHR more efficiently. To our delight, introduction of a hydroxyl group improved the selectivity for CDC25B. The m-hydroxyl derivative showed increased inhibitory activity for CDC25B, but similar inhibition profiles were not seen in the cases of the p-OMe derivative 8e and m-NMe2 derivative 8f. Although substitution of the p-position with a hydroxyl group (9d) was not effective for further improvement of the selectivity, interestingly, the o-hydroxyl derivative 10d (RE44) showed much less potent inhibition of VHR and stronger inhibition of CDC25B. Such a substituent effect was not observed with methoxy and dimethylamino groups at the p- and o-positions (9e, 9f, 10e, and 10f). Finally, the inhibitory selectivity of compound 10d for CDC25B over CDC25A and VHR was found to be the highest among the compounds tested. Compound 10d should be a promising candidate as a biological tool for studying the functions of CDC25B, if it operates at the cell level.

Both CDC25s and VHR phosphatases are involved in the regulation of the cell cycle,22 and inhibition of the enzymatic activity of these proteins is expected to have an antiproliferative effect on cultured cells. Therefore, we tested the inhibitory effect of all of the synthesized compounds on proliferation of HL-60 cells. All compounds inhibited the proliferation of HL-60 cells, and the IC50 values of most of the compounds were around 10 μM. However, it is noteworthy that compounds 8d, 9d, and 10d possessing a hydroxyl group on the aromatic ring, which showed greater inhibitory potency for CDC25B over CDC25A and VHR was found to be the highest among the compounds tested. Compound 10d should be a promising candidate as a biological tool for studying the functions of CDC25B, if it operates at the cell level.

With these DSP inhibitors having distinct selectivity for CDC25A/B and VHR in hand, we next evaluated the effect of compounds 7 (RE1: dual CDC25A/B and VHR inhibitor), 8a (RE12: selective VHR inhibitor), and 10d (RE44: selective CDC25A/B inhibitor) on the cell-cycle progression of tsFT210 cells.22 The temperature-sensitive FT210 cell line has been widely used for cellular studies of CDC25s inhibitors. Although tsFT210 cells can grow normally at the permissive temperature of 32 ºC and show a normal cell-cycle distribution, the cell cycle can be easily arrested at mid to late G2 phase, because...
two point mutations on the cdc2 gene of this cell line cause inactivation of the gene product, CDK1, at the restrictive temperature of 39 °C. In fact, as shown in Figure 2Aa, incubation of tsFT210 cells at 39 °C for 17 h resulted in synchronization at G2 phase. The populations of the various phases of the cell cycle are summarized in the Supporting Information. Release of the G2-synchronized cells was performed by incubation for 4 h at 32 °C in the presence or absence of compounds (Figure 2Ab−l). Treatment of 7 and 10d predominantly maintained the G2/M arrest of tsFT210 cells in a concentration-dependent manner (Figure 2Ad−fj−l) as compared with the vehicle control (Figure 2Ab). Moreover, compound 8a, which is a selective inhibitor of VHR without inhibitory potency for CDC25s in vitro, had little effect on tsFT210 cells (Figure 2Ag−i), as expected. These results suggested that inhibition potency for CDC25 phosphatases is well correlated with G2/M-arresting activity in tsFT210 cells.

Table 1. Inhibition of CDC25s and VHR by RE Derivatives

| compd | CDC25A IC50 (SD) (μM) | CDC25B IC50 (SD) (μM) | CDC25C IC50 (SD) (μM) | VHR IC50 (SD) (μM) | MKP-1 IC50 (SD) (μM) | MKP-3 IC50 (SD) (μM) | PTP1B IC50 (SD) (μM) | growth inhibition of HL-60 cells IC50 (range) (μM) |
|-------|------------------------|------------------------|------------------------|---------------------|----------------------|----------------------|----------------------|----------------------------------|
| 7    | 16.6 (1.3)             | 8.42 (0.53)            | ND                     | 11.4 (0.47)         | ND                   | ND                   | ND                   | 9.51 (1.97)                      |
| 8a   | ND                     | ND                     | ND                     | 1.63 (0.23)         | ND                   | ND                   | ND                   | 9.29 (0.05)                     |
| 8b   | ND                     | ND                     | ND                     | 1.43 (0.06)         | ND                   | ND                   | ND                   | 12.0 (3.50)                     |
| 8c   | 6.22 (0.27)            | 5.13 (0.42)            | 78.2 (3.5)             | 4.69 (0.67)         | ND                   | ND                   | ND                   | 10.8 (1.23)                     |
| 8d   | 15.4 (3.3)             | 3.15 (0.46)            | ND                     | 11.1 (0.36)         | ND                   | ND                   | ND                   | 2.09 (0.06)                     |
| 8e   | 10.2 (0.14)            | 8.31 (1.6)             | ND                     | 5.53 (0.79)         | ND                   | ND                   | ND                   | 8.87 (2.66)                     |
| 8f   | 11.4 (1.3)             | 6.55 (0.39)            | ND                     | 5.13 (0.70)         | ND                   | ND                   | ND                   | 5.92 (0.66)                     |
| 9a   | 7.35 (0.44)            | 3.33 (0.11)            | 56.7 (3.0)             | 2.68 (0.27)         | ND                   | ND                   | ND                   | 19.1 (2.94)                     |
| 9b   | 6.25 (0.40)            | 5.52 (1.1)             | ND                     | 3.63 (0.27)         | ND                   | ND                   | ND                   | 18.2 (2.36)                     |
| 9c   | 10.7 (1.2)             | 13.1 (4.0)             | ND                     | 8.41 (1.1)          | ND                   | ND                   | ND                   | 8.25 (0.07)                     |
| 9d   | 6.86 (1.4)             | 4.23 (0.33)            | 44.1 (1.7)             | 9.93 (0.27)         | ND                   | ND                   | ND                   | 2.45 (0.01)                     |
| 9e   | 11.7 (0.33)            | 23.8 (1.1)             | ND                     | 6.77 (1.3)          | ND                   | ND                   | ND                   | 17.8 (0.52)                     |
| 9f   | 8.67 (0.82)            | 6.25 (0.37)            | ND                     | 12.0 (1.2)          | ND                   | ND                   | ND                   | 10.9 (0.53)                     |
| 10a  | 12.7 (0.43)            | 8.61 (0.55)            | ND                     | 8.99 (0.13)         | ND                   | ND                   | ND                   | 7.09 (0.26)                     |
| 10b  | 12.3 (0.62)            | 13.5 (0.19)            | ND                     | 10.2 (2.3)          | ND                   | ND                   | ND                   | 11.4 (2.81)                     |
| 10c  | 12.6 (0.19)            | 7.53 (1.6)             | 47.1 (11.4)            | 8.93 (0.73)         | ND                   | ND                   | ND                   | 7.88 (0.20)                     |
| 10d  | 13.5 (3.4)             | 4.26 (0.17)            | ND                     | 24.9 (2.2)          | ND                   | ND                   | ND                   | 2.44 (0.16)                     |
| 10e  | 19.0 (1.2)             | 12.1 (0.90)            | ND                     | 6.60 (0.97)         | ND                   | ND                   | ND                   | 11.1 (2.22)                     |
| 10f  | 8.50 (0.88)            | 5.75 (0.43)            | ND                     | 5.19 (0.65)         | ND                   | ND                   | ND                   | 7.03 (0.36)                     |

aRef 19. bIC50 values were determined from three independent experiments. cIC50 values were determined from a single experiment. dIC50 values were determined from two independent experiments. ND: IC50 > 100 μM.

Figure 2. (A) Cell-cycle analysis of tsFT210 cells in the absence or presence of test compounds. (a) G2/M-arrested cells after a temperature shift for 17 h at 39 °C. (b) DMSO-treated cells after a temperature shift for 4 h at 32 °C. (c) Cells treated with 100 nM nocodazole. (d−l) Cells treated with 5−50 μM RE derivatives 7, 8a, and 10d. (B) Effect of RE derivatives (7, 8a, and 10d) on CDK1 phosphorylation status.
in vitro, 7 and 10d concentration dependently inhibited the dephosphorylation of CDK1 (lanes d–f and lanes j–l). In contrast, 8a did not inhibit the dephosphorylation of CDK1 even at 50 μM (lanes g–i). These results indicate that 7 and 10d acted as CDC25s inhibitors at the cell level and confirm that small differences in the chemical structure of RE derivatives markedly influence the selectivity of inhibition of CDC25s at the cell level. Moreover, potent cytotoxic effects of 7 and 10d lacking CDC25C inhibitory activity suggest that CDC25C may not be important for the G2/M transition in tsFT210 cells. It is noteworthy that sub-G1 phase cells, which are indicative of apoptotic cell death, were not increased by high concentrations of the RE derivatives (Figure 2A and Supporting Information). Thus, the cytotoxicity of these compounds appears to be low, which would be very advantageous for the use of these inhibitors in cellular studies on CDC25s functions.

As mentioned above, ROS generation and electrophilic reactivity are important in the inhibition of CDC25s by quinoid 1.13 A decrease of inhibitory potency of 1 was observed when the concentration of DTT was increased in the assay buffer used for the evaluation of inhibitory activity against CDC25s, probably due to direct reaction of DTT with ROS generated by 1 and/or with electrophilic 1 itself. RE derivatives have a characteristic enamine structure conjugated with two carbonyl groups and are not expected to generate ROS or to react with thiol functionality as an electrophile. However, to completely exclude the possibility of ROS generation or an electrophilic mechanism for the CDC25s inhibition by RE derivatives, we examined the effect of DTT concentration on the inhibitory potency of RE derivatives. In accordance with the previous report,13 a higher concentration of DTT resulted in an increase of IC50 value of 1 for CDC25A/B (Figure 3A), although only a slight change of enzymatic activity of CDC25A/B itself was observed in the presence of a large amount of DTT. In contrast, the inhibitory activities of 7 and 10d for CDC25s were independent of DTT concentration. These results support the idea that neither electrophilic addition of RE derivatives to the thiol group in the catalytic site nor ROS generation is involved in the inhibition of CDC25s by RE derivatives. Finally, we measured the ROS level of cells untreated or treated with inhibitors, utilizing the ROS indicator H2DCFDA.20 Upon pretreatment of tsFT210 cells with H2DCFDA at the restrictive temperature of 39 °C for 1 h, followed by treatment with 1 (5 μM or 15 μM) at the permissive temperature of 32 °C for 30 min, a 3- (5 μM 1) or 4.4-fold (15 μM 1) increase of fluorescence intensity as compared to the vehicle control was observed; this is characteristic of an oxidative burst (Figure 3B). In contrast, 7, 8a, and 10d did not induce a significant change of the fluorescence intensity even at the concentration of 50 μM (Figure 3B). These results strongly suggest that RE derivatives do not generate ROS, and thus, participation of ROS in the cell cycle-arresting effect of RE derivatives can be excluded.

In conclusion, we have discovered a CDC25A/B-selective inhibitor, RE44 (10d), by screening of an RE-focused library with a nonacidic and nonelectrophilic enamine core structure. Moreover, it was demonstrated that small structural differences of RE derivatives could control the selectivity of inhibition for DSPs, and the enzyme selectivity was correlated with the activity in tsFT210 cells. Although the cellular activity of RE44 was moderate24 and the specificity over VHR and CDC25A is not complete, it is noteworthy that RE44 did not inhibit CDC25C even at 100 μM. This significant subtype selectivity among CDC25s should make RE44 a useful tool for biological research concerning CDC25A/B, especially in combination with the VHR-selective inhibitor RE12. These inhibitors are also expected to be new lead molecules for candidate therapeutic agents. Work to enhance the cellular activity and to develop more specific inhibitors able to discriminate the subtypes of CDC25s is under way.

ASSOCIATED CONTENT

Supporting Information
Synthesis of compounds, experimental procedure, characterization of new compounds, 1H NMR, 13C NMR, and biological experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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