A novel histone deacetylase inhibitor MPT0L184 dysregulates cell-cycle checkpoints and initiates unscheduled mitotic signaling

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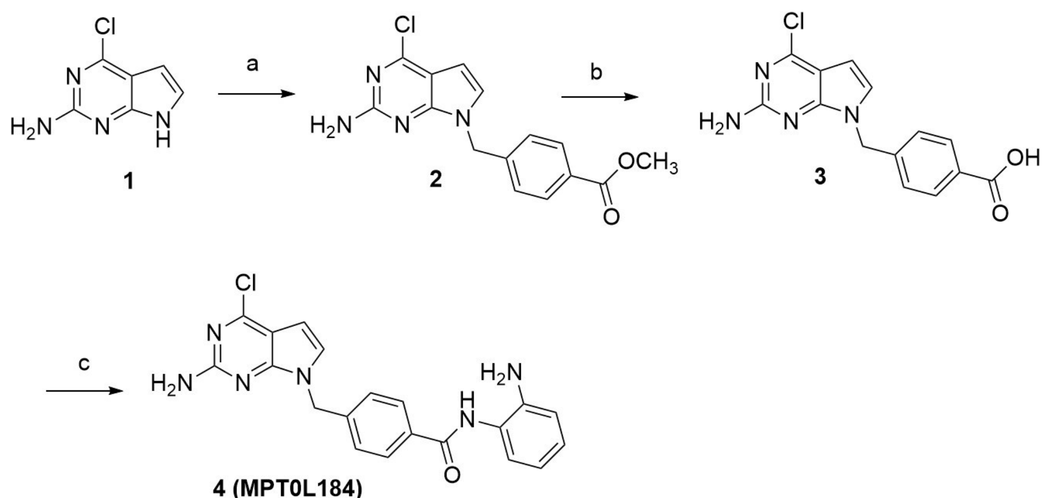
Supplementary materials and methods

Chemical synthesis of MPT0L184

Scheme



Reagents and conditions a) Methyl 4-(bromomethyl)benzoate, K_2CO_3, DMF, 60 °C; b) LiOH, dioxane, rt; c) benzene-1,2-diamine, EDC, HOBt, DIPEA, DMF, rt.

Nuclear magnetic resonance spectra were obtained with Bruker DRX-500 spectrometer (operating at 500 MHz), with chemical shift in parts per million (ppm, d) downfield from TMS as an internal standard. High-resolution mass spectra (HRMS) were measured with a JEOL (JMS-700) electron impact (EI) mass spectrometer. All reactions were carried out under an atmosphere of dry nitrogen.

Synthesis of 4-(2-Amino-4-chloro-pyrrolo[2,3-d]pyrimidin-7-ylmethyl)-benzoic acid methyl ester (**2**)

A mixture of 4-chloro-7H-pyrrolo[2,3-d] pyrimidin-2-amine (**1**) (1 g, 5.93 mmol), methyl 4-(bromomethyl) benzoate (1.35 g, 5.93 mmol) and potassium carbonate (1.22 g, 8.89 mmol) in DMF was stirred at 60 °C for 4 h. The reaction mixture was quenched with H_2O and extracted with EtOAc (50 mL x 3). The combined organic layer was dried over anhydrous $MgSO_4$, concentrated under reduced pressure and purified by silica gel chromatography to give compound **2** in 73 % yield; ^1H NMR (300 MHz, CD_3OD): 7.89 (d, J = 8.1 Hz, 2H), 7.28 (d, J = 8.1 Hz, 2H), 7·08 (d, J = 3.9 Hz, 1H), 6.31 (d, J = 3.9 Hz, 1H), 5.23 (s, 2H), 3.79 (s, 3H).

Synthesis of 4-(2-Amino-4-chloro-pyrrolo[2,3-d]pyrimidin-7-ylmethyl)-benzoic acid (**3**)

A mixture of **2** (1.5 g, 4.73 mmol), 1 M LiOH aq. (10 ml) and dioxane (20 mL) was stirred at room temperature for 2 h. The reaction was concentrated under reduced pressure and H_2O was added. The mixture was acidified with 3N HCl and extracted with EtOAc (50 mL x 3). The combined organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to yield the acid (**3**) in 96% yield; ^{1}H NMR (300 MHz, CD_3OD): 7.98 (d, J = 8.1 Hz, 2H), 7.29 (d, J = 8.1 Hz, 2H), 7.06 (d, J = 3.9 Hz, 1H), 6.39 (d, J = 3.9 Hz, 1H), 5.29 (s, 2H).

<u>Synthesis of 4-(2-Amino-4-chloro-pyrrolo[2,3-d]pyrimidin-7-ylmethyl)-N-(2-amino-phenyl)-benzamide (**4; MPT0L184**)</u>

A mixture of **3** (1 g, 3.30 mmol), EDC.HCl (1.26 g, 6.60 mmol), HOBt (0.668 g, 4.95 mmol), benzene-1,2-diamine (0.356 g, 3.30 mmol) and DIPEA (1.43 ml, 8.24 mmol) in DMF (5 mL) was stirred at room temperature for 5 h. The reaction mixture was then quenched with H_2O and extracted with EtOAc (50 mL x 3). The combined organic layer was dried over anhydrous $MgSO_4$, concentrated under reduced pressure and purified by silica gel chromatography (hexane: EtOAc = 1:1) to give **4** in 71 % yield; mp: 215 - 216 °C; ^{1}H NMR (500 MHz, CD_3OD): 9.55 (s, 1H), 7.88 (d, J = 8.0 Hz, 2H), 7.25 (d, J = 8.0 Hz, 2H), 7.21 (d, J = 3.5 Hz, 1H), 7.11 (d, J = 7.5 Hz, 1H), 6.93 (m, 1H), 6.73 (dd, J = 1.0 and 8.0 Hz, 1H), 6.64 (s, 2H), 6.55 (t, J = 6.5 Hz, 1H), 6.33 (d, J = 3.5 Hz, 1H), 5.31 (s, 2H), 4.83 (s, 2H). ^{13}C (75 MHz, DMSO-d_6): 165.50, 159.97, 154.05, 151.82, 143.57, 142.05, 141.52, 134.35, 133.85, 131.70, 128.37, 127.33, 126.94, 123.69, 116.71, 116.56, 109.09, 99.53, 47.34. HRMS (ESI) for $C_{20}H_{18}ClN_6O$ $(M + H)^+$: calcd, 393.1231; found, 393.1235.

Western blot analysis

Cell extracts were lysed in Laemmli sample buffer (LSB; 60 mM Tris (pH 6.8), 2% sodium dodecylsulfate (SDS) and 10% glycerol). Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto nitrocellulose membranes. Proteins were probed using specific antibodies as follows: anti-poly(ADP ribose) polymerase-1 (PARP-1) (Santa Cruz Biotechnology, sc-7150), anti-caspase-3 (Novus, NB100-56708), anti-mitotic protein monoclonal 2 (MPM2) (Millipore Cat# 05-368, RRID:AB_309698), anti-cyclin B1 pS126 (Abcam Cat# ab55184, RRID:AB_879764), anti-cyclin B1 Santa Cruz Biotechnology Cat# sc-752, RRID:AB_2072134), anti-CDK1 pY15 (GeneTex, Cat# GTX128155, RRID:AB_2877127), anti-CDK1 (Santa Cruz Biotechnology Cat# sc-54, RRID:AB_627224), anti-H3 pS10 (Millipore Cat# 06-570, RRID:AB_310177), anti-H3 (Abcam Cat# ab1791, RRID:AB_302613), anti-WEE1 (Santa Cruz Biotechnology Cat# sc-5285, RRID:AB_628447), anti-CHK1 (Santa Cruz Biotechnology Cat# sc-

8408, RRID:AB_627257), anti-KAP1 pS824 (Abcam Cat# ab70369, RRID:AB_1209417), anti-CHK2 pT68 (Cell Signaling Technology Cat# 2661, RRID:AB_331479), anti-CHK1 pS345 (Cell Signaling Technology Cat# 2348, RRID:AB_331212), anti-RPA2 pS33 (Bethyl Cat# A300-246A, RRID:AB_2180847), anti-RPA2 pS4/S8 (Bethyl Cat# A300-245A, RRID:AB_210547), anti-γH2AX (Millipore Cat# 05-636, RRID:AB_309864), anti-H2A (Abcam Cat# ab18255, RRID:AB_470265), anti-SMC3 acK105/106 (Millipore, Cat# MABE1073, RRID:AB_2877126), anti-SMC3 (Bethyl Cat# A300-060A, RRID:AB_67579), anti-α-tubulin acK40 (Sigma-Aldrich Cat# T7451, RRID:AB_609894), anti-α-Tubulin (Sigma-Aldrich Cat# T5168, RRID:AB_477579), anti-H3 acK9 (Millipore Cat# 07-352, RRID:AB_310544), anti-H4 acK5/8/12/16 (Millipore Cat# 06-866, RRID:AB_310270), anti-H4 (Abcam Cat# ab7311, RRID:AB_305837), anti-actin (Millipore Cat# MAB1501, RRID:AB_2223041), anti-p21 (Santa Cruz Biotechnology Cat# sc-6246, RRID:AB_628073), anti-p53 (Santa Cruz Biotechnology Cat# sc-6243, RRID:AB_653753), anti-Aurora A (GeneTex Cat# GTX104620, RRID:AB_1949708), anti-Aurora B (BD Biosciences Cat# 611082, RRID:AB_2227708), anti-CDC25A (Bethyl Cat# A300-075A, RRID:AB_143271) and anti-CDC25C (Santa Cruz Biotechnology Cat# sc-55513, RRID:AB_2275797). Horseradish peroxidase (HRP)-conjugated goat anti-mouse (Jackson ImmunoResearch Labs Cat# 115-035-003, RRID:AB_10015289) and -rabbit (Jackson ImmunoResearch Labs Cat# 111-035-003, RRID:AB_2313567) antibodies were purchased from Jackson ImmunoResearch Labs. Images were collect using ImageQuant LAS 4000. To avoid incomplete removal of probed antibodies that may interfere with the interpretation of results, data of indicated experiments were collected from different sets of gel electrophoresis with equal loading of the same samples.

MTS assay

Cells grown on 96-well plate were treated with compounds for 72 hours, followed by incubation with 0·2 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; BioVision, 2805) at 37°C for two hours. The amount of surviving cells was by measuring the absorbance at 490 nm (Perkin Elmer Victor^3 1420 Multilabel Counter). The concentration for half maximal growth inhibitory (IC_{50}) was calculated by CompuSyn.

Annexin V binding assay

Cells grown on 6-cm dishes were treated with compounds for 48 hours. Cells were collected and incubated with annexin V-FITC for 15 minutes at room temperature. The

fluorescence intensities were measured using BD FACSCalibur Flow Cytometry System and were plotted using FlowJo.

Thymidine-nocodazole synchronization

Cells were firstly synchronized in the early-S phase by treating with 4 mM thymidine for 24 hours. 4 hours after the thymidine release, cells were then treated with 150 ng/ml of nocodazole for 12 hours to trap cells in mitosis. Mitotic cells were then collected by shaking and tapping of the flask and washed thoroughly, followed by incubation with the culture medium for relieving the nocodazole block.

Immunofluorescence staining

Cells grown on coverslips were treated with compounds. At each designated time point, cells were fixed with 2% formaldehyde in PBS at room temperature for 15 minutes and then permeablized with 0.2 % Triton X-100 in PBS for 5 minutes, followed by blocking with the blocking buffer (3% bovine serum albumin in PBST). Cells were incubated with anti-cyclin B1 (Santa Cruz Biotechnology, sc-752) at 4℃ for overnight, washed and then incubated with Alexa Fluor 488-conjugated goal anti-rabbit (Jackson ImmunoResearch Labs, 111-545-114) at room temperature for 1 hour. Finally, cells were mounted with Mowiol 4-88 (Sigma, 81381) containing 5 μg/ml of 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; Sigma, D9542) for nuclear staining. Images were collected using an OLYMPUS IX71 fitted with an UCPlanFL N 40x/0·60 Ph2 objective lens. The final composite images were created using Adobe Photoshop 7.0.

***In vitro* WEE1 activity assay**

WEE1 inhibition assay was performed by Eurofins Cerep-France. Human recombinant WEE1 was incubated with 10 μM of MTP0L184, Ulight-ARTKQTARKSTGGKAPRKQLAGCG and ATP for 120 minutes at room temperature. Kinase activity was determined by measuring the levels of phospho-Ulight-ARTKQTARKSTGGKAPRKQLAGCG.

***In vitro* HSP90 binding assay**

MDA-MB-231 cells were lysed in NP40 buffer (50 mM Tris-Cl, pH8.0, 150 mM NaCl, 0.5% NP-40, 0.2 mM EDTA and protease inhibitor (Sigma, P8340)). Lysates were then incubated with indicated compounds and streptavidin beads (Millipore, E5529) coated with biotin-labeled geldanamycin (Enzo, BML-EI341-0001) for 3 hours at 4℃. Beads were washed three times with wash buffer (50 mM Tris-Cl, pH8.0, 200

mM NaCl, 0.5% NP-40, 0.2 mM EDTA and protease inhibitor) and pulled-down proteins were then analyzed by Western blot.