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Identification and Validation of a Potent Type II Inhibitor of Inactive Polo-like Kinase 1

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The search for new therapeutic strategies is one of the main research fields in translational cancer research. The serine/threonine kinase polo-like kinase 1 (Plk1)[1] attracts great attention in the field of cancer therapy because it exhibits generally elevated activity in cancer cells[2,3] and is a negative prognostic factor for cancer patients.[4] The importance of Plk1 activity as a measure for the aggressiveness of a tumor results from its important role in mitotic checkpoints.[5–8] Plk1 inhibition by antisense oligonucleotides, small interfering RNAs, antibodies, or dominant-negative mutants has resulted in reduced Plk1 expression and activity in vitro and in vivo.[8–15] A first generation of Plk1 inhibitors targeting the active conformation has entered clinical trials.[16–18] Here, we present the structure-based identification and biochemical validation of a novel potent (IC_{50} = 200 nM) inhibitor of inactive Plk1 as a potential starting point for lead structure optimization.

The high degree of conservation of kinase structure due to the same catalytic mechanism, the same cosubstrate (ATP) and similar protein folding poses the problem of inhibitor selectivity.[19] Kinases undergo conformational changes between the active and the inactive conformation by switching crucial structural elements: the αC helix, the activation loop with the conserved DFG motif as anchor, and the glycine-rich P loop (Figure 1, figure S1 in the Supporting Information). An additional hydrophobic pocket (allosteric site), in which amino acid residues are less conserved, is accessible in the inactive conformation.[19] As a consequence, inhibitors of kinases in the inactive conformation (type II inhibitors) are more selective over other kinases than inhibitors of the active conformation (type I).[19]

We performed structure-based virtual screening for potential type II Plk1 inhibitors using a comparative protein model (homology model) in the absence of known reference ligands—a strategy that has been successful in other hit and lead finding projects already.[20–22] To cope with the structural ambiguities of the homology model, we combined pharmacophore screening and automated ligand docking methods,[23,24] and transferred this concept to a model of the inactive conformation of Plk1.

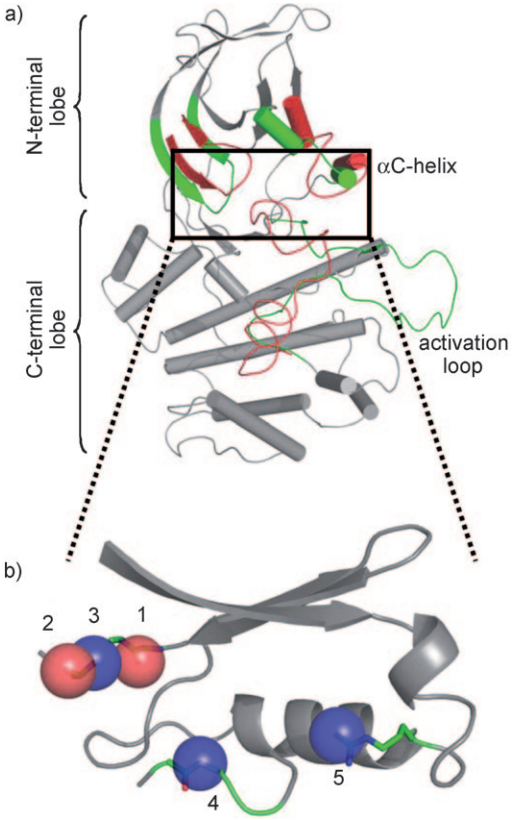

Figure 1. Plk1 structure and homology model. a) Plk1 crystal structure (PDB 2OU7,[25] gray) with activation loop, αC-helix, and P loop (green), superimposed with the homology model (red; inactive conformation). b) Enlarged binding site of Plk1 homology model with the five potential pharmacophore points (1: Glu 131, 2: Cys 133, 3: Cys 133, 4: Asp 194, 5: Arg 95).

Our homology model of Plk1 reveals some distinct structural differences in the binding site between the inactive and active conformation (Figure 1a, cf. Supporting Information): movement of the activation loop (DFG-out), shift of the αC helix to open additional space in the hydrophobic pocket, movement of the P loop between beta-sheets β1 and β2. The resulting potential ligand-binding site of the inactive conformation is long and narrow, and might be able to accommodate stretched-out ligands like most known type II inhibitors[19] that exhibit a common interaction profile with inactive kinase.[19] Ligand interactions with the hinge region and the DFG motif are crucial for stabilizing an inactive kinase conformation. Therefore, we decided to develop three pharmacophore models with different interaction points in all three regions (Table 1). In total, we considered five potential pharmacophoric points within the binding pocket of Plk1 in the inactive conformation (Figure 1b).

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Table 1. Features of the different pharmacophore models.

	Hydrogen-bond acceptors		Hydrogen-bond donors		
	Glu 131	Cys 133	Cys 133	Asp 194	Arg 95
model 1	–	+	–	+	+
model 2	+	–	–	+	+
model 3	–	–	+	+	+

For candidate identification, we followed a conventional three-step virtual screening strategy. First we performed pharmacophore matching with all three pharmacophore queries using UNITY Flexsearch from SYBYL 7.3 (Tripos Inc., St. Louis, USA). Each model contained one pharmacophore point in the hinge region of Plk1 (hydrogen-bond acceptors: Glu 131 and Cys 133, hydrogen-bond donor: Cys 133). Distance tolerance was set to 0.5 Å. Asp 194 resides within the DFG motif, while Arg 95 lies at the end of the hydrophobic pocket and was included to screen for substrates filling the whole pocket. Pharmacophore model I retrieved 162, model II 1120, and model III 810 compounds from a large compilation of commercially available substances. Then, we reduced the number of these primary virtual hits by property filters, which we derived from known type II kinase inhibitors. We used twice the standard deviation[26] of the considered properties as threshold values for compound elimination (Table 2). As a result, 40 virtual hits remained from pharmacophore matching with model I, 404 from model II, and 238 from model III.

Table 2. Properties of known potent type II kinase inhibitors and thresholds used for compound pre-screening.

	MW	log*P*[a]	n_acc[b]	n_don[c]	globularity	TPSA[d] [Å]
Mean	496	3.4	5.2	1.8	0.032	97
SD[e]	70	1.3	1.6	1.17	0.019	23
Median	511	3.2	4.5	2	0.029	89
Min[f]	360	1	3	1	0	52
Max[f]	640	6	8	3	0.06	104

[a] log*P* (o/w) = lipophilicity (computed). [b] n_acc = number of acceptors. [c] n_don = number of donors. [d] TPSA = topological polar surface area. [e] SD = standard deviation. [f] Threshold minimal (min) and maximal (max) values.

In the third virtual screening step, we proceeded with automatic ligand docking of the remaining candidates using the software GOLD 3.2 in combination with the ChemScore scoring function[27] to probe shape complementarity between ligand and receptor and to avoid potential steric clashes, instead of including steric excluded volume criteria in the pharmacophore queries (cf. Supporting Information). The distribution of ChemScore values of the top-ranking 30 compounds of each model motivated us to discard all compounds suggested by model I (Figure 2). From the remaining candidates, we visually inspected the highest scoring docking poses for plausibility and selected 13 structurally diverse compounds for subsequent cell-based and biochemical testing.

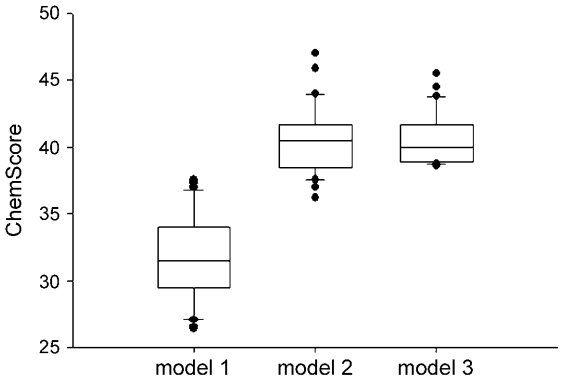
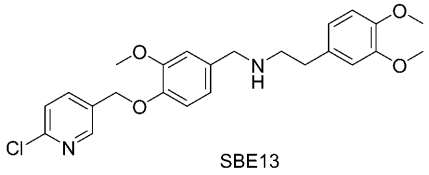


Figure 2. Box plots of ChemScore distributions. This graphical summary shows the mean ChemScores derived from docking of the top 30 scored hits from each pharmacophore model with GOLD, their distribution and standard deviations.

One of these compounds, vanillin-derived SBE13, was able to significantly reduce cell proliferation (Figure 3; 10 μM, $p < 0.01$; 33 μM, $p < 0.05$; 66 μM, $p < 0.01$; 100 μM, $p < 0.01$; $EC_{50} = 18$ μM) and induce apoptosis in HeLa cells (Figure 4).



SBE13

The docking pose of SBE13 in the homology model (figure S2 in the Supporting Information) suggests an interaction with Arg 95, and thus spans the whole hydrophobic pocket, which is anticipated for a type II inhibitor.[19] Another characteristic feature of type II inhibitors is the interaction with the hinge region and with the Asp in the DFG motif.[19] This interaction (with Asp 194, and Cys 133) is also formed by SBE13 as

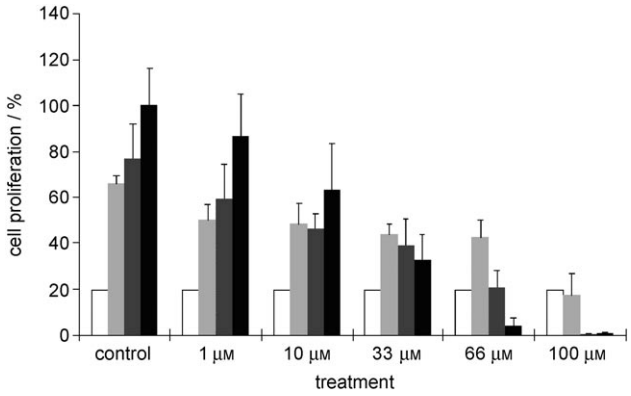


Figure 3. Effect of SBE13 on the proliferation of HeLa cells. Measurements were taken at 0 (□), 24 (▒), 48 (■) and 72 (■) hours after treatment with varying concentrations of SBE13, and cell proliferation is expressed as a percentage (%) of the control (100%). Controls were incubated with normal culture medium alone.

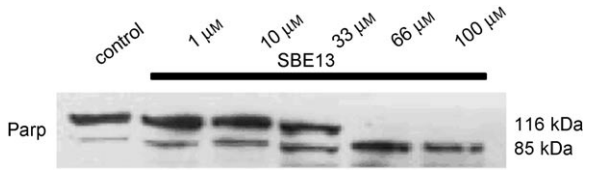


Figure 4. Effect of SBE13 treatment on apoptosis in HeLa cells. Controls were incubated with normal culture medium. To determine the full length Parp protein and the cleavage product in apoptotic cells, Western blot analyses targeting Parp were carried out.

indicated by the docking study. The ability of SBE13 to inhibit Plk1 activity was further analyzed in synchronized HeLa cells[16,17] after 13 h release in the presence of SBE13. For this purpose, we performed kinase assays using immunoprecipitated Plk1. A strong decrease of kinase activity by SBE13 was observed (Figure 5, $IC_{50} = 200$ pM). To test the selectivity of SBE13

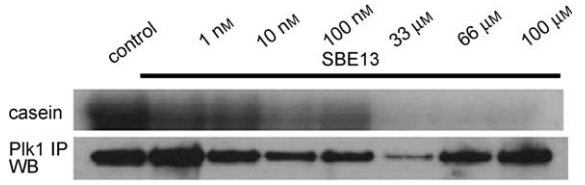


Figure 5. Kinase assays in HeLa cells after SBE13 treatment. Kinase activity of immunoprecipitated protein after double thymidine block and 13 h release with different SBE13 concentrations. A Western blot (WB) of immunoprecipitated Plk1 demonstrates that equal amounts of Plk1 were subjected to kinase assays.

for Plk1 we probed its inhibitory effect on aurora A kinase, Plk2, and Plk3. No decrease in aurora A kinase activity was observed, and only marginally decreased Plk2 ($IC_{50} > 66$ μM) or Plk3 ($IC_{50} = 875$ nM) kinase activity was detected after 13 h release in the presence of SBE13 ($p > 0.05$). The residual Plk1 activity (~20% at lower SBE13 concentrations), as observed in the kinase assays, might be sufficient for cell proliferation, as demonstrated by the higher EC_{50} values in proliferation studies.

Taken together, we identified a novel inhibitor of cancer cell proliferation, which might exert its effect via Plk1 inhibition. This first-in-class Plk1 inhibitor displays 1000-fold selectivity within the Plk family as demonstrated by only marginally decreased activity of Plk2 and Plk3 and an absence of effect on aurora A activity. Our study demonstrates that carefully designed structure-based virtual screening can help identify novel type II kinase inhibitors as potential antitumor therapeutics with minimal experimental effort.

Experimental Section

Cell Culture: The cancer cell line HeLa was obtained from DSMZ (Braunschweig, Germany), fetal calf serum (FCS) from PAA Laboratories (Cölbe, Germany), MEM from Sigma–Aldrich (Taufkirchen, Germany), phosphate buffered saline (PBS), glutamine, and trypsin from Invitrogen (Karlsruhe, Germany). Cells were treated with the Plk1 inhibitors one day after subculturing and seeded out in 175 cm^{-2} flasks. Control cells were incubated with normal culture medium. Concentrations of test compounds ranged from 1 μM to 100 μM.

Synchronization of cancer cells: Kinase assays were done after synchronization using the double thymidine protocol.[16,17] In brief, cells were treated with thymidine (2 mM, Sigma–Aldrich, Taufkirchen, Germany) for 16 h, released for 8 h, followed by incubation with thymidine for 16 h, and thereafter released in the presence of the inhibitor SBE13.

Kinase assays: To assay Plk1 kinase activity, cells were lysed after 13 h release in the presence of SBE13 after double thymidine block,[16,17] and kinase was immunoprecipitated from lysates using antibodies, as described.[14] In brief, for each immunoprecipitation 800 μg of total protein were incubated with Plk1 antibody cocktail (1.5 μg, Zymed, Karlsruhe, Germany) for 2 h at 4 °C on a rotator. Immunoprecipitated protein was collected using Protein A/G Agarose beads (SantaCruz Biotechnologies Inc., Heidelberg, Germany). Plk1 immunoprecipitates were incubated with casein (1 μg, Sigma–Aldrich, Taufkirchen, Germany) and with [γ-^{32}P]ATP (1 μCi) for 30 min at 37 °C in kinase buffer. Products from the kinase assays were fractionated on 10% bis-tris-polyacrylamide gels, and phosphorylated substrate was visualized by autoradiography after an exposure of 12–36 h. Equal amounts of immunoprecipitates were subjected to Western blot analysis to confirm equal loading of Plk1 protein in kinase reactions.

Statistical methods: All experiments were performed at least in triplicate. All treatments were compared with untreated control cells. Statistical analysis was performed with two-way ANOVA (GraphPad Prism, GraphPad Software Inc., San Diego, USA) to consider random effects as described.[13] IC_{50} values were calculated from the kinase assay experiments assuming the kinase activity of untreated control cells as 100%.

Homology model: Full details on the generation and description of the homology model are provided in the Supporting Information.

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