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Design and synthesis of macrocyclic inhibitors of phosphatase Cdc25B

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Dedicated to Professor Dr. Ulrich Eder on the occasion of his 65th birthday

Abstract—Based on molecular modeling studies, macrocyclic inhibitors of phosphatase cdc25B were synthetically derived from steroids. A preliminary SAR for this new template was elaborated. A series of compounds shows inhibition of cdc25B in the low micromolar range and good selectivity versus other phosphatases. The compounds did not show a significant antiproliferative effect in MaTu or HaCaT cells.

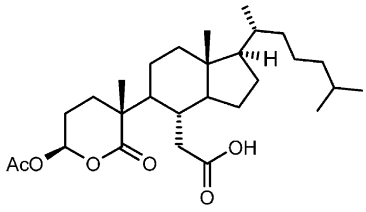
1. Introduction

Cdc25 phosphatases have essential functional roles in progression and regulation of eukaryotic cell proliferation.[1] In human cells, three isoforms have been identified, cdc25A, B and C, each responsible for activation of defined CDK/cyclin complexes during specific phases of the cell division cycle (G1/S transition, S phase progression, G2/M transition).[2] Neutralization of cdc25 by microinjection of antibodies results in cell cycle arrest, indicating the essential functional role.

Cdc25A and B are potential oncogenes: (i) Cdc25A and/or B (mRNA or protein) are overexpressed in a large number of human carcinomas (including breast, lung, colorectal, gastric, prostate, head and neck, ovary, lymphomas and melanomas) and tumor cell lines,[3] (ii) cotransfection of cdc25A or B, respectively, with activated H-RAS into embryonic fibroblasts results in transformation of these cells and tumor formation in nude mice,[4] (iii) transgenic mice overexpressing cdc25B in the mammary gland develop hyperplasia or show increased susceptibility to chemically-induced mammary tumors,[5] (iv) furthermore, transcriptional expression of both isoforms is activated by the proto-oncogene c-myc.[6] Chemical inhibitors of cdc25 phosphatase, therefore, offer the potential for interfering with the proliferation of tumor cells.

2. Design

Among the small molecule inhibitors of cdc25 protein phosphatases known from the literature are several compounds of steroidal origin.[7] Peng et al. reported on the synthesis of compound **1** which possesses moderate inhibitory activity against human cdc25A.[8,9]



1

IC_{50} (cdc25A) = 8.7 μM[8]

As a part of a program for the evaluation of novel templates for natural product based libraries we are interested in macrocyclic structures of type **2** which are also derived from steroids.[10,11]

Keywords: Steroids; Macrocycles; Phosphatases; Molecular modeling; Templates.
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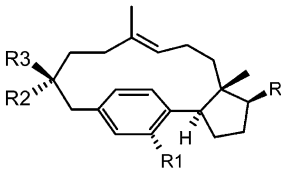
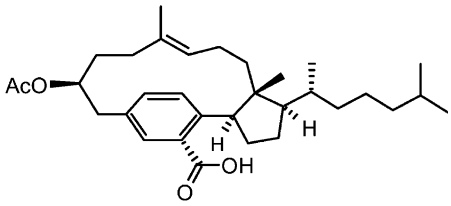


2

This extremely rigid structure is still comparable to the parent steroid regarding conformational flexibility and position of functional groups. At the same time it offers a high potential for structural diversification. Together with the corresponding residues it leads to compound **3**.



3

IC_{50} (cdc25B) = 5.6 μM

Since the ring conformations of these compounds have been elucidated in great detail by small-molecule crystal structures,[11] only the orientations of the attached side chains have to be modeled. Superposition of energy-minimized conformers of compound **3** and the Peng-inhibitor **1** shows striking similarities in three-dimensional space for the rigid parts of the molecules. Cleavage of the ring systems in the steroid leaves the relative orientation of the acetate group and the cyclopentane ring fairly unaffected. Moreover, the exocyclic carboxylate groups of **1** and **3** appear to superimpose reasonably well. With the two six-membered rings in **1** matching the opening of the macrocylic ring after the superposition, the two compounds occupy very similar volumes (Fig. 1).

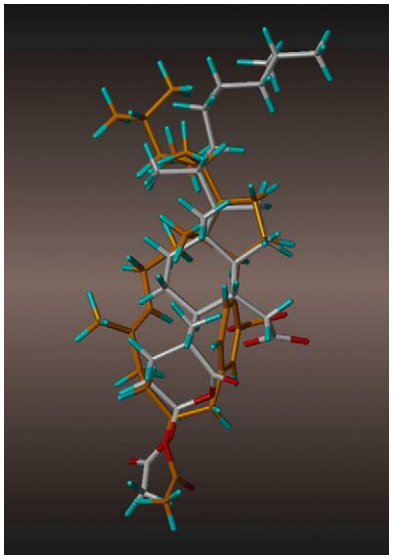


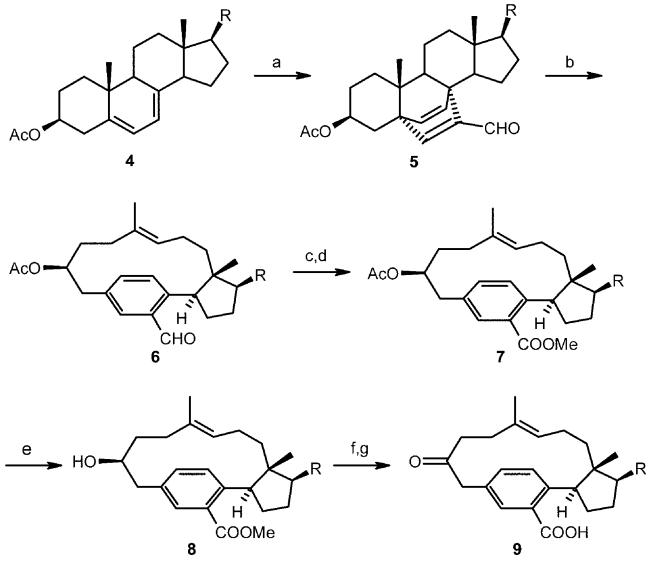
Figure 1. Superimposition of energy-minimized conformers of the steroid-derived compound **1** (shown in white) and the macrocyclic structure **3** (shown in orange).

The structural similarity gave rise to the assumption that there also might be a similarity in biological activity. This indeed was confirmed by initial biological tests: compound **3** inhibited phosphatase cdc25B with an IC_{50} of 5.6 μM, the same range as **1** interacted with cdc25A. Due to this first encouraging result we embarked upon the synthesis of a series of simple analogues of **3**.

The goal of this study was to establish SAR to identify pharmacophoric groups crucial for activity and specificity in order to generate a new lead on the basis of our novel template structure.

3. Synthesis

The synthesis of a representative structure is outlined in Scheme 1. Starting material was 3-*O*-acetyl-7-dehydrocholesterol **4** which is readily available from commercial 7-dehydrocholesterol. Following the Winterfeldt protocol,[12] the diene **4** was treated with propiolaldehyde and BF_3–Et_2O. The resulting Diels–Alder adduct **5** was then heated to reflux in xylene to give the retro-Diels–Alder-product **6** in 90% yield. The aldehyde in **6** gives way to numerous possibilities for derivatizations. To obtain the favorable carboxyl moiety it was oxidized to **3** by sodium chlorite. The carboxylic acid could be protected as a methyl ester (**7**) by treatment with TMS–diazomethane. Cleavage of the acetate with $NaOCH_3$ yielded alcohol **8**. At this stage the derivatization of the hydroxyl group is easily possible. For example, a PCC-oxidation to the keto group and a subsequent hydrolysis of the methyl ester gave the carboxylic acid **9**.



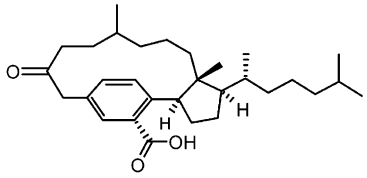
Scheme 1. R = cholesterol side chain. Reagents and conditions: (a) propiolaldehyde, BF_3–Et_2O, CH_2Cl_2, 0 °C to rt; (b) xylene, reflux, 90% (2 steps); (c) $NaClO_2$, NaH_2PO_4, amylene, *t*BuOH/H_2O; (d) (TMS)CHN_2, MeOH/toluene, rt, 85% (2 steps); (e) $NaOCH_3$ (5% in MeOH); (f) PCC, CH_2Cl_2; (g) LiOH in MeOH, 52% (3 steps).

4. Results and discussion

A first series of 28 macrocyclic compounds of type **2** was synthesized. The substances were evaluated as inhibitors of the recombinant human cdc25B phosphatase. Phosphatase activity of a purified GST-cdc25B fusion protein was determined by measuring the rate of dephosphorylation of the fluorigenic, generic phosphatase substrate fluorescein diphosphate in 384-well microtiter plates.[13] The compounds were preincubated with the enzyme for 1 h and subsequently the reaction was initiated by addition of the substrate (5 μM). Fluorescence intensity was determined in the linear range of the reaction after 1 h of incubation at room temperature.

For a first selectivity check the compounds were also tested against the phosphatases PTP1B, PTPα and PP2A. These phosphatases are involved in insulin signaling, activation of Src familiy kinases, and regulation of a variety of cellular signaling processes, respectively.[14]

Several compounds show inhibition of cdc25B in the low micromolar range and an at least 3-fold selectivity versus the reference phosphatases. The results are shown in Table 1. So far the most potent compound **13** with an IC_{50} of 3.4 μM features a carboxylic acid moiety at the aromatic ring (R^1) and a keto function (R^2/R^3) while the double bond of **2** is hydrogenated (mixture of isomers at new stereogenic center).



13

These features still match the properties of the Peng-compound **1**. Compound **13** is representative for the preliminary SAR-information that can be derived from the data: The carboxylic acid (R^1) seems to be crucial for the activity (Table 1, entry 1–11). Only a carbaldehyde is also tolerated in this position (Table 1, entry 12–14). R^2/R^3 may be varied over a wide range. Many polar functionalities of different steric demands are tolerated in this position. A hydrogenation of the double bond has no significant influence on the potency.

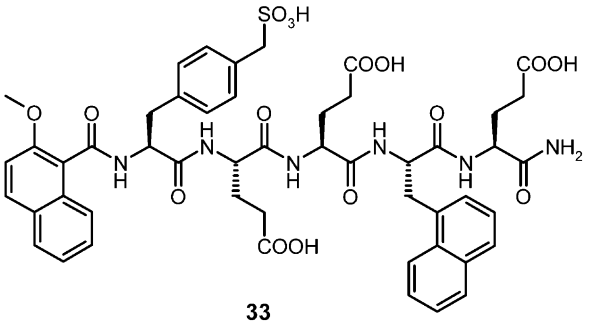
The first generation series of macrocycles thus shows positive results on the enzyme level. However, no compound with a potency of better than 3 μM could be identified so far.

At this stage, we wanted to analyze to what degree our SAR data could be reconciled with the available structural data of the target protein. A patent application of BASF includes an X-ray structure of human cdc25B co-crystallized with the peptide ligand **33**.[15]

Table 1. Inhibition of cdc25B by selected macrocyclic compounds of type **2** and selectivity towards reference phosphatases

Entry	Compd	R^1	R^2	R^3	Double bond	IC_{50} (μM)[a]	Selectivity (μM)[a]		
						Cdc25B	PTP1B	PP2A	PTP α
1	**10**	COOH	H	OH		7.9	>30	>30	>30
2	**11**		H	OH	Hydrogenated	4.9	>30	22	>30
3	**3**		H	OAc		5.6	>30	>30	>30
4	**12**		H	OAc	Hydrogenated	7.8	>30	>30	>30
5	**9**		=O			8.5	>30	>30	>30
6	**13**		=O		Hydrogenated	3.4	>30	ca. 25	>30
7	**14**		H	$CH_3OC(O)C(O)O$		11	>30	>30	>30
8	**15**		H	$CH_3C(O)CH_2C(O)O$		7.5	>30	>30	>30
9	**16**		PhO	H		7.1	>30	>30	>30
10	**17**[b]		OH	$CH_2CH=CH_2$		8.4	>30	>30	>30
11	**18**[b]		$CH_2CH=CH_2$	OH		9.3	>30	>30	>30
12	**19**	CHO	H	OH		11	>30	>30	>30
13	**6**		H	OAc		4.9	>30	>30	>30
14	**20**		PhO	H		3.5	>30	9.6	>30
15	**21**	CH_2OH	H	OH		>30	>30	>30	>30
16	**22**		H	OH	Hydrogenated	>30	>30	>30	>30
17	**23**		H	OAc		>30	>30	>30	>30
18	**8**	$COOCH_3$	H	OH		>30	>30	>30	>30
19	**7**		H	OAc		>30	>30	>30	>30
20	**24**			=O		>30	>30	>30	>30
21	**25**	CH_2–morpholine	H	OAc	Hydrogenated	>30	>30	>30	>30
22	**26**	$C(O)CH_3$	H	OH		>30	>30	>30	>30
23	**27**		H	OAc		>30	>30	>30	>30
24	**28**	$CH(OH)CH_3$	H	OH		>30	>30	>30	>30
25	**29**		H	OAc		>30	>30	>30	>30
26	**30**	CH_2OCH_3	H	OAc		29	>30	>30	>30
27	**31**	CH_3	H	OH	Hydrogenated	>30	>30	>30	>30
28	**32**		H	OAc	Hydrogenated	11	>30	>30	>30

[a] Values are determined by phosphatase assays using fluorescein diphosphate as substrate (at K_m concentration).
[b] Stereochemistry of **17** and **18** might be vice versa.



33

This is the only available protein structure characterizing the complexed state of the active site of cdc25B. Interestingly, the molecular shapes of the protein-bound peptide conformation and our most potent inhibitor **13** show some similarity as well, as illustrated by the superposition in Figure 2. The macrocyclic ring forces the molecule core into a similarly compact shape as was found for the peptide. If this superposition is significant, the carbonyl moiety of **13** (rather than the carboxylate moiety) ends up in the position of the phosphate-mimicking sulfonic group. Including the protein structure into the comparison, this appears reasonable since the carboxylate could instead interact with one of the basic side chains that flank the binding site (Arg544, Arg482). When comparing different cdc25B structures[16,17] these residues show a high degree of flexibility, indicating their potential to adapt to a particular ligand.

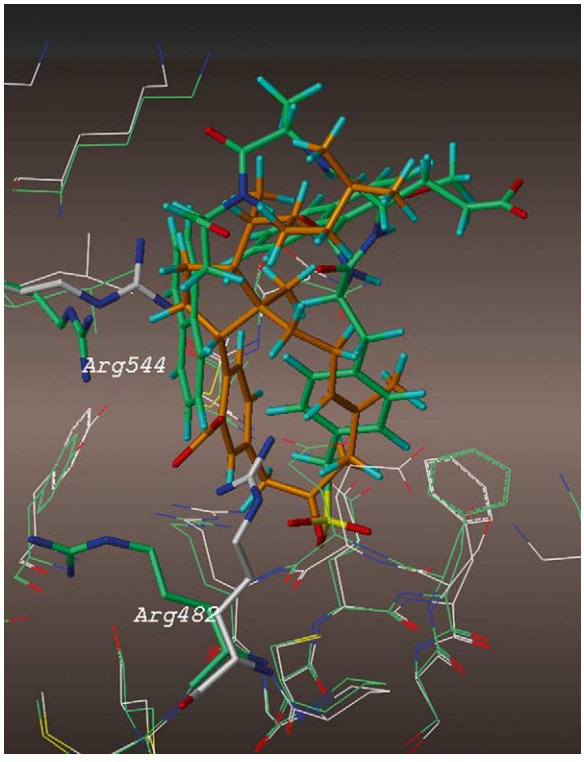


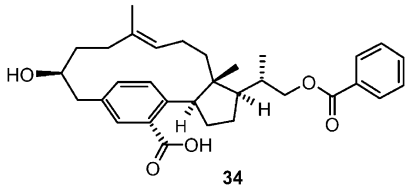
Figure 2. Superimposition of an energy-minimized conformer of compound **13** (shown in orange) and the peptide ligand **33** bound in the active site of human cdc25B protein (shown in green). In order to illustrate some of the flexibility of the pocket, a second protein structure (PDB[19] entry 1 cwt, shown in white) was added (Cα atoms superimposition). The Arg-residues flanking the binding site, which represent potential interaction partners for the carboxylate group in compound **13**, feature particularly flexible side chains. The orientation shown here suggests a bidentate saltbridge to Arg482. However, other interactions appear equally possible.

The substrate binding pockets of dual specificity phosphatases like cdc25B are generally more shallow and open than those of pure tyrosine phosphatases (as e.g., PTP1B). When trying to further explore feasible binding modes for the macrocyclic compound **13**, it was therefore not surprising that no reasonable solution could be found using standard docking algorithms. However, when employing the FlexX-Pharm Module[18] and forcing one oxygen atom into the inner phosphate binding pocket, the proposed binding modes for all different cdc25B structures we used involved the carbonyl moiety as anchor group. Based on the currently available structural data it appears therefore unlikely that the carboxylate group acts as the phosphate mimicking group. This would require the macrocylce to enter the pocket via its longitudinal side, leading to steric clashes with the known protein models.

However, in a vertical orientation of the macrocyclic scaffold, various rotations around its longitudinal axis still appear possible, impeding the determination of a distinct binding mode.

It should be stressed that the picture we have of the protein flexibility can be incomplete and distinctively different binding modes may be possible upon structural adaptation of the protein. Moreover, the constraints applied during docking rely on the assumption that the compound binds to the H-bond donors in the phosphate binding pocket. While this assumption appears reasonable, we do not have any experimental proof of it yet. Our assay protocol does not reveal whether the macrocyclic compounds are phosphate-competitive or not. Some evidence for the hypothesis that the inhibitors bind to some other surface patch is given by the report that at least one macrocyclic compound in the series by Peng et al.[9c] turned out to be a non-competitive phosphatase inhibitor.

The above described binding mode questions the importance of the long alkyl side chain. This group appears largely exposed to solvent, suggesting that it could be removed or replaced by more polar groups. A first compound which backs this hypothesis is **34** in which the cholesterol side chain is replaced by a 22-OBz-group.



34

For this compound an IC_{50} of 6.5 μM was determined. So more polar substituents seem to be tolerated in this position. This provides a valuable hint for the design of further compounds. The unfavorable lipophilicity of the current series might cause the lack of efficacy observed so far in cell-based assays.

5. Conclusion

In summary we have shown that novel macrocyclic compounds of type **2** are inhibitors of the cell cycle phosphatase cdc25B in the micromolar range. Most of the aspects of the current SAR can be rationalized by our modeling efforts, utilizing both ligand-based and protein structure-based approaches. The goal of future activities is the improvement of cell efficacy, for example by modification of the side chain towards reduced lipophilicity.

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