CDC25 Phosphatase Inhibitors: An Update

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Abstract: The cell division cycle 25 (CDC25) family of proteins is a group of highly conserved dual-specificity phosphatases. They are key regulators of normal cell division and the cell response to DNA damage, and play a fundamental role in transitions between cell cycle phases during normal cell division, *via* the activation of CdK/cyclin complexes. Their abnormal expression, detected in a number of tumors, often correlated with a poor clinical prognosis, implies that their dysregulation is involved in malignant transformation. Thus, inhibition of these proteins represents an attractive therapeutic target in oncology, as evidenced from many patents and papers published on the subject in recent years. Hence, this review aims to provide an overview of recent developments in the field of CDC25 phosphatase inhibitor design since 2008.

Keywords: Antiproliferative agents, cancer, CDC25 inhibitors, cell cycle, dual-specificity phosphatases.

INTRODUCTION

One of the most common post-translational modifications encountered in proteins is reversible protein phosphorylation [1]. This is a fundamental mechanism in the control of life-sustaining processes in cellular organisms. Indeed, enzymes that catalyze phosphate monoester hydrolysis (phosphatases) operate in an intricate balance with those that catalyse phosphorylation (kinases), and are therefore vitally important in cell signaling and regulation.

Two distinct families of protein phosphatases are known to exist [2-4]. These are the serine/threonine phosphatases (PSTPases), which dephosphorylate substrates in a single-step reaction *via* a metal-activated water molecule; and the protein tyrosine phosphatases (PTPases), which dephosphorylate pY in a two-step reaction [5-6]. The first step in pY dephosphorylation is the nucleophilic attack of the phosphate ester substrate by a conserved cysteine, and may involve H^+ transfer from a general acid to the leaving group and the formation of the thiophosphorylated intermediate. In the second step, the intermediate is hydrolyzed and the free enzyme regenerated.

PTPs also include a subfamily of dual-specificity PTPs (DSPs), which hydrolyze both pY and S/T phosphoesters [5], and thereby play important roles in regulating various intracellular activities and mechanisms associated with human diseases [7]. Among these, the cell division cycle 25 (CDC25) phosphatases are a subfamily of DSPs that are vital to cell cycle regulation. Indeed, aberrant levels of tyrosine phosphorylation, deriving from an imbalance of either the levels or activities of protein tyrosine kinases or protein tyrosine phosphatases have been implicated in a host of human disorders, including cancer, diabetes, Alzheimer's disease, Parkinson's disease, infection, and obesity [8].

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CDC25 FUNCTION AND STRUCTURE

CDC25 phosphatases are found in all eukaryotic organisms except plants [9]. In mammalian cells, three isoforms have been identified: CDC25A, CDC25B and CDC25C [10-12]. Orthologs of these isoforms have been found in *Xenopus laevis* (CDC25A and CDC25C) and chickens (*Gallus gallus*; CDC25A and CDC25B). Among different species, the catalytic domains of CDC25 proteins are quite well conserved with respect to their regulatory regions. In contrast, these are far more diverse and more frequently subject to alternative splicing events, which can generate at least two variants of CDC25A [13], and five each of CDC25B [14-15] and CDC25C [13,16-18].

Since the discovery of the three human CDC25 phosphatases in the early 90's [11-12,19], their involvement in control of the cell cycle has been extensively documented. The currently emerging picture, thoroughly described in recent reviews written by the Ducommun group [16,20], suggests that all three CDC25 phosphatases act at various cell cycle points to control the activity of specific Cdk/cyclin complex subpopulations by dephosphorylation of the pY and pT residues. Thus, CDC25A seems to be implicated in the control of G_1/S and G_2/M transitions, whereas CDC25B and CDC25C appear to play a role in the regulation of G_2/M transition [21].

The transitions between each cell cycle phase must be strictly regulated in order to maintain genomic stability. However, this genomic equilibrium can be upset by hyperactivity of these phosphatases, which are associated with checkpoint bypass [20]. Indeed, experimental data show that overexpression of CDC25B (but not CDC25C) rapidly pushes S- or G_2-phase cells into mitosis with incompletely replicated DNA [20], and that overexpression of CDC25A can induce mitotic events [21, 23, 24]. CDC25 phosphatases

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are also central targets and regulators of the G_2/M checkpoint mechanisms, including the ATM/ATR pathways, activated in response to DNA injury [25].

In the context of the progression of cell division, the A and B isoforms of CDC25 have been reported as potential oncogenes [26], being overexpressed in more than ten types of human cancer, including prostate [27] and breast [28] cancers, as well as vulvar squamous cell carcinomas [29]. In contrast, CDC25C is expressed at a far lower level in a limited number of tumors [30]. However, the full-length CDC25C as well as an alternative CDC25C splice variant were found to display increased mRNA levels in 50% of prostate cancers, as compared with 17% in normal prostate tissue [18]. This suggests that CDC25C could play an important role in prostate cancer progression and could therefore be used to monitor and predict the aggressiveness of this disease.

CDC25 proteins are 470–566 residues in length, and are composed of two principal domains. The C-terminal catalytic domains of the CDC25A, B, and C proteins are highly conserved with respect to their N-terminal regulatory regions, which are globally more divergent. The N-terminal domain contains many sites of phosphorylation involved in the regulation of the activity of the phosphatase, as well as protein stability and the association of CDC25 with its regulatory partners [24]. This amino-terminal moiety also contains signal peptides (NLS and NES sequences) that control the intracellular localization of the CDC25 phosphatases. The C-terminal domain encloses the catalytic pocket of these proteins.

The HCX_5R motif, which defines the catalytic site of all PTPs [31], is a significant region of homology between the CDC25s [0.8Å root-mean-square deviation (RMSD) in their 148 C^{α}-coordinates, with the exception of the disordered C-terminal α-helix in isoform A] and other PTPs. Several high-resolution structural modifications in the catalytic domain of CDC25B have been determined, including single residue mutations [32] or different oxidation states of the catalytic cysteine [33]. These structures show minor conformational differences in the side chains of solvent-exposed residues. In the catalytic signature HCX_5R, H is a highly conserved histidine residue, C is the catalytic cysteine, X_5 are five residues that form a loop in which all of the amide hydrogen molecules are bound to the phosphate of the substrate, and R is a highly conserved arginine required for binding to the phosphorylated amino acid of the substrate.

Examination of the structure of CDC25s has revealed that, in contrast to other protein phosphatases, their active site is surprisingly flat and extremely shallow, with a lack of auxiliary loops and no obvious features for mediating substrate recognition, which suggests a broad protein interface [34-35]. Indeed, substrate recognition has been proposed to rely on hotspot residues (R488, R492 and Y497 on CDC25B) located 20-30 Å from the active site [21,36]. In particular, Lazo *et al*. have documented a specific ~200-fold reduction in the apparent rate of association of the substrate (Cdk2/cyclinA) by mutants of the hotspot residues R488, R492 and Y497 (Fig. (**1A**)). Interestingly, the R492-D206 interaction was found to make the largest contribution (3.8 kcal/mol) to the total free binding energy, followed by the interaction between R488 and D206 (2.8 kcal/mol). Furthermore, structural characterization of this region revealed several features that support the feasibility of developing CDC25B-Cdk2/cyclinA interaction inhibitors. Indeed, a pocket located adjacent to the hotspot arginines was found to remain unoccupied by Cdk2, and was suggested by computational models to serve as an anchor for small-molecule binding. The hotspot residues and this nearby pocket, highlighted in dark gray in Fig. (**1A**), provided approximately 350 $Å^2$ of solvent-accessible surface area (220 $Å^2$ non-polar and 130 $Å^2$ polar) [37], and featured several potential hydrogen bond acceptors and donors. The volume of the pocket was shown to be approximately 200 $Å^3$, large enough to accommodate a fragment-like molecule (MW<250). Amino-acid variations at two positions in the pocket, F386 (CDC25A: Y344 and CDC25C: C290) and M505 (CDC25A: L362 and CDC25C: L409), further supported the feasibility of anchoring selective inhibitors at this site. Importantly, tests using small-molecule substrates have ensured that these hotspot mutations do not specifically affect phosphatase structure or reactivity [38].

Other important structural information regarding CDC25s have been recently reported by Arantes [39], who, using MD simulations, bioinformatics analysis, and computer-generated conformational ensembles, demonstrated that the last 30–40 residues in the C-terminus of CDC25B are either partially unfolded or disordered in solution (Fig. (**1B**)). Moreover, using three structural models with varying levels of flexibility, a rigid model corresponding to the crystal structure, a semi-flexible model corresponding to a set of MD snapshots, and a fully flexible model built with statistical rigor for backbone configurations, this author analyzed the effect of the C-terminal flexibility of the enzyme upon binding of two known inhibitors, 6-chloro-7-(2-morpholin-4-yl-ethylamino) quinoline-5,8-dione (NSC 663284) (**1**, Chart (**1**)), a partial mixed competitive inhibitor [40], and 2,5-dihydroxy-3-(7–(2-methyl-benzyl)-1*H*-indol-3-yl)[1,4]benzoquinone (**2**), a reversible inhibitor [41]. Two different binding modes were identified for compound (**1**): one, atop the P-loop, for reversible, competitive binding, and another, in the shallow pocket beside the active site, for irreversible oxidation. However, data available for compound (**2**) did not allow unambiguous identification of a clear binding mode.

The main effect of modeling backbone flexibility was found to be the formation of transient cavities (residues D392-H395, Y400, Y428, P444-L445, K509-G510, K513-E514 and M531-N532) or compact hydrophobic units on the surface of the stable, folded protein core (Fig. (**1B**)). These cavities were shown to be unexposed or unavailable for ligand binding in rigid and densely packed structures, but particularly important for binding to bulkier ligands with hydrophobic moieties.

As a whole, this information could facilitate the rational drug design of new potential CDC25 inhibitors, and could help us to speculate on the mechanisms of small-molecule complexation to partially unfolded or locally disordered proteins.

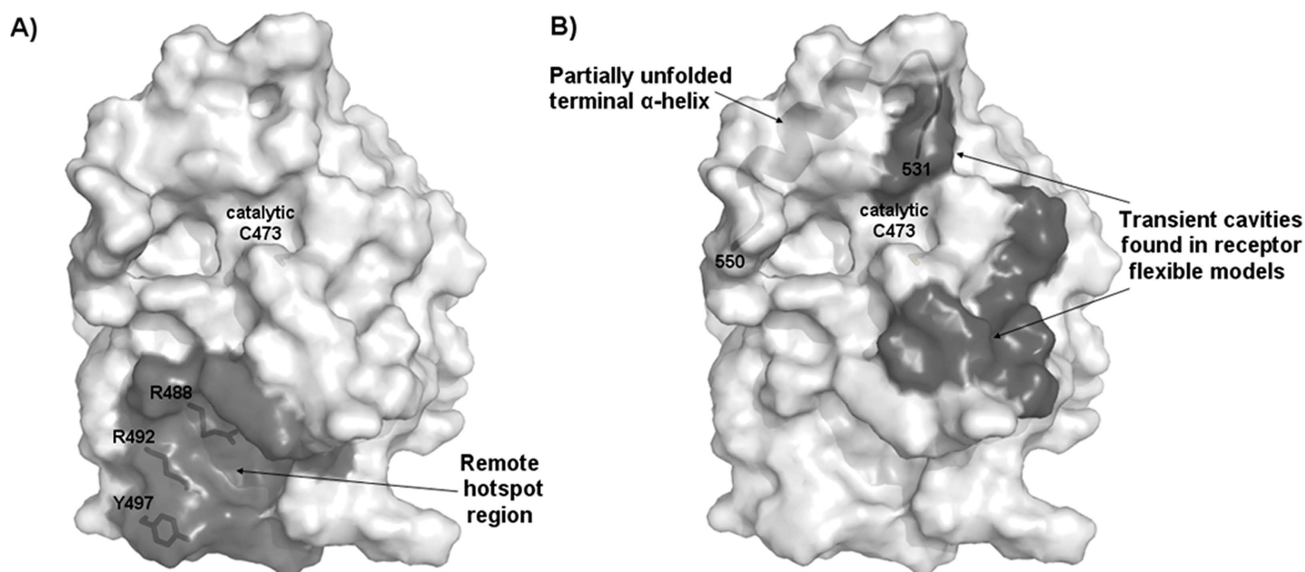

Fig. (1). Surface of the CDC25B phosphatase catalytic domain, as seen from the side that interacts with its substrate [36]. **A**) Hotspot residues (R488, R492 and Y497), which govern the association with the protein substrate, are shown in gray sticks. The remote hotspot region is highlighted in dark gray [8, 38]. **B**) Partially unfolded or disordered α terminal helix (residues 531-550) is shown in light gray cartoon. Protein regions that form transient cavities in the CDC25B flexible models [39] are highlighted in dark gray.

CDC25 INHIBITORS

A number of structurally diverse but effective inhibitors of the CDC25 phosphatases have thus far been identified [1-41]. Most of the CDC25 inhibitors reported in the literature have been discovered *via* either the isolation of new scaffolds by HTS [42] or the generation of improved derivatives of pre-existing inhibitor scaffolds [41,43-44]. In order to gain structural insight into their inhibitory mechanisms, binding modes of the newly discovered CDC25 inhibitors have been addressed using docking simulations in their active sites [45-47].

This review is intended to provide a summary of research into this subject published in the period encompassing 2008 and mid-2011. Specifically, we have chosen to focus on advances in the identification and the SARs of structurally novel CDC25 phosphatase inhibitors. For a discussion of earlier research, the reader is referred to a number of excellent reviews published previously [48-50].

First and foremost, in 2009, Zhou *et al*. [51] discovered the novel ortho-quinonoid CDC25B inhibitor LGH00031 (**3**, Chart (**1**) and Table **1**) by HTS. The compound was found to inhibit CDC25B irreversibly in a time- and dose-dependent manner, to have an IC_{50} of 0.97 μmol/L and to impair the proliferation of A549 (IC_{50}: 0.328 μmol/L), HeLa (IC_{50}: 0.290 μmol/L) and HCT116 cells (IC_{50}: 0.143 μmol/L). Its activity against CDC25B *in vitro* was found to be mediated by ROS derived from oxygen and DTT. Indeed, in the presence of DTT *in vitro*, LGH00031 (**3**) rapidly underwent reduction to the corresponding semi-quinone anion radicals, a process that is probably mediated by dihydrolipoic acid in cells. *In vitro*, however, the resulting ROS were seen to catalyze this step by oxidation of the cysteine at the active site of CDC25B to the sulfonic acid. Correspondingly, cell cycle arrest, a loss of cell viability and reductions in the level of cyclin B1 protein and phosphorylation of Cdk1 Y15 were noted.

In the same year, Park *et al*. [52] identified 32 novel CDC25 phosphatase inhibitors with micromolar activity by means of a structure-based *de novo* method applied to two previously identified inhibitor scaffolds: compounds (**4**) (5-methylene-2-phenylamino-thiazol-4-one) and (**5**) (1-phenyl-2-(4H-[1,2,4]triazole-3-yl-sulfanyl)-ethanone) [53]. Among the 107 derivatives of compound (**4**) tested for inhibitory activity in this study, 19 were found to have IC_{50} values of less than 10 μM for at least one of the two phosphatases, CDC25A or CDC25B. This study also highlighted the fact that halogen atoms and methyl groups appear to be suitable substituents on the phenyl ring of compound (**4**). Compound (**6**), in Chart (**1**), was found to be the most active, showing IC_{50} values of 5.1 and 1.2 μM for CDC25A and CDC25B, respectively (Table **1**). Docking studies evidenced that the thiazol-4-one ring of (**6**) receives two hydrogen bonds from the backbone amidic nitrogen atoms in the active site of CDC25B, whereas the guanidinium group of R436 in the active site of CDC25A played the role of hydrogen-bond donor with respect to the inhibitor thiazol-4-one moiety. The phenyl ring of (**6**) was found to reside at the entrance to the active site in CDC25A, but deep in the active site of CDC25B, establishing a hydrophobic contact with the side chains of Y428, R479, and M531. Therefore, the involvement of stronger hydrophobic interactions in the CDC25B/(**6**) complex with respect to CDC25A/(**6**) was invoked to explain the differences in IC_{50} values of the derivatives of scaffold (**4**) towards the two CDC25 phosphatases.

Thirteen derivatives of compound (**5**) exhibited IC_{50} values lower than 10 μM for at least one of the two CDC25 phosphatases. As in the case of scaffold (**4**), most of the derivatives exhibited a higher inhibitory activity for

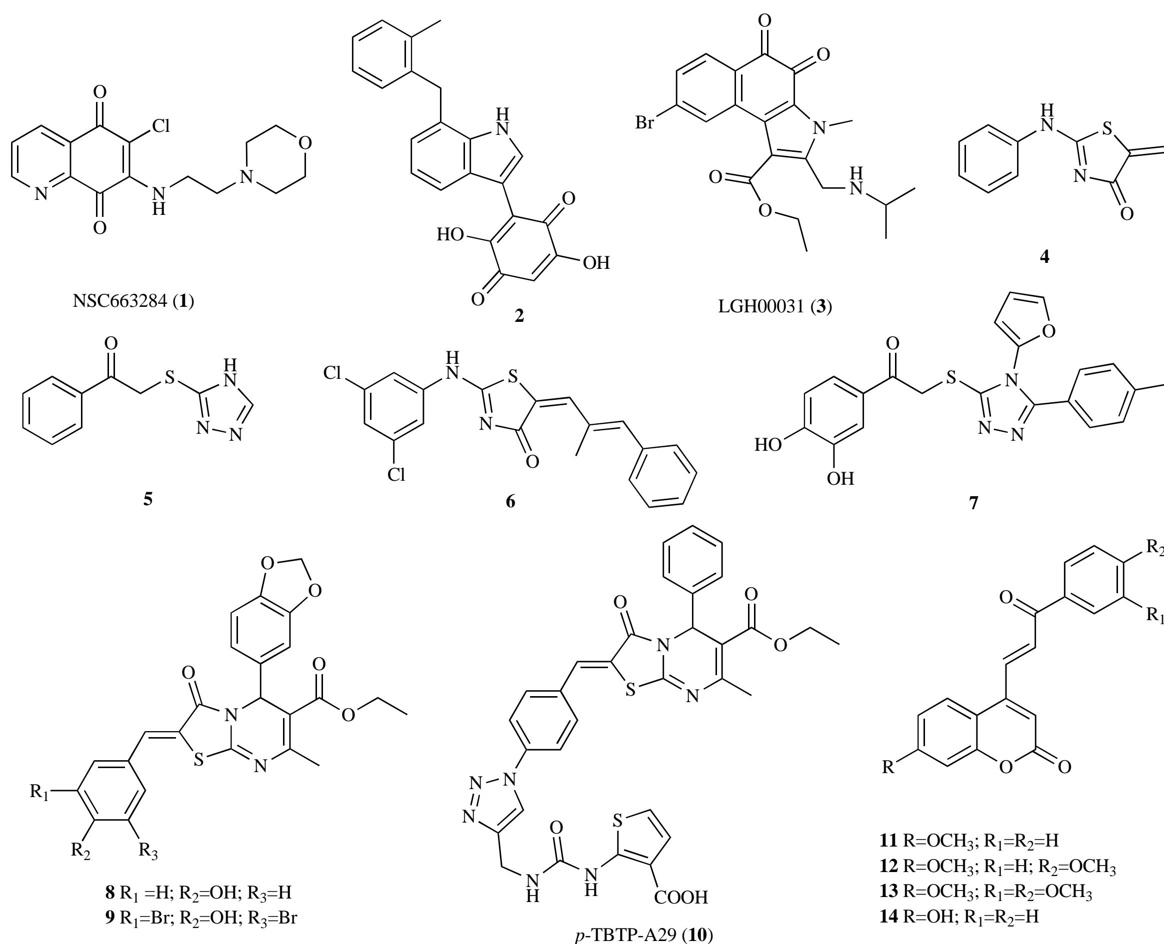

Chart 1. Chemical structures of synthetic CDC25 inhibitors **1-14**.

Table1. CDC25 Inhibitors with Reported Antitumor Activity

Cpd	(IC_{50} μM)			Cell Growth Inhibition (IC_{50} μM)						Ref.
	CDC25A	CDC25B	CDC25C	MCF7	HeLa	MDA-MDB435	A549	HCT116	LNCap	
1		0.64	-	~35	-	0.2	-	-	-	[40]
2	25	18	30	-	-	-	-	-	-	[41]
3	1.25	0.97	-	-	0.3	-	0.328	0.143	-	[51]
4	<10	<10	-	-	-	-	-	-		[52,53]
5	<10	<10	-	-	-	-	-	-	-	[52,53]
6	5.1	1.2	-	-	-	-	-	-	-	[52]
7	4.7	2.3	-	-	-	-	-	-	-	[52]
8	-	13.5	-	-	15.8[a]				-	[54]
9	-	4.5[b]	-	-	-	-	-	-	3.4	[55]
10	-	3	-	-	-	-	-	-	-	[56]
11	-	16.1[c]	21.2[c]	-	-	-	-	-	-	[57]
12	26.7[c]	-	27.9[c]	-	-	-	-	-	-	[57]
13	39.8[c]	-	26.2[c]	-	-	-	-	-	-	[57]
14	28	-	26	-	-	-	-	-	-	[57]

[a]Value obtained by a clogenic assay.
[b]Value reported on CDC25B3 isoform.
[c]Value expressed as percentage of inhibition.

CDC25B than for CDC25A. Compound (**7**), illustrated in Chart (**1**), exhibited the greatest inhibitory activity of the series, with IC_{50} values of 4.7 μM towards CDC25A and 2.3 μM towards CDC25B, respectively (Table **1**). Computational models showed that both phenolic oxygens of (**7**) formed three hydrogen bonds with the backbone amidic nitrogen atoms and side chain of C430 in the CDC25A active site. In contrast, only one of the two phenolic oxygens was involved in a bifurcated hydrogen bond with the carboxylate groups of E431 and E474 in the active site of CDC25B. The authors also observed that the carbonyl oxygen of (**7**) received a hydrogen bond from a backbone amide group of CDC25B, whereas it was exposed to bulk solvent in the CDC25A/(**7**) complex. This difference was cited as a determining factor behind the slightly higher inhibitory activity of (**7**) against CDC25B with respect to CDC25A.

In 2009, in order to improve the inhibitory potency of thiazolopyrimidine derivative (**8**) (IC_{50} = 13 μM, Table **1**), previously identified by *in silico/in vitro* screening experiments on the CDC25B isoform [54], Kolb *et al.* [55] synthesized and evaluated a library of 45 thiazolopyrimidine derivatives for inhibitory activity towards CDC25B. *In vitro*, 14 of these compounds inhibited CDC25B with IC_{50} of < 20 μM; the most efficient inhibitor, the 3,5-dibromo-4-hydroxyphenyl derivative (**9**), had a potency of 4.5 μM. Kinetics studies revealed that all of these compounds exhibit mixed-inhibition profiles, suggesting possible interactions with both the catalytic site and the inhibitor-binding pocket. Furthermore, (**9**) was able to reverse the bypass of genotoxicity-induced G2 arrest on CDC25B overexpression, indicating that this compound targets the dual-specificity phosphatase in cultured cells. Subsequently, the cytotoxic activities of the 14 active compounds were determined against two human cancer cell lines, MiaPaCa-2 and LNCaP. The results showed that LNCaP cells tend to be more sensitive than the MIA PaCa-2 line to thiazolopyrimidines. Indeed, compound (**9**) displayed the best cytotoxic potency against LNCaP with an IC_{50} value of 3.4 μM, but it was observed to be inactive against MiaPaCa-2. Two orientations were proposed for (**9**), the first of which featured the 3,5-di-bromo-4-hydroxyphenyl moiety stacked against F475, next to the catalytic site, and the phenyldioxolane moiety orientated towards the catalytic cysteine. In this situation, compound (**9**) could form four strong hydrogen bonds with the protein (with atoms of the catalytic pocket and the side chain of N532, none with R482 and R544) and could also be involved in significant hydrophobic contacts and aromatic stacking interactions. Alternatively, the 3,5-dibromo-4-hydroxyphenyl moiety could point toward the swimming pool area, without being able to achieve full insertion into the cavity. In this case, (**9**) interacts with the protein through five hydrogen bonds; indeed, several hydrophobic/aromatic contacts were noted.

In the same year, Duval *et al.* [56] used parallel click chemistry for the rapid unmasking of novel thiazolopyrimidines as CDC25 phosphatase inhibitors, using compounds (**8**) and (**9**) as a starting point [53-54]. Analysis of the SARs in this series suggested that an extended conjugation through a substituted 2-thiocinnamide system is a critical feature for CDC25 inhibition. Thus, the rational design of inactive azide precursors and the use of alkynes as pharmacomodulating elements allowed these researchers to generate an 87-member triazole library with good to quantitative yields and high purities. This permitted the direct biological screening of reaction mixtures for bioactive cycloadducts, thereby providing an efficient short-cut to the standard drug-discovery approach. Using this strategy, several novel micromolar inhibitors with an unexpected triazolobenzylidene-thiazolopyrimidine (TBTP) skeleton were rapidly identified, along with key SARs in this series. *p*-TBTP-A29 (**10**) was found to be the most potent CDC25B inhibitor of the series with an IC_{50} value of 3 μM (Table **1**).

In 2010, in the attempt to identify new scaffolds able to inhibit CDC25 phosphatases, Valente *et al.* [57] discovered several novel coumarin-based compounds. The design of these compounds was prompted by the observation that the thiazolopyrimidine (**9**) is cytotoxic to HeLa [54] and prostatic LNCaP cells [56], and that several flavonolignan compounds isolated from silymarin provoked strong cell-cycle arrest in PC3 cells, as well as differential effects on the levels of some cell-cycle regulator cyclins, including CDC25A, CDC25B and CDC25C [58]. Hence, the authors synthesized a series of coumarin derivatives and tested them against all three CDC25 isoenzymes. The introduction of a benzoylvinyl moiety at the C4 position of the coumarin nucleus (chalcone-coumarin **11-14**) gave an interesting degree of inhibition against both CDC25A and CDC25C with values up to 94.2% and 79.3% against (**11**) and 94.3% and 94.2% against (**14**), respectively, compounds (**11**) and (**14**) being the most potent inhibitors in this assay. In a reversibility assay of CDC25A inhibition, compound (**11**) displayed a pattern of time-dependent inhibition, and the maximal inhibitory effect was observed during a 90-min pre-incubation period (35% of residual activity). As the inhibitory activity persisted after the dilution procedure, it was concluded that compound (**11**) acts as an irreversible inhibitor of the CDC25 phosphatases *in vitro*.

Another significant screening was carried out by the University of Pittsburgh Molecular Library Screening Center (Pittsburgh, PA), which searched the National Institutes of Health (NIH) compound library for new CDC25B inhibitors [59]. Seventy-nine (0.12%) of the 65,239 compounds screened at 10 μM met the active criterion of ≥50% inhibition of CDC25B activity, and 25 (31.6%) of these were confirmed as CDC25B inhibitors with IC_{50} values of <50 μM. Nine CDC25B inhibitors did not appear to affect CDC25B through a mechanism involving oxidation, as they did not generate detectable amounts of H_2O_2 in the presence of DTT and their CDC25B IC_{50} values were not significantly affected either by exchanging the DTT for β-mercaptoethanol or reduced glutathione or by adding catalase to the assay. Among the other compounds, however, two bisfuran-containing hits [(PubChem substance identifiers 4258795 (**15**) and 4260465 (**16**)], shown in Chart (**2**), significantly inhibited the growth of human MBA-MD-435 breast and PC-3 prostate cancer cell lines. To confirm the structure and biological activity of (**16**), the compound was re-synthesized along with two of its analogs [(PubChem SIDs 26683753 (**17**) and 26683756 (**18**)]. Both the substitutions in the two analogs were not tolerated, and only

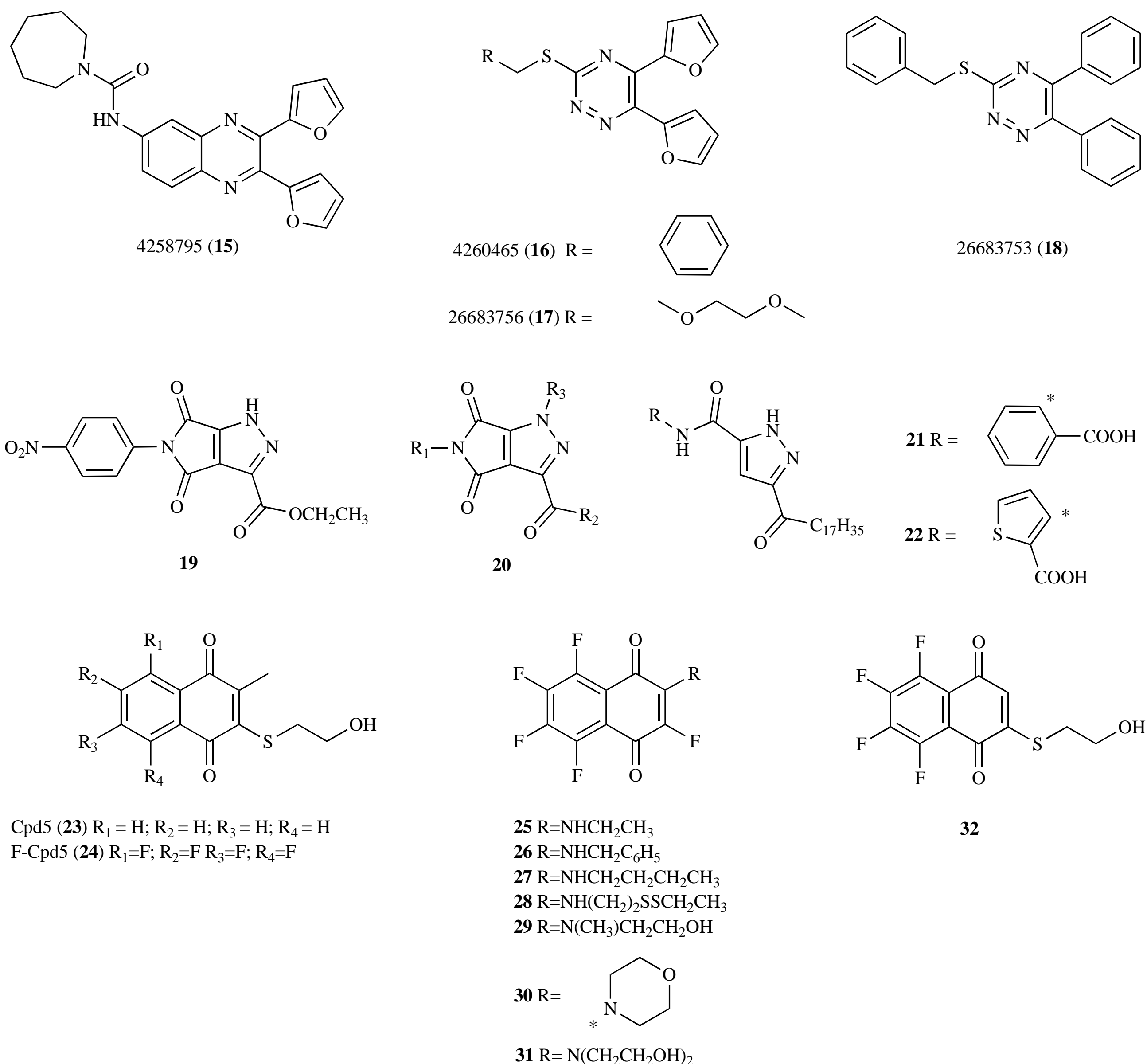

Chart 2. Chemical structures of synthetic CDC25 inhibitors **15-32**.

the re-synthesized compound (**16**) inhibited CDC25B activity *in vitro* (IC_{50} = 13.83 µM, Table **2**) and significantly inhibited the growth of the MBA-MD-435 breast and PC-3 prostate cancer cell lines (IC_{50} = 20.16 µM and 24.87 µM, respectively).

In addition, Chen *et al.* [60] recently reported the discovery and structural optimization of N-substituted maleimide fused-pyrazole analogs as a novel series of CDC25B inhibitors with submicromolar *in vitro* inhibitory potency against CDC25B. The original hit, compound (**19**), which contains a maleimide fused-pyrazole core, was obtained by high-throughput screening of an in-house compound library against a recombinant CDC25B, expressed and purified in the laboratory. This compound exhibited moderate CDC25B inhibitory activity (IC_{50} = 32µM), and, therefore, in order to elucidate the SARs and improve the potency, the authors prepared a series of compounds with scaffold (**20**), in which they changed the substituted group R_1 on the phenyl ring, R_2 on the carbonyl group, and R_3 on the nitrogen of the pyrazole ring.

Preliminary structural optimization of this series provided compounds (**21**) (IC_{50} = 0.16 µM) and (**22**) (IC_{50} = 0.12 µM), which showed, respectively, 200- and 270-fold potency with respect to the original hit, compound (**19**), as well as improved physical properties. Enzyme kinetic studies showed that compound (**22**) binds reversibly to CDC25B and inhibits it in a mix-type mode.

Although most quinones have been reported to inhibit CDC25 by sulfhydryl arylation at the quinone moiety, due to their redox properties they can also generate ROS [61], which may be toxic to normal tissues and thus reduce their therapeutic utility. However, one strategy for overcoming the intrinsic toxicity of quinones might be to use derivatives that

Table 2. CDC25 Inhibitors with Reported Antitumor Activity

Cpd	(IC_{50} μM)			Cell Growth Inhibition (IC_{50} μM)						Ref.
	CDC25A	CDC25B	CDC25C	Hep3B	MCF7	PC3	MDA-MDB435	RPMI 8226	LMTK	
15	-	11.6	-	-	-	-	-	-		[59]
16	-	13.8	-	-	-	24.9	20.2	-	-	[59]
17	-	-	-	-	-	-	-	-	-	[59]
18	-	-	-	-	-	-	-	-	-	[59]
19	-	32	-	-	-	-	-	-	-	[60]
20	-	-	-	-	-	-	-	-	-	[60]
21	-	0.16	-	-	-	-	-	-	-	[60]
22	-	0.12	-	-	-	-	-	-	-	[60]
23	5	2	2	-	-	-	-	-	-	[64]
24	0.8	1	50	2	173	-	-	14.8		[64]
25	-	-	-	-	2.5	-	-	0.7	3.2	[66]
26	-	-	-	-	0.8	-	-	0.8	4.0	[66]
27	-	-	-	-	2.2	-	-	2.5	17.2	[67]
28	-	-	-	-	5.8	-	-	4.0	46.6	[67]
29	-	-	-	-	5.8	-	-	1.2	11.7	[68]
30	-	-	-	-	5.7	-	-	6.0	30.0	[68]
31	-	-	-	-	7.5	-	-	5.1	52.7	[68]
32	-	-	-	-	12.8	-	-	2.9	25.0	[68]

are more stable in their reduced state and are therefore less likely to initiate the formation of radicals that cause indiscriminate damage to cells. Interestingly, fluorinated derivatives of 1,4-naphthoquinones were found to be less likely to form ROS; of the substances tested, the most potent inhibitor of CDC25A and CDC25B phosphatases was Cpd5 (**23**) [62] and its fluorinated version, F-Cpd5 (**24**) [63-65]. Indeed, F-Cpd5 (**24**) was calculated to have much higher reduction potential than (**23**) and predicted not to generate ROS [63]. Moreover, it was three times more potent than (**23**) in inhibiting Hep3B cell growth [63-65]. Furthermore, it exhibited a 12-fold reduction in mitogen-induced DNA synthesis inhibition in normal rat hepatocytes with respect to Hep3B cells [64].

Based on these observations, Zakharova *et al*. [66-68] published three consecutive papers reporting the synthesis of various polyfluoro-1,4-naphthoquinone derivatives, including details of their cytotoxicity in human myeloma, human mammary adenocarcinoma, mouse fibroblasts and primary mouse fibroblast cells, as well as their mutagenic and antioxidant properties in a Salmonella tester strain. Since the structure of the synthesized compounds resembled that of F-Cpd5 (**24**), the authors hypothesized similar inhibitory effects on the CDC25 phosphatases and elected not to evaluate them in conventional binding assays.

In the first of these papers [66], the authors reported compounds 2-ethylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**25**) and 2-phenylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**26**) as the most active, exerting a stronger cytotoxic effect against cancer cells in comparison with normal mammalian cells and efficiently protecting bacterial cells from mutagenesis in both the presence and absence of H_2O_2. In fact, all investigated properties of these two compounds were shown to be superior to those of F-Cpd5, as evidenced in Table **2**. In the second paper [67], the authors showed that the most cytotoxic compounds were 2-n-butylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**27**) and (2,2'-dithiodi-2)-3,5,6,7,8-pentafluoro-1,4-naphthoquinone-2-ylamino)ethane (**28**). Finally, in the third article [68], the authors demonstrated that 2-[2-hydroxyethyl (methyl)amino]-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**29**), 2-morpholino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**30**), 2-[bis-(2-hydroxyethyl)amino]-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**31**), and 2-[(2-hydroxy)ethylsulfanyl)]-5,6,7,8-tetrafluoro-1,4-naphthoquinone (**32**) had more potent cytotoxic effects against cancer as compared to normal mammalian cells. In addition, (**29**), (**31**) and (**32**) showed the best protective effects against H_2O_2-dependent mutagenesis in bacterial cells.

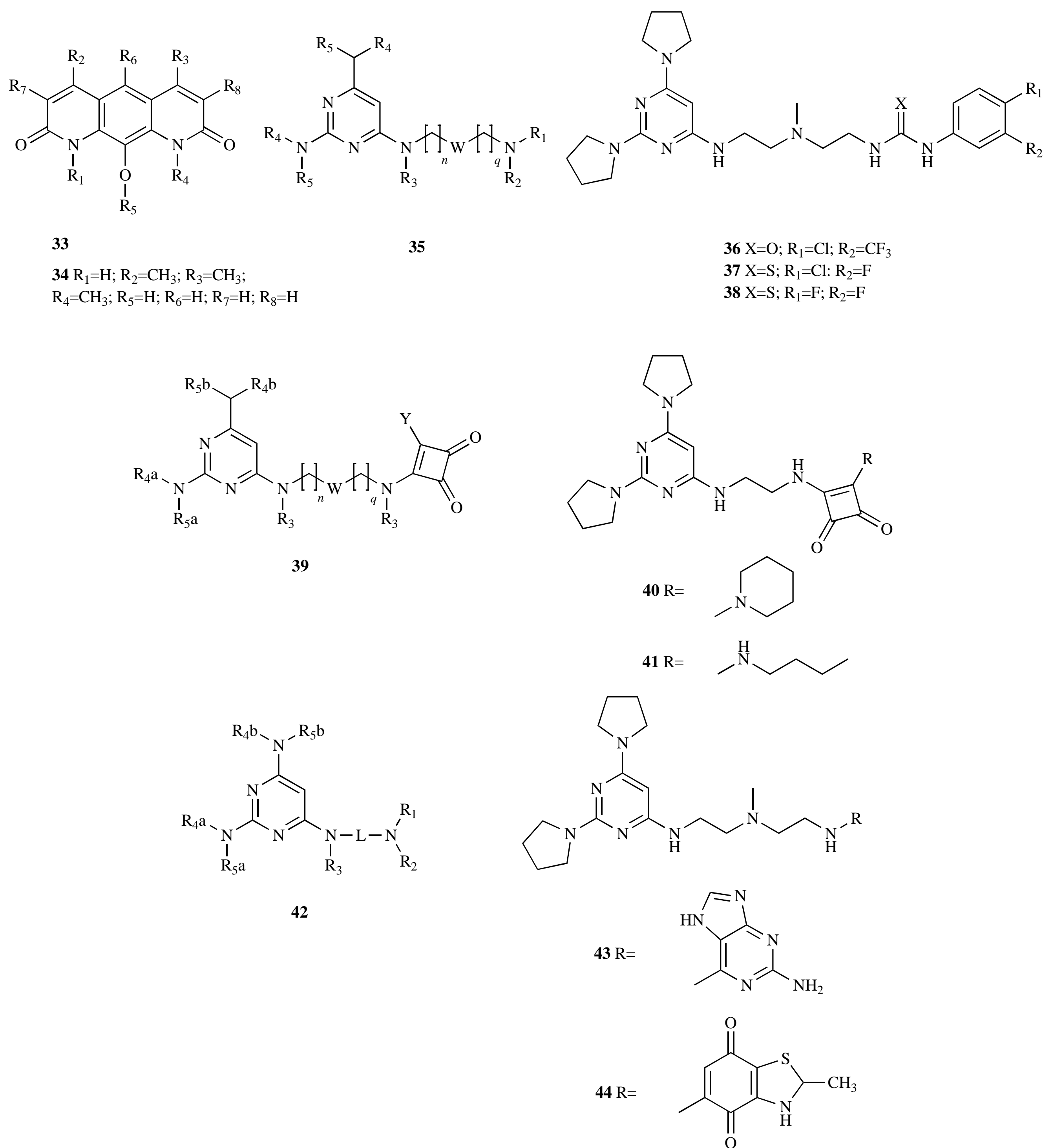

Chart 3. Chemical structures of synthetic CDC25 inhibitors **33-44**.

Interest in CDC25 phosphatases as promising targets for cancer therapy has also prompted the publication of many patents. In December 2009, Nussbaum *et al*. and InterMed Discovery GmbH, as inventors and applicants, respectively, published details of the synthesis and pharmaceutical potential as CDC25 inhibitors of a series of new pyridoquinolones [69]. Other diazaquinomycin-like pyridoquinones were found to be antibacterial and anticancer agents [70], and several derivatives of diazaquinomycin (dihydrodiazanthracenes, aza- and diaza-anthraquinonic derivatives) were shown to exhibit anti-tumoral activity [71-72]. In contrast, neither pyridoquinones nor pyridoquinolones have been reported to exhibit inhibitory activity towards CDC25.

The effective compounds have the general formula (**33**, Chart (**3**)), but variable substituents: R_1 is -H, -C_{1-6} alkyl, -C(=O)-C_{1-6} -alkyl, -C(=O)-O-C_{1-8} -alkyl, -C(=O)-NH-C_{1-8},

Table 3. CDC25 Inhibitors with Reported Antitumor Activity

Cpd	(IC_{50} µM)			Cell Growth Inhibition (IC_{50} µM)					Ref.
	CDC25A	CDC25B	CDC25C	HCT116	DU145	A549	H460	MIAPaCa	
33	-	-	-	-	-	-	-	-	[69]
34	0.15	0.23	0.18	0.1	0.1	0.1	<0.3	-	[69]
35	-	-	-	-	-	-	-	-	[73]
36	-	-	<100	-	500	-	-	500	[73]
37	-	-	<100	-	500	-	-	500	[73]
38	-	-	<100	-	500	-	-	500	[73]
39	-	-	-	-	-	-	-	-	[74]
40	-	-	<1	-	2	-	-	2	[74]
41	-	-	<1	-	2	-	-	2	[74]
42	-	-	<1	-	-	-	-	-	[76]
43	-	-	<1	-	-	-	-	-	[76]
44	-	-	<1	-	-	-	-	-	[76]

etc. (see patent for more comprehensive details of the substituents), R_2 and R_3 are, independent of each other, -H, -C_{1-8}-alkyl, -phenyl, -C_{1-8} -alkyl-phenyl, -C(=O)-C_{1-8}-alkyl, etc., R_4 is -H, -C_{1-8} -alkyl, -phenyl, -C_{1-8} -alkyl-phenyl, etc., R_4 and R_5 are -H, -C_{1-8} -alkyl, -phenyl, -C_{1-8} -alkyl-phenyl, etc., R_6 is -H, -C_{1-6} -alkyl, -phenyl,-C_{1-6}-alkyl-phenyl, R_7 and R_8 are, independent of each other, -H, -C_{1-8}-alkyl, -C(=O)-C_{1-8}-alkyl, -C(=O)-O-C_{1-8}-alkyl etc. One of the most interesting products, compound (**34**), was thoroughly characterized regarding its selectivity for CDC25 sub-types A, B, and C. After 20 minutes of pre-incubation in inhibition assays, the compound showed IC_{50} values of 150 nM against CDC25A, 230 nM against CDC25B and 180 nM against CDC25C, respectively. In addition, a cell proliferation assay of compound (**34**) showed its antiproliferative efficacy in several cell lines *in vitro*, with IC_{50} values of 100 nM for HCT116, DU145 and A549 cell lines, respectively, and < 300 nM for the H460 cell line (Table **3**).

During 2010, other international patents, US/2010/ 137275A, US/2010/0173910 and WO/2010/130900A [73-76], fruit of research conducted by the Prevost group, which identified a series of triaminopyrimidines derivatives as a new class of CDC25 inhibitors, were published. In the first patent, US/2010/137275A [73], the authors reported a method of preparing a series of novel triaminopyrimidines with the general formula (**35**)**,** in which R_1, R_2, W, R_3, R_4, and R_5 are variable groups (Chart (**3**)). These derivatives were described as novel CDC25 inhibitors, useful in the prevention of cancerous and non-cancerous proliferative lesions, neurodegenerative disorders, parasitic diseases, viral infections, autoimmune diseases, graft rejection, inflammatory diseases, allergies and alopecia, as well as in the prevention of oocyte maturation. The most interesting compounds, N-[4-chloro-3-(trifluoromethyl)phenyl]-N'-{2-[{2-[(2,6-dipyrrolidin-1-ylpyrimidin-4-yl)amino]ethyl}(methyl)amino]ethyl}urea (**36**), N-(4-chloro-3-fluorophenyl)-N'-{2-[{2-[(2,6-dipyrrolidin-1-ylpyrimidin-4-yl)amino]ethyl}(methyl)amino]ethyl}thiourea (**37**) and N-(3,4-difluoro-phenyl)-N'-{2-[{2-[(2,6-dipyrrolidin-1-ylpyrimidin-4-yl)amino]ethyl}(methyl)amino]ethyl}thiourea (**38**) displayed IC_{50} values of less than or equal to 100µM towards the purified recombinant CDC25C enzyme. Moreover, when, on day 1, MIAPaCa-2 and Du145 cancer cell lines were treated with increasing concentrations, up to 10 µM, of each test compound for 96 hours, IC_{50} values of less than 500 µM were revealed (Table **3**).

The subsequent patent, US/01/00173910 [74], put forward by Liberatore *et al.* as inventors and Ipdrn Phstmsd D.S.S. as the applicant, described a series of cyclobutenedione triaminopyrimidine derivatives with the general formula (**39**)**,** where Y, R_3, W, R_{4a}, R_{5a}, R_{4b}, R_{5b}, n and m are variable (see patent for detailed information about all substituents). The applicant had discovered that triaminopyrimidine derivatives with the general formula (**39**) showed an inhibitory activity towards the purified recombinant CDC25C enzyme by an assay described in the patent application WO/01/44467 [75]. Hence, the authors concluded that the compounds could be useful as pharmaceutical inhibitors of tumor and normal cell proliferation, alone or in combination with other treatments, and could be employed in the treatment of neurodegenerative diseases. Experimental data reported in the patent highlighted the fact that eighteen of these had IC_{50} values below or equal to 1 µM regarding inhibition of the phosphatase activity of CDC25C. Furthermore, several had an IC_{50} of 2 µM of less against the proliferation of MIA PaCa-2 human pancreatic and DU145 human prostate cancer carcinoma cell lines (Table **3**). Two representative examples from this series, 3-({2-[(2,6-dipyrrolidin-1-ylpyrimidin-4-yl)amino]ethyl}amino)-4-piperidin-1-ylcyclobut-3-ene-1,2-

dione (**40**) and 3-(butylamino)-4-({2-[(2,6-dipyrrolidin-1-ylpyrimidin-4-yl)amino]ethyl}amino)cyclobut-3-ene-1,2-dione, (**41**), are illustrated in Chart (**3**).

Finally, the patent WO/2010/130900A [76] described another series of triaminopyrimidines with the general formula (**42**). In this case too, R_1 R_2 L, R_3 R_{4a} and R_{5a} are variable substituents. In the invention, a method of synthesis, the therapeutic compositions and the potential of these compounds as medicaments are also reported. Eleven compounds from this series showed an IC_{50} towards the purified CDC25C phosphatase of less than or equal to 1µM. Two of these eleven compounds, N^6-(2-((2-(2,6-di (pyrrolidin-1-yl)pyrimidin-4-ylamino)ethyl)(methyl)amino) ethyl)-7H-purine-2,6-diamine (**43**) and 5-(2-((2-(2,6-di (pyrrolidin-1-yl)pyrimidin-4-ylamino)ethyl)(methyl)amino) ethylamino)-2-methyl-2,3-dihydrobenzo[d]thiazole-4,7-dione (**44**), are illustrated in Chart (**3**) as examples.

CONCLUDING REMARKS AND FUTURE PROSPECTS

The aim of this review was to summarize the present state-of-the-art regarding the design and SARs of CDC25 phosphatase inhibitors. Inhibitors published before 2008 have only been included if new aspects have appeared in the meantime. For a complete picture, the reader is referred to our former reviews [48-50].

The discovery of new CDC25 inhibitors is extremely significant for cancer therapy due to the critical roles that these phosphatases play in regulating cell-cycle progression and DNA damage-induced checkpoint arrest and recovery [20,24,26,77,78]. Elucidation of the crystal structures of the catalytic domains of CDC25A and CDC25B has revealed that they both possess a shallow solvent-exposed active site and no obvious sites for substrate recognition or inhibitor binding [21,32,79]. In fact, CDC25-protein substrate recognition appears to occur through a broad protein–protein interaction surface [21,32,79], making the molecular modeling of inhibitors exceedingly challenging.

Nevertheless, a number of potent CDC25 inhibitors have now been synthesized or isolated from natural product extracts, and although many of these exhibit selectivity against other dual-specificity phosphatases or PTPs, most inhibit all three CDC25 isoforms. The majority of the known CDC25 inhibitors are quinone-based structures, phosphate surrogates or electrophiles. The mechanism of action of many quinonoid compounds involves the oxidation and inactivation of the CDC25 active site cysteine by ROS, either generated through *in vitro* redox cycling of these compounds in the presence of DTT or induced within cells by exposure to these compounds [80-82]. In fact, some quinone compounds have been shown to mediate their inhibitory action *via* covalent modification the CDC25 active site cysteines or vicinal serines [77,78].

However, despite the large number of potent quinoid CDC25 inhibitors with antiproliferative activity against tumor cell lines discovered, very few of these have demonstrated *in vivo* activity in human tumor cell xenograft animal models. Hence, the ultimate goal of developing a bioavailable inhibitor of CDC25 phosphatases, therefore, a novel drug for anticancer therapy is yet to be achieved, and targeting of these enzymes remains a tough challenge for medicinal chemists. Nonetheless, the ongoing work in this field is continuously providing us with a deeper understanding of the structure and function of CDC25 phosphatases, and thereby promising great hope for the future.

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ABBREVIATIONS

CDC25 = Cell Division Cycle 25
PSTPs = Serine/Threonine Protein Phosphatases
PTPs = Protein Tyrosine Phosphatases
DSPs = Dual-Specificity Phosphatases
NLS = Nuclear Localization Sequences
NES = Nuclear Exclusion Sequences
CdK= Cyclin Dependent Kinase
MD = Molecular Dynamics
P-loop = Phosphate-Binding Loop
SAR = Structure-Activity Relationship
HTS = High Throughput Screening
ROS = Reactive Oxygen Species
DTT = Dithiothreitol
MIA PaCa-2 = Human Pancreatic Cancer Cell Line
Du145 = Human Prostate Cancer Cell Line
HCT116 = Human Colon Cancer Cell Line
A549 = Human Lung Adenocarcinoma Cell Line
H460 = Human Non-Small Cell Lung Cancer Cell Line
HeLa = Immortal Cell Line
MDA-MDB435 = Human Breast Cancer Cell Line
LNCap = Human Prostatic Carcinoma Cell Line
MCF7 = Human Breast Adenocarcinoma Cell Line
Hep3B = Human Epatoma Cell Line
PC3 = Human Prostate Cancer Cell Line
RPMI 8226 = Human Myeloma Cell Line
LMTK = Transformed Mouse Fibroblast Cell Line

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