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Synthesis and biological evaluation of novel coumarin-based inhibitors of Cdc25 phosphatases

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ABSTRACT

The cell division cycle 25 (Cdc25) family of proteins are dual specificity phosphatases that activate cyclin-dependent kinase (CDK) complexes, which in turn regulate progression through the cell division cycle. Overexpression of Cdc25 proteins has been reported in a wide variety of cancers; their inhibition may thus represent a novel approach for the development of anticancer therapeutics. Herein we report new coumarin-based scaffolds endowed with a selective inhibition against Cdc25A and Cdc25C, being **6a** and **6d** the most efficient inhibitors and worthy of further investigation as anticancer agents.

Cell division cycle 25 (Cdc25) phosphatases are key actors in eukaryotic cell cycle control. The three human Cdc25 (Cdc25A, Cdc25B, and Cdc25C) are dual specificity phosphatases responsible for dephosphorylating Cdk/cyclins on pThr14 and/or pTyr15 residues, so activating Cdk/cyclin during normal cell cycle progression.[1] So far, Cdk–cyclin complexes are the only known substrates for Cdc25 phosphatases.[2]

Cdc25A seems to be implicated in the control of G1/S and G2/M transitions, whereas Cdc25B and Cdc25C seems to be more implicated in regulation of G2/M transition.[3] The transitions between each cell cycle phase must be strictly regulated in order to maintain genomic stability. Over activity of these phosphatases is associated with checkpoint bypass and upset this genomic equilibrium.[1] Thus, experimental data show that overexpression of Cdc25B (but not Cdc25C) rapidly pushes S or G2 phase cells into mitosis with incompletely replicated DNA[4] and overexpression of Cdc25A can induces mitotic events.[3,5,6] Cdc25 phosphatases are also central targets and regulators of the G2/M checkpoint mechanisms activated in response to DNA injury including ATM/ATR pathways.[7]

Cdc25 phosphatases can thus serve as central regulators of the cell cycle with the role of driving each state of cell division. The expression and activity of these enzymes are finely regulated by multiple mechanisms including post-translational modifications, interactions with regulatory partners, control of their intracellular localization (all Cdc25s shuttle between the nucleus and the cytoplasm during the cell cycle[6]), and cell cycle-regulated degradation. Accordingly, increased expression of Cdc25A and Cdc25B is found in many high-grade tumors and is correlated with poor prognosis in human cancers.[8] Cell division cycle 25 proteins are reported to be overexpressed in primary tumor samples from patients with breast, prostate, ovarian, endometrial, colorectal, oesophageal, thyroid, gastric and hepatocellular cancers, glioma, neuroblastoma or non-Hodgkin lymphoma.[1] Until now, a lot of inhibitors have been tested for Cdc25 inhibition.[9–11] Three non-quinone and quinone based compounds (BN 82002, BN82685 and IRC083864) are reported as active on, respectively HeLa, miaPaCa2 and LNCap cell lines with an in vivo activity in xenografted mice[12–14] (Fig. 1).

Nevertheless, in 2009 a new class of thiazolopyrimidines (Fig. 1), like Cdc25B inhibitors were disclosed by parallel click

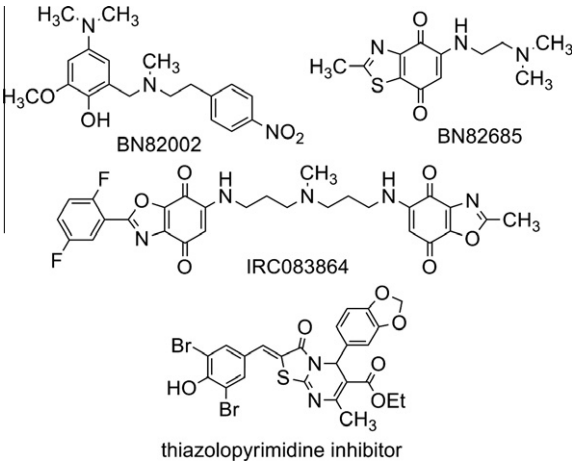

Figure 1. Cdc25 phosphatases inhibitors active in cultured cells.

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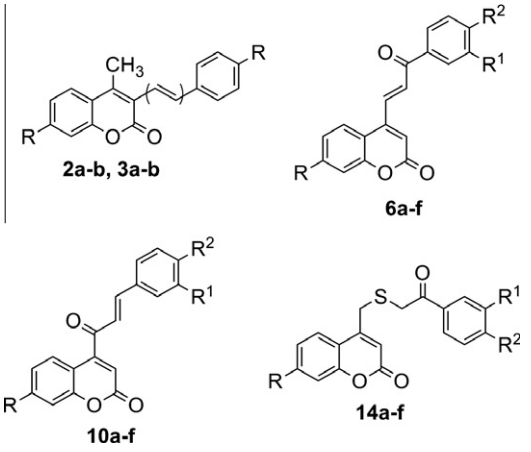
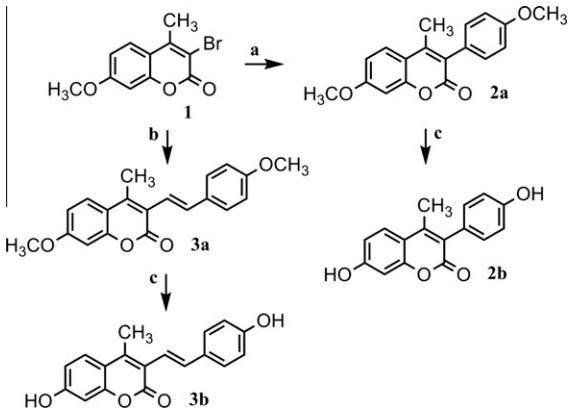


Figure 2. Novel coumarin-based inhibitors of Cdc25 phosphatases.

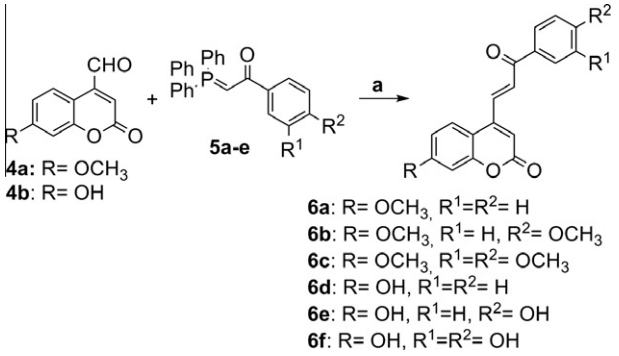
chemistry and in situ screening and shown to display cytotoxic properties against HeLa cells[15] and against prostatic LNCaP cells.[16] Furthermore, recently several flavonolignan compounds isolated from silymarin showed a strong cell cycle arrest in PC3 cells and differential effect of these compounds on the levels of some cell cycle regulators-cyclins among these Cdc25A, Cdc25B and Cdc25C.[17] Finally it has been reported in *Caenorhabditis elegans* CDC-25.1 was inhibited by flavones.[18] So, prompted by these findings and by the structural basis of the flavone nucleus, and with the attempt to identify new scaffolds able to inhibit Cdc25 phosphatases, we developed the synthesis of novel coumarin-based small molecules (Fig. 2) and we tested such derivatives against all of three Cdc25 isoenzymes (Fig. 2).

Our approach started with the introduction of 4-methoxy(hydroxy)phenyl and 4-methoxy(hydroxy)styryl groups at C3 position of the 4-methylcoumarin (compounds **2a–b**, **3a–b**). Compound **1**[19] underwent a Suzuki coupling reacting with 4-methoxyphenylboronic acid, palladium acetate, potassium hydrogen phosphate to afford compound **2a**,[20] whereas, through Heck coupling reacting with 4-methoxy styrene and palladium chloride, gave compound **3a**. Finally, carrying out onto the methoxy derivatives **2a** and **3a** a cleavage with boron tribromide **2b**[20] and **3b**, respectively, were obtained (Scheme 1).

The synthesis of the chalcone–coumarin (benzoylvinyl coumarin) derivatives **6a–f** was performed through Wittig condensation between aldehydes **4a**[21] or **4b**[22] and phosphoranes **5a–e**, previously prepared according to methods described in literature.[23]



Scheme 1. Reagents and conditions: (a) *p*-MeO-Ph-boronic acid, K_2HPO_4, $Pd(OAc)_2$, MeOH, 60 °C, 2 h, 85%; (b) *p*-MeO-styrene, $PdCl_2$, Et_3N, reflux, 12 h, 78%; (c) BBr_3, CH_2Cl_2, −20 °C, overnight, 70%.

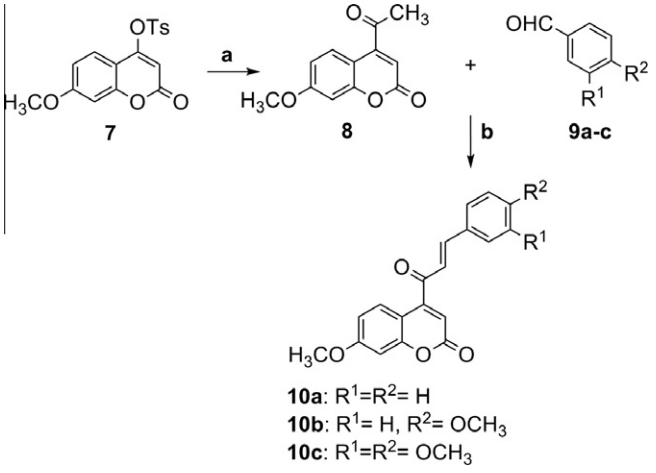


Scheme 2. Reagents and conditions: (a) dry DMF, 0 °C, 1 h, 85–90%.

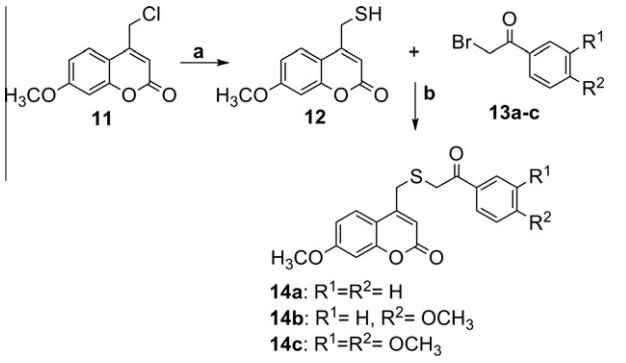
(Scheme 2) The reverse chalcone–coumarin (cinnamoyl coumarin) 10a–**c** were prepared with a different synthetic approach: indeed, through α-carbon regioselective Heck coupling onto the 4-tosylate intermediate **7**[24] as reported,[25] followed by acidic hydrolysis, 4-acetylcoumarin **8**[26] was provided. The final compounds **10a–c** were furnished by aldolic condensation of **8** with the opportune aldehydes **9a**–**c** (Scheme 3).

At last we investigated the introduction of a sulfur atom between coumarin and benzoyl moieties, as shown by derivatives **14a–c**, which were synthesized reacting 4-chloromethylcoumarin **11**, prepared according to the method previously by us reported,[27] with thiourea and, after hydrolysis, yielded 4-mercaptomethylcoumarin **12**. Finally **12** was converted to **14a–c** through alkylation with the opportune ω-bromoacetophenones **13a**–**c** (Scheme 4).

Human glutathione-*S*-transferase (GST)–Cdc25 recombinant enzymes were used to evaluate the inhibitory potential of compounds. Each isoenzyme was prepared as previously described.[10,28] Briefly, the GST-tagged Cdc25s were expressed in a bacterial expression system via an IPTG induction. After lysis of bacteria, purification on GSH-Agarose column allowed to obtain pure GST–Cdc25 recombinant proteins. Recombinant Cdc25A and Cdc25 C are full length enzymes whereas Cdc25 B is truncated one (active site only). The enzymatic activity was measured by a dephosphorylation assay with 3-*O*-methyl fluorescein phosphate as described.[29] The results are expressed as percentage of inhibition of Cdc25 phosphatase activity in presence of the tested compounds. All compounds were tested at 100 μM final concentration, except for BN82002 (Sigma–Aldrich), used as reference drug,



Scheme 3. Reagents and conditions: (a) (1) *n*-butyl vinyl ether, DIPEA, DPPF, $Pd_2(dba)_3$, dioxane, 85 °C, 22 h, 75% (2) 2 N HCl, EtOH, rt, 3 h, 80%; (b) piperidine, glacial acetic acid, EtOH, 70 °C, 3 h, 80–85%.



Scheme 4. Reagents and conditions: (a) (1) NH_2CSNH_2, EtOH/Et_2O, reflux, overnight, (2) 2 N NaOH, rt, 10 min, (3) 2 N HCl, rt,75%; (b) KOH, EtOH, rt, 15 min, 85–90%.

which was tested at 10 μM (Table 1). Inhibition values percentage of all tested derivatives are reported in Table 1.

With the exception of compounds **6a**, **6b**, **6d** and **14a** displaying a weak inhibition of Cdc25B (35.0%, 21.3%, 23.2% and 16.1%, respectively), these derivatives did not show affinity for recombinant Cdc25 B. This result can be explained by the fact that this recombinant protein is truncated and limited to its active site. Considering first series of compounds (**2a–b**, **3a–b**), 3-phenyl derivatives (**2a)** inhibited feebly Ccd25 A and Cdc25 C or showed selectivity of inhibition regarding Cdc25A (**2b**); when we introduced a vinyl group between phenyl ring and coumarin ring the capability of inhibition increased twofold against Cdc25A (from 20.5% (**2a**) to 45.3% (**3a**)) and almost threefold against Cdc25 C (from 17.9% (**2a**) to 48.4% (**3a**)).

In series of compounds **6**, the introduction of benzoylvinyl moiety at C4 position of coumarin nucleus (chalcone–coumarin **6a–f**) gave us an interesting increase of inhibition against both Cdc25A and Cdc25 C up to 94.2% or 79.3% (**6a**) and 94.3% or 94.2% (**6d**), respectively, being compounds **6a** and **6d** the most potent inhibitors in this assay. The substitution of the methoxy group with hydroxy group onto the coumarin moiety didn't improve the potency, nevertheless it provided an higher inhibition for Cdc25 C (from 79.3% (**6a**) to 94.2% (**6d**)). The addition of just one or two methoxy or hydroxy groups onto the benzoyl moiety decreased the inhibitory activity for Cdc25A and Cdc25 C.

When we shifted the carbonyl group from phenyl ring to the coumarin moiety, obtaining reverse chalcone–coumarin compounds (**10a–c**), the inhibitory activity reduced up to half (**10a**) or it was almost completely lost (**10b–c**) against all enzymes. The insertion of a sulfur atom between coumarin moiety and benzoyl group, as described for compounds **14a–c**, did not ameliorate the activity, but, interestingly, the trend was upset: indeed the addition of one or two methoxy groups onto the phenyl ring increased the inhibition and, in particular, against Cdc25A until almost 40% (**14c**).

These results were confirmed by establishing IC_{50} values (concentrations inhibiting 50% of enzyme activity) for the most potent compounds (**6a–f**). Statistical calculations were performed using generalized Poisson regression model, as described by Maul.[31] (Table 2)

Furthermore we examined the reversible fixation of these small coumarin-based molecules. Cdc25A phosphatase was pre-incubated for several times with compound **6a** at active concentration of 100 μM. Then, the reaction mixture was diluted 100-fold to obtain a final non inhibitory concentration of 1 μM (data not shown). It is assumed that the mechanism of action should be considered as irreversible if the inhibitory effects persist after dilution[13] (Fig. 3).

Table 1
Inhibitory activity (expressed as percentage of inhibition) of compounds **2a**–**b**, **3a**–**b**, **6a**–**f**, **10a**–**c** and **14a**–**c** tested at 100 μM against Cdc25A, Cdc25B and Cdc25C phosphatases[a]

Compds[30]	Cdc25A	Phosphatases Cdc25B	Cdc25C
2a	20.5 (±11.0)	ND	17.9 (±0.8)
2b	22.0 (±10.4)	ND	ND
3a	45.3 (±7.6)	ND	48.4 (±1.3)
3b	27.5 (±1.6)	8.5 (±3.1)	15.6 (±6.9)
6a	94.2 (±7.3)	35.0 (±5.5)	79.3 (±2.8)
6b	66.4 (±3.8)	21.3 (±4.2)	64.8 (±4.1)
6c	61.5 (±3.0)	6.5 (±4.5)	69 (±2)
6d	94.3 (±7.9)	23.2 (±10.5)	94.2 (±7.6)
6e	55.0 (±10.1)	9.2 (±7.0)	23.9 (±10.3)
6f	68.7 (±4.9)	ND	43.7 (±10.5)
10a	45.0 (±8.0)	ND	31.3 (± 3.5)
10b	ND	ND	4.8 (±2.3)
10c	7.4 (±9.9)	9.1 (±2.5)	ND
14a	ND	16.1 (±3.3)	21.2 (±7.2)
14b	26.7 (±3.8)	ND	27.9 (±4.5)
14c	39.8 (±9.7)	ND	26.2 (±6.8)
BN82002	87.9 (±4.6)	86.3 (±2.1)	92.9 (±9.6)

ND: not detectable
[a] Values are means of three independent experiments, standard deviation is given in round brackets.

Table 2
Inhibitory activity (IC_{50} values) against Cdc25A and against Cdc25C phosphatases of compounds **6a**–**f**[a]

Compds	Phosphatases (IC_{50}, μM)	
	Cdc25A	Cdc25C
6a	27	49
6b	70	47
6c	78	67
6d	28	26
6e	96	>150
6f	62	72

[a] The IC_{50} values were determined by testing seven different concentrations of compounds (from 0 to 150 μM). For each compound, each concentration was separately tested in three independent microplates, at the rate of three wells per microplate. The statistical evaluation of IC_{50} was made with a software specially designed for calculating the median inhibitory concentration for toxicity tests.[31]

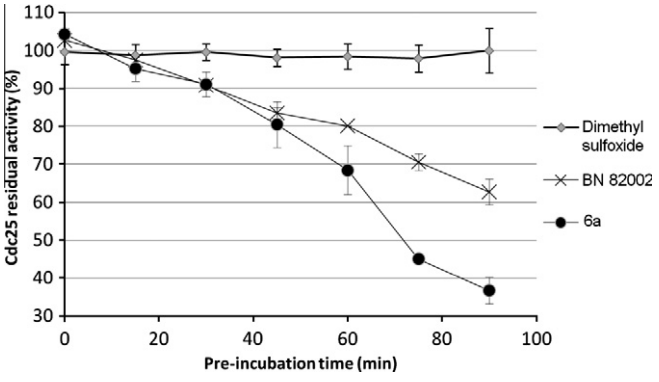


Figure 3. Reversibility assay of Cdc25A inhibition by **6a**. DMSO was used as control and BN82002 as reference for irreversible inhibition. Cdc25A was pre-incubated with inhibitory concentrations of **6a** (100 μM), and BN82002 (10 μM) for various time intervals (0–90 min). After incubation, the reaction mixture was diluted 100-fold to reach inactive concentrations of **6a** (1 μM) and BN82002 (0.1 μM). The phosphatase activity was determined by adding the substrate OMFP in each well containing 900 ng of Cdc25A after dilution. Data are representative of three independent experiments (M ± SD).

Compound **6a** displayed a time-dependent inhibition of Cdc25A, and the maximal inhibitory effect is observed for a 90 min pre-incubation (35% of residual activity) as indicated in Figure 3. As the inhibitory activity persisted after the dilution procedure, compound **6a** could be considered as acting as irreversible inhibitor of the Cdc25 phosphatases in vitro.

In summary, we have identified new coumarin-based derivatives as Cdc25 phosphatases inhibitors, among these the chalcone–coumarin series **6a–f** showed the most interesting results, being **6a** and **6d** endowed with lowest IC_{50} as reported in Table 2: 27 and 28 μM against Cdc25A, respectively. Compounds **6a** and **6b** can be considered two new lead compounds for further work leading to optimization, but also for *in cell* evaluation that will be reported in due course.

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29. The enzymatic activity of the GST–Cdc25 recombinant enzyme was performed in 96-well plates in [50 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA and 0.1% SAB, pH 8,1] buffer containing 3-*O*-methylfluorescein phosphate 500 μM as substrate. The Gst–Cdc25 proteins, diluted in assay buffer, were used at a final concentration of 1 μg/well. After 2 h at 30 °C, 3-*O*-methylfluorescein fluorescent emission was measured with a CytoFluor system Perspective Applied Biosystems; excitation filter: 475 nm and emission filter: 510 nm.
30. (a) All compounds were characterized by HRMS (ESI) and NMR analysis. (b) Analytical data for compounds **6a** and **6d**: for **6a** ^{1}H NMR (250 MHz, DMSO-d_6) δ ppm 3.90 (s, 3H, OCH_3), 6.94–7.05 (m, 3H, coumarin protons), 7.55–7.70 (m, 3H, benzene protons), 7.88–8.20 (m, 5H, coumarin proton, benzene protons, –CH=C*H*COPh and –C*H*=CHCOPh); ^{13}C NMR (250 MHz, $CDCl_3$) δ ppm 55.85, 98.20, 101.35, 109.85, 111.55, 112.70, 125.66, 128.70 (2C), 129.04 (2C), 129.62, 133.77, 136.45, 136.96, 148.78, 155.69, 160.96, 163.18, 179.61, 188.82; HRMS (ESI) *m*/*z* 329.09 $[M+Na]^+$; for **6d** ^{1}H NMR (250 MHz, DMSO-d_6) δ ppm 6.76–6.85 (m, 3H, coumarin protons), 7.55–7.79 (m, 4H, benzene protons and coumarin proton), 7.90–7.96 (d, 1H, –CH=C*H*COPh), 8.07–8.20 (m, 3H, benzene protons and –C*H*=CHCOPh), 10.70 (bs, 1H, O*H*); ^{13}C NMR (250 MHz, DMSO-d_6) δ ppm 102.67, 108.03, 110.05, 113.26, 126.30, 128.85 (2C), 128.88 (2C), 130.31, 133.75, 135.28, 136.65, 147.93, 155.22, 160.36, 161.52, 188.78; HRMS (ESI) *m*/*z* 315.06 $[M+Na]^+$.
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