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

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A B S T R A C T

Aberrant alteration of epigenetic information disturbs chromatin structure and gene function, thereby facilitating cancer development. Several drugs targeting histone deacetylases (HDACs), a group of epigenetic enzymes, have been approved for treating hematologic malignancies in the clinic. However, patients who suffer from solid tumors often respond poorly to these drugs. In this study, we report a selective entinostat derivative, MPT0L184, with potent cancer-killing activity in both cell-based and mouse xenograft models. A time-course analysis of cell-cycle progression revealed that MPT0L184 treatment elicited an early onset of mitosis but prevented the division of cells with duplicated chromosomes. We show that MPT0L184 possessed potent inhibitory activity toward HDAC1 and 2, and its HDAC-inhibitory activity was required for initiating premature mitotic signaling. HDAC inhibition by MPT0L184 reduced WEE1 expression at the transcription level. In addition, MPT0L184 treatment also downregulated ATR-mediated CHK1 phosphorylation independent of HDAC inhibition. Furthermore, gastric cancer cells resistant to HDAC inhibitors were vulnerable to MPT0L184. Taken together, our study discovers MPT0L184 as a novel HDAC inhibitor that can trigger premature mitosis and potentially counteract drug resistance of cancers.

1. Introduction

Genome integrity and cell identity are maintained through faithful propagation of genetic and epigenetic information to daughter cells in a rigorously scheduled cell cycle. Alterations of these information can challenge gene functions and cell fates, thus leading to severe diseases like cancers. Histone acetylation, one of epigenetic modifications, largely contributes to regulation of genome functions and cell-cycle progression [1]. Histone deacetylases (HDACs) are responsible for erasing the acetylation of histones and non-histone substrates marked by histone acetyltransferases (HATs). These enzymes are deregulated in many types of cancers and often associated with poor prognosis [1,2]. Pharmacological targeting of HDACs causes protein hyperacetylation, leading to increased levels of reactive oxygen species (ROSs), genome instability, DNA damage, cell-cycle arrest, and apoptosis [3]. Four HDAC inhibitors (HDACis) have been approved by the US Food and Drug Administration (FDA) for treating cutaneous T-cell lymphomas (CTCLs), peripheral T-cell lymphomas (PTCLs), and multiple myelomas (MMs) [4]. In addition, the Chinese FDA has also approved tucidinostat, an analog of the class I HDACi entinostat, for relapsed or refractory PTCLs [4]. Although several HDACis are being examined in clinical trials to combat solid tumors in combination with chemo agents or targeted
therapeutics, responses of patients vary considerably when HDACis are used alone [4,5]. Moreover, severe adverse effects and the poor pharmacokinetics further challenged the clinical use of these HDACis [6,7]. The discovery of novel HDACis with improved cancer-killing efficacy and/or additive activity that can overcome HDACi resistance should thus be beneficial against solid tumors.

Serine/threonine cyclin-dependent kinases (CDKs) are master factors driving chromatin replication and cell division. In cancer cells, the regulatory mechanisms that coordinately govern CDK activity are often perturbed. This results in disordered genome replication and separation, ultimately leading to the accumulation of genomic instability that further facilitates tumorigenesis [8,9]. Several CDK inhibitors have been approved or are under investigation either alone or in combination with cancer therapeutic agents including aromatase and HDAC inhibitors [10–12]. On the contrary, recently emerging therapeutics have focused on further removing brakes of cell-cycle progression to enhance CDK activity and therefore engender deleterious genomic catastrophe in cancer cells [8,9]. The activity of the mitotic CDK, CDK1, is strictly controlled by its phosphorylation status. CDK-activating kinases phosphorylate CDK1 at T161 and increase its activity, while the G2 checkpoint kinases, WEE1 and membrane-associated tyrosine- and threonine-specific kinase (MYT1), maintain CDK1 activity at a low level through respective phosphorylations at Y15 and T14/Y15 [13]. Erasing these inhibitory phosphorylations by the cell division cycle 25 (CDC25) family of phosphatases is a prerequisite for the timely activation of CDK1 to initiate mitotic events [14]. In addition, the DNA integrity checkpoint kinase, CHK1, restrains CDK activity through phosphorylating CDC25, which results in degradation or nuclear export of CDC25, and directly enhancing WEE1 activity [15]. Inactivation of either CHK1 or WEE1 causes hyper-activation of CDKs and thus triggers unscheduled chromatin replication and mitotic onset, leading to genomic catastrophe [8,9]. Specific inhibitors targeting CHK1 and WEE1 have been developed and evaluated in the clinic for cancer therapy. However, many of these trials have been terminated due to severe side-effects [16,17].

Our medicinal chemistry program is inclined towards the development of potent class I HDACis via structural optimization of entinostat [18,19]. One of the derivatives, MPT0L184, has particularly attracted our attention because of its potent cytotoxicity and unique biological activity. Structurally, MPT0L184 comprises a substituted isostere of the purine ring, namely 7H-pyrrolo[2,3-d]pyrimidine, as the cap motif and N-benzyl linker to tether the cap group with the 2-aminooanilide moiety (Fig. 1). The isosteric replacement of the purine ring was envisioned to ease the complexities associated with the synthetic routes to purine-based compounds. In addition, the insertion of the purine isostere also rendered the scope to ascertain the impact of the nitrogen atom replacement in the fused bicyclic ring on the bioactivity. We show that MPT0L184 perturbs cell-cycle checkpoint activities and elicits unscheduled onset of mitotic events. MPT0L184 exhibits potent toxicity in a mouse model and various cancer cell lines including HDACi-resistant cells, providing a potential HDACi for cancer therapeutics.

2. Materials and methods

2.1. Cell culture

Human breast cancer MDA-MB-231 cell line used in cell-based experiments and osteosarcoma U-2 OS cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco, 12800-017). Human gastric cancer YCC11 and YCC3/7 cell lines were cultured in minimum essential medium alpha (MEM; Gibco, 11900-024). The human leukemic KG-1, colon cancer DLD-1, and non-small cell lung cancer NCI-H1299 cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, 23400-021). The human T-cell lymphoma HuT78 cell line was cultured in Iscove’s modified Dulbecco’s medium (IMDM; Sigma, I3390). Above cell lines were cultured in a humidified incubator at 37°C with an atmosphere of 5% CO2. The MDA-MB-231 cell line used in the xenograft mouse study was cultured in Leibovitz’s L-15 medium (Gibco, 41300-039) in a humidified incubator at 37°C without CO2. All media contained 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/glutamine. YCC cell lines were kindly provided by Dr. Sun Young Rha (Songdang Institute for Cancer Research). NCI-H1299 and U-2 OS cell lines were obtained from American Type Culture Collection (ATCC), and MDA-MB-231, DLD-1, KG-1, and HuT78 cell lines were obtained from Bioresource Collection and Research Centre (BCRC). Cell lines were used for no more than 6 weeks after thawing and have been

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Fig. 1. Structures of entinostat and its derivatives.
Cells grown on 96-well plates were treated with compounds for 48 or 72 h, followed by incubation with 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, M2128) at 37 ◦C for 4 h. Formazan crystals were then dissolved in dimethyl sulfoxide (DMSO). The number of surviving attached cells was determined by measuring the absorbance at 490 nm (Perkin Elmer Victor3 1420 Multilabel Counter). The concentration for half-maximal growth inhibition (IC50) was calculated with CompuSyn.

2.3. Cell viability assay

Cells were treated with various compounds. At each time point, cells were stained with trypan-blue dye and subjected to cell counting using a Vi-CELL XR Viability Analyzer (Beckman Coulter).

2.4. Terminal uridine nick-end labeling (TUNEL) assay

Cells grown on coverslips were treated with compounds for 48 h and then fixed with 4% formaldehyde, followed by permeabilization with 0.1% Triton X-100 in PBS. DNA breaks were probed with an In Situ Cell Death Detection Kit (Roche, 11684795910) according to the manufacturer’s instructions and nuclei were stained with 5 μg/ml of DAPI (Sigma, D9542). Images were collected using a Molecular Devices ImageXpress Micro XL microscope fitted with an 10x Plan Fluor objective lens.

2.5. Xenograft mouse model

Human MDA-MB-231 breast cancer cells (10^6) used for implantation were subcutaneously (s.c.) injected into 12-week-old female BALB/c female nude mice. After the tumor volume had reached about 200 mm³, mice were treated with the vehicle control, 20 mg/kg paclitaxel (by an intraperitoneal injection (i.p.), every 4 days), 50 mg/kg of MPT0L184 (by an i.p. injection, every day) or 100 mg/kg of MPT0L184 (by an i.p. injection, every day). Tumor volumes were monitored twice weekly. The study ended when mean tumor volume in control group approached ~ 1000 mm³. Tumor volumes were calculated as (width^2 x length)/2. Animal experiments were performed in accordance with relevant guidelines and regulations and followed ethical standards. Protocols have been reviewed and approved by the Animal Use and Management Committee of Taipei Medical University (TMU LAC-2017-0199). Statistical method was performed using Student’s t-test analysis. All data are presented as the mean ± standard error of the mean (S.E.M) of 5–6 mice in each group.

2.6. Flow cytometry

Cells treated with various compounds were isolated and centrifuged at 1200 rpm for 5 min, followed by fixation with 70% cold-ethanol. Fixed cells were washed with phosphate-buffered saline (PBS) containing 1% fetal bovine serum (FBS), followed by staining with 0.05 mg/ml of propidium iodide (PI; Sigma, P4170) in PBS containing 0.25 mg/ml of ribonuclease A (RNase A; Sigma, R6513) at 37 ◦C for 30 min. The DNA content was measured using a BD FACScalibur Flow Cytometry System and cell-cycle profiles were plotted using FlowJo.

2.7. DNA/nuclear morphology analysis

Cells treated with various compounds were isolated and centrifuged at 1200 rpm for 5 min. Collected cells were then re-suspended in culture medium and spun onto glass slides using a CytoSpin™ 4 Cyto centrifuge (Thermo) at 10^3 rpm for 15 min, followed by fixation with cold-methanol. Nuclei were stained with 5 μg/ml of 4’,6-diamidino-2-phenylindole (DAPI) and examined by a traditional fluorescence microscope (Olympus, IX81) fitted with an UPlanSapo 40x/0.90 objective lens. The final composite images were created using Adobe Photoshop 7.0.

2.8. Synchronization

Cells were treated with 4 mM thymidine for 20–24 h to accumulate cells at the early-S phase. Cells were then washed strictly and incubated with culture medium to relieve the thymidine block.

2.9. Quantitative polymerase-chain-reaction (qPCR)

RNA was extracted and purified using a RNeasy Plus Mini Kit (Qiagen, 74136). Complementary DNAs (cDNAs) were synthesized using SuperScript IV Reverse Transcriptase (Invitrogen, 108090010). A SYBR Green I Master Kit (Roche, 04707516001) was used for the real-time PCR performed with a LightCycler 480 II (Roche) with the following conditions: 95 ◦C for denaturation, 60 ◦C for annealing, and 72 ◦C for extension. The sequences of primers were: WEE1 5’-ACAGTGG-TATGTGCTGC-3’ (sense) and 5’-TGCCATTGCTAGGTC-3’ (anti-sense); S26 5’-TGGATAATAAACACTAGGAACGC-3’ (sense) and 5’-GGCTTCAAGACCGGCAA-3’ (anti-sense).

2.10. In vitro HDAC activity assay

An HDAC inhibition assay was performed by Eurofins Panlabs Discovery Services and Reaction Biology Corp. Briefly, individual human recombinant HDAC was incubated with a series of concentrations of MPT0L184 or entinostat at 25 ◦C for 15 min and then mixed with the Fluor-de-Lys deacetylase substrate for an additional hour. IC50 values were calculated with CompuSyn.

2.11. Cellular HDAC1 activity assay

Cellular HDAC1 activity was measured using a HDAC1 Immunoprecipitation & Activity Assay Kit (BioVision, K342-25) according to the manufacturer’s instructions. Briefly, HDAC1 was immuno-isolated from the whole lysates of MDA-MB-231 cells treated with indicated compounds for 4 h, followed by incubation with the HDAC1 substrate. The fluorescence was measure by a Spectramax M2 microplate reader (Molecular Devices) at Ex/Em = 380/500 nm.

2.12. Chemical synthesis of MPT0L184, Western blot, annexin V binding assay, MTS assay, thymidine-nocodazole synchronization, immunofluorescence staining, WEE1 activity assay, and HSP90 binding assay

Please refer to the Supplementary Information of the online version of this article at Biomedicine & Pharmacotherapy.
3. Results

3.1. MPT0L184 exhibits potent growth-inhibitory activity in vitro and in vivo

We first determined the cytotoxicity of MPT0L184 by treating cells with compounds for 48 h, followed by a viability analysis. Entinostat, the Chinese FDA-approved tucidinostat, and two entinostat derivatives, MPT0L185 and MPT0L198 [18] (Fig. 1), were exploited for comparison. Among the tested compounds, MPT0L184 exhibited the highest toxicity in triple-negative breast cancer MDA-MB-231 cells, with an IC\textsubscript{50} value of 3.98 ± 0.51 μM, whereas entinostat (6.18 ± 0.37 μM) and tucidinostat (7.53 ± 1.08 μM) were less poisonous (Fig. 2A). MPT0L184 also effectively decreased the viability of colorectal adenocarcinoma DLD-1 and non-small cell lung cancer carcinoma NCI-H1299 cells (Fig. 2B), suggesting that the cytotoxicity of MPT0L184 is not specific to a subset of solid tumors. Intriguingly, MPT0L184, entinostat, and tucidinostat showed higher virulence to the acute myelogenous leukemia KG-1 cell line but not to cutaneous T-cell lymphoma HuT78 cells compared to the FDA-approved HDACi vorinostat (Supplementary Fig. 1A) [4,18].

![Fig. 2. MPT0L184 exhibits potent inhibitory activity on cell proliferation in vitro and tumor growth in vivo.](image-url)

(A) MTT analysis of MDA-MB-231 cells treated with indicated entinostat derivatives for 48 h. Percentages of surviving attached cells were calculated and results of four biological replicates are shown with means and SD (n = 12). (B) MTT analyses of DLD-1 (left) and NCI-H1299 (right) cells treated with MPT0L184, entinostat, or tucidinostat for 48 h. Percentages of surviving attached cells were calculated and representative results from one of two biological replicates are shown with means and SD (n = 3). (C) Trypan-blue exclusion assay of MDA-MB-231 cells treated with 16 μM of MPT0L184, entinostat, or tucidinostat. Numbers of viable cells (left) and death rate (right) were calculated and representative results from one of two biological replicates are shown with means and SD (n = 3). (D) DNA fragmentation analysis by a TUNEL assay for MDA-MB-231 cells treated with 8 or 16 μM of MPT0L184 or entinostat for 48 h. Percentages of TUNEL-positive cells were calculated and results of three biological replicates are shown with means and SD (n = 3). (E) Western blot analysis of apoptotic markers in MDA-MB-231 cells treated with 16 μM of MPT0L184 or entinostat for 48 h. Actin was used as a loading control. Representative results from one of three biological replicates are shown. p-Casp-3: Pro-caspase-3; a-Casp-3: Active caspase-3. (F) Anti-tumor efficacy of MPT0L184 in a human MDA-MB-231 breast cancer xenograft model. Female BALB/c nude mice were subcutaneously injected with MDA-MB-231 cells and treated with vehicle, 20 mg/kg of paclitaxel, 50 mg/kg of MPT0L184, or 100 mg/kg of MPT0L184. The tumor volume (left) and mouse bodyweight (right) were recorded twice per week. ip, intraperitoneal injection; q4d, once every 4 days; qd, once every day. *P < 0.05; **P < 0.001.
next performed a trypan-blue exclusion assay to monitor cell growth in response to compound treatment. We found that MPT0L184 stopped cell proliferation immediately after treatment, while treatment with entinostat or tucidinostat only attenuated overall cell growth rate during the initial 24 h (Fig. 2C; Supplementary Fig. 1B). After 48 h of treatment, more than 40% of cells with MPT0L184 treatment had died, while over 80% of cells treated with entinostat or tucidinostat were still alive (Fig. 2C).

Given the high death rate of MPT0L184-treated cells, we compared the efficiency of MPT0L184 and entinostat in inducing apoptosis. We observed that MPT0L184 treatment substantially elevated the number of cells in the sub-G₁ phase in a dose-dependent manner (Supplementary Fig. 1C). The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay further demonstrated that MPT0L184 was effective in inducing DNA fragmentation, a late event of apoptosis (Fig. 2D). In addition, we found that MPT0L184 treatment significantly increased the active forms of poly(ADP-ribose) polymerase-1 and caspase-3, indicative of apoptotic pathway activation (Fig. 2E). We further used annexin V to probe cells with membrane phosphatidylserine externalization, an event that specifically occurs at the early stage of apoptosis. Our data showed that MPT0L184 treatment significantly increased the number of cells positive for annexin V (Supplementary Fig. 1D).

Notably, these phenomena were more pronounced in cells treated with MPT0L184 compared to that with entinostat. These results indicate that MPT0L184 is a potent entinostat analog that kills cancer cells through apoptosis.

To further evaluate the anti-cancer activity of MPT0L184 in vivo, we examined the suppressing efficiency of tumor formation in a xenograft mouse model. Female nude mice inoculated with MDA-MB-231 cells were intraperitoneally administered MPT0L184 (50 and 100 mg/kg, qd), and the tumor growth rate was monitored twice per week. Paclitaxel (20 mg/kg, qd), a chemotherapeutic agent commonly used to treat triple-negative breast cancer patients, was used as a positive control. We found that MPT0L184 administration significantly suppressed the increase in tumor volume in a dose-dependent manner (Fig. 2F). Moreover, the loss of bodyweight was not evidenced in mice with MPT0L184 treatment (Fig. 2F), suggesting that the exploited dosages of MPT0L184 have low toxicity toward the animals. These results support the potential of MPT0L184 to be developed as an anti-cancer drug.

3.2. MPT0L184 induces cell-cycle arrest in the G2 phase

We and others have shown that entinostat and its derivatives can elicit cell-cycle arrest in the G₁ phase (Fig. 3A and [18,20]). Consistently, treatment with MPT0L184 at the early stage of the G₁ phase prohibited cells from entering the S phase (Supplementary Fig. 2). Nevertheless, in the asynchronized condition, cells treated with MPT0L184 accumulated in the G₂/M phase (Fig. 3A), suggesting that MPT0L184 exerts a unique activity that prevents cell division. We further examined the level of mitotic proteins in cells with MPT0L184 treatment and used colchicine, a microtubule destabilizing agent, as a control for comparison. Cells treated with colchicine exhibited mitotic features, including increased protein levels of Aurora A/B and phosphorylations of cyclin B1 at S126, histone H3 at S10, and mitotic proteins probed by the mitotic protein monoclonal 2 (MPM2) antibody (Fig. 3B). Unexpectedly, these mitotic markers were decreased or absent in cells treated with MPT0L184 (Fig. 3B), suggesting that the long treatment of MPT0L184 may prevent cells from entering mitosis. We then analyzed chromosome morphology that becomes condensed in prophase-to-metaphase. After 24 h of treatment with various

Fig. 3. MPT0L184 induces cell-cycle arrest in the G₂ phase. (A) Cell-cycle profiles of MDA-MB-231 cells treated with 16 μM of entinostat or MPT0L184 for 24 h. Representative results from one of two biological replicates are shown. (B) Western blot analysis of mitotic markers in MDA-MB-231 cells treated with 16 μM of MPT0L184 or 0.2 μM of colchicine for 24 h. α-Tubulin was used as a loading control. Representative results from one of two biological replicates are shown. *non-specific. Data were collected from different sets of gel electrophoresis with equal loading of the same samples. (C) DNA/nuclear morphology analysis of MDA-MB-231 cells treated with 20 μM of MPT0L184, 2 μM of vinorelbine, 0.2 μM of colchicine, 0.33 μM of nocodazole or 1 μM of paclitaxel for 24 h. Representative images (upper) and means with SD of percentages of each pattern calculated from three biological replicates (n = 3) are shown (lower). Note that the exposure time for each image varies to highlight the differential patterns.
tubulin-binding agents, we observed an accumulation of cells with condensed chromosomes, whereas this was not seen when cells were treated with MPT0L184 (Fig. 3C). These results indicate that MPT0L184 treatment leads to the accumulation of cells in the G2 phase.

3.3. MPT0L184 triggers premature mitosis

To unravel the mechanism of action of MPT0L184, we set up a time-course experiment to examine the expression dynamics of mitotic markers. We synchronized cells to the early-S phase (T0) with thymidine and analyzed cell-cycle progression and abundances of mitotic markers at different time points after release from the thymidine block (Fig. 4A; Supplementary Fig. 3A). To reduce the effect of compound treatment on DNA replication, we treated cells with MPT0L184 4 h after release (T4), at which the majority of cells had reached the late-S to G2 phase (Supplementary Fig. 3A). In untreated cells, we detected increased phosphorylation levels of histone H3 and cyclin B1 8 h post-release (T8, Fig. 4A), indicating that a portion of cells was proceeding to the mitotic stage. The levels of these two mitotic markers peaked at T10 and dropped by T12 when the majority of cells had completed mitosis and entered the next G1 phase (Fig. 4A; Supplementary Fig. 3A). These phenomena were slightly delayed in colchicine-treated cells, and the phosphorylations were retained at least until T24 due to the mitotic block (Fig. 4A; Supplementary Fig. 3A). Surprisingly, these two mitotic markers in cells treated with MPT0L184 appeared at T6, 2 h after treatment, and remained at a high level for at least 6 h (T12), accompanied by the cell-cycle arrest in the G2 phase (Fig. 4A; Supplementary Fig. 3A). We also observed an early reduction in CDK1 phosphorylation at Y15, an increased number of cells with condensed chromosomes, and cyclin B1 translocation to the nucleus (Fig. 4A and B; Supplementary Fig. 3B), suggesting an early activation of CDK1. Consistent with findings in asynchronized cells (Fig. 3B), H3 phosphorylation, cyclin B1 protein, and condensed chromosomes were significantly reduced or absent in cells after long-term treatment with MPT0L184 (T24), concomitant with an elevated number of cells with fragmented nuclei (Fig. 4A and B). Importantly, we also detected activation of mitotic signaling by MPT0L184 in cells persistently arrested in the S phase by the replication inhibitor hydroxyurea or a high dose of thymidine, although the effects were not as prominent as that observed in asynchronized cells (Fig. 4C; Supplementary Fig. 3C). These results indicate that treatment with MPT0L184 initiates unscheduled mitosis onset.

Fig. 4. MPT0L184 triggers premature mitosis. (A) Western blot analysis of mitotic markers in synchronized MDA-MB-231 cells treated with 20 μM of MPT0L184. The experimental design (upper) and representative results from one of two biological replicates (lower) are shown. 0.2 μM of colchicine was used as a positive control for accumulation of cells in mitosis. (B) DNA/nuclear morphology analysis as in Fig. 3C for synchronized MDA-MB-231 cells treated with 20 μM of MPT0L184 for 4 or 20 h. Percentages of each pattern calculated from three biological replicates are shown with means and SD (n = 3). An unpaired t-test was used for the statistical analysis. *P < 0.05; **P < 0.01. (C) Western blot analysis of mitotic markers in S-phase-arrested MDA-MB-231 cells treated with 20 μM of MPT0L184 for 6 h. The experimental design (upper) and representative results from one of two biological replicates (lower) are shown. Data were collected from different sets of gel electrophoresis with equal loading of the same samples. Asyn: asynchronized; Thy: thymidine; HU: hydroxyurea.
3.4. HDAC-inhibitory activity is required for MPT0L184 to induces premature mitosis

The substitution of the purine isostere did not involve any change on the zinc-binding motif (2-aminobenzamide) (Fig. 1), suggesting that MPT0L184 retains the HDAC-inhibitory activity. We thus compared the HDAC-inhibitory activity of MPT0L184 to that of entinostat, MPT0L185, and MPT0L198 (Fig. 1; [18]). We showed that MPT0L184 exhibited non-differential activity as did entinostat and MPT0L185 in enhancing histone acetylation in cells (Supplementary Fig. 4A). In addition, acetylation levels of structural maintenance of chromosomes protein 3 (SMC3) at K105/106 and α-tubulin at K40, whose deacetylations are respectively mediated by HDAC8 and HDAC6, were not significantly changed in response to MPT0L184 treatment (Supplementary Fig. 4A).

An in vitro HDAC scan further confirmed that MPT0L184 displayed the same specificity as entinostat toward HDACs, targeting class I HDACs (1/2/3) (Fig. 5A and data not shown). Intriguingly, when compared to entinostat, MPT0L184 was more potent at suppressing HDAC1 and 2, but less effective at inhibiting HDAC3 (Fig. 5A). To evaluate the HDAC inhibitory activity of MPT0L184 in cells, we isolated cellular HDAC1 from treated cells and measured the HDAC activity fluorometrically. We showed that MPT0L184 exhibited a higher inhibitory activity toward HDAC1 in cells compared to entinostat (Fig. 5B). HDAC1 and 2 are reported to negatively regulate p21 expression by associating with its promoter in cells [21,22]. In line with this, we found that MPT0L184 dramatically increased p21 level, and this effect was more pronounced than that of entinostat (Fig. 5C). Moreover, hyperactivation of mitotic signaling and p21 expression by MPT0L184 was independent of p53, as these events were also evidenced in p53-null NCI-H1299 cells (Supplementary Fig. 4B). These results support that MPT0L184 possesses high inhibitory activity toward HDAC1 and 2 in cells.

To assess the contribution of HDAC inhibition to MPT0L184-initiating premature mitosis, we generated MPT1A343, an MPT0L184 analog lacking the HDAC-inhibitory function, by the substitution of aniline (Supplementary Fig. 4C). We found that treatment with MPT1A343 failed to increase histone H3 acetylation and enhance p21 expression (Fig. 5C; Supplementary Fig. 4A). Importantly, MPT1A343 was insufficient to elicit mitotic signaling or perturb cell-cycle progression (Fig. 5D; Supplementary Fig. 4D). Moreover, the high virulence of MPT0L184 requires its HDAC-inhibitory activity, as MPT1A343 exhibited relatively low toxicity (Fig. 5E). These data strongly suggest that MPT0L184 triggers precocious mitosis, at least in part, through HDAC inhibition.

3.5. MPT0L184 impairs checkpoint functions

CDKs are key regulators of cell-cycle progression and their activities are tightly controlled via the reversible inhibitory phosphorylation at Y15 by WEE1/MIT1 kinase and CDC25 phosphatases [13]. CHK1 inhibition stabilizes CDC25A and thus leads to CDK1 hyperactivation and premature mitosis [23-25]. We showed that MPT0L184 and the CHK1 inhibitor UCN-01 failed to enhance mitotic signaling in the presence of the CDK inhibitor roscovitine (Fig. 6A), indicating that CDK activity is crucial for inducing premature mitosis by both compounds. We observed that CDK1Y15 phosphorylation was significantly reduced at the earlier time points after MPT0L184 treatment (Fig. 4A). However, levels of cellular phosphatases CDC25A/C remarkably decreased in cells treated with MPT0L184, and we did not detect an inhibitory activity of MPT0L184 toward WEE1 in vitro (Supplementary Fig. 5A and B). Inhibition of heat-shock protein 90 (HSP90) has been shown to down-regulate protein stability of WEE1 and CHK1, thereby leading to perturbation of mitosis onset [26,27]. Given that MPT0L184 shares the 7H-pyrrolo[2,3-d]pyrimidine group with BIIB021 (Supplementary Fig. 5C), an HSP90 inhibitor under phase I/II clinical evaluation, it is

![Fig. 5. HDAC-inhibitory activity is required for MPT0L184 to induce premature mitosis and p21 expression.](image)
possible that MPT0L184 behaves as an HSP90 inhibitor. We thus examined protein levels of WEE1 and CHK1 in MPT0L184-treated cells. As expected, we observed that HSP90 inhibition by STA-9090 or BIIB021 significantly reduced protein levels of CHK1 and WEE1, concomitant with increased H3 phosphorylation and moderately reduced CDK1 Y15 phosphorylation (Fig. 6B; Supplementary Fig. 5D). In contrast, MPT0L184 treatment resulted in a reduction of WEE1 expression and CDK1 Y15 phosphorylation, but had only a negligible impact on CHK1 level (Fig. 6B; Supplementary Fig. 5D). Notably, MPT0L184 was more potent at inducing mitotic signals, as reflected by higher H3S10 phosphorylation, than the HSP90 inhibitors (Fig. 6B; Supplementary Fig. 5D). The quantitative polymerase-chain-reaction (qPCR) analysis further revealed that both MPT0L184 and entinostat, but not STA-9090, suppressed WEE1 expression at the transcription level (Fig. 6C). In addition, MPT0L184 was unable to compete with the interaction of geldanamycin, another HSP90 inhibitor, with HSP90 protein in vitro (Supplementary Fig. 5E), arguing that MPT0L184 lacks HSP90-binding activity [28]. These results indicate that MPT0L184 suppresses WEE1 expression and induces premature mitosis independent of HSP90. We further tested if MPT0L184 treatment would influence the sensitivity of MDA-MB-231 cells to adavosertib, a WEE1 inhibitor under clinical evaluation. We found that a 2-h pretreatment of MPT0L184/entinostat resulted in a reduction of WEE1 and CHK1 in synchronized MDA-MB-231 cells treated with 16 μM of BIIB021, STA-9090, MPT0L184, or entinostat for 6 h. Importantly, reduced CHK1/345 phosphorylation, but not low WEE1 expression, was also observed in cells with MPT1A343 treatment (Fig. 6E). This evidence argues that the substitution of aniline does not completely ruin MPT0L184 activities. Thus, our data indicate that HDAC inhibition by MPT0L184 is required for WEE1 downregulation but not the sole mechanism for suppression of ATR/CHK1 signaling.

3.6. HDACi-resistant cancer cells are sensitive to MPT0L184

Our findings revealed that MPT0L184 possesses dual functions of suppressing HDAC activity and initiating unscheduled mitotic signaling. We therefore tested if cancer cells resistant to HDACis are susceptible to MPT0L184. Consistent with the previous study [29], the YCC3/7 gastric cancer cell line was more resistant to entinostat compared to the sensitive cell line YCC11 (Fig. 7A). In YCC11 cells, MPT0L184 was more toxic than entinostat at higher concentrations, but the IC_{50} values of two compounds were indistinguishable (Fig. 7A). It is noteworthy that MPT0L184 showed comparable toxicity toward two cell lines (Fig. 7A), indicating that HDACi-resistant cells are vulnerable to MPT0L184. We also observed the hyper-activation of mitotic signals in YCC3/7 cells treated with MPT0L184 but not entinostat (Fig. 7B). Importantly, inactivation of CDK by roscovitine eliminated the mitotic signal induced by MPT0L184 and dampened the cytotoxicity of MPT0L184 in a dose-dependent manner (Fig. 7B and C). These results suggest that CDK hyperactivation that trigger premature mitosis may overcome HDACi-resistant cancer cell mechanisms.
4. Discussion

Our study discovers MPT0L184 as a novel HDAC inhibitor (HDACi) which possesses high inhibitory activity toward HDAC1/2 and can elicit unscheduled onset of mitosis. MPT0L184 disturbs checkpoint functions by downregulating WEE1 expression and ATR/CHK1 signaling, which potentially augments CDK activity and thus leads to mitosis dysregulation. Moreover, cancer cells with HDACi resistance were susceptible to MPT0L184, arguing that MPT0L184 could be used as an alternative HDACi to counteract drug resistance.

WEE1 and CHK1 are two key gatekeeper kinases that control CDK1 activity at the G2/M boundary [9]. Our data revealed that the WEE1-mediated inhibitory phosphorylation of CDK1 at Y15 was obviously reduced in cells with MPT0L184 treatment (Fig. 4A). We also observed reduced WEE1 expression at the transcription level (Fig. 6B and C), plausibly resulted from enhanced expression of epigenetic regulators that negatively regulate WEE1 expression [30–33]. However, WEE1 downregulation did not seem to be the prime determinant of CDK1 upregulation, as entinostat also impeded expression of WEE1 to a similar level as MPT0L184 but did not activate CDK1 (Fig. 6B and C). Given that MPT0L184 did not act as a WEE1 inhibitor and levels of CDC25 phosphatases were concurrently reduced (Supplementary Fig. 5A and B), other mechanisms antagonizing WEE1 function must be abrogated by MPT0L184. It is reported that WEE1 inhibition can induce a compensatory response that activates ATR/CHK1, which in turn suppresses CDC25s and activates WEE1 through phosphorylations, leading to inactivation of CDK1 and thus cell-cycle arrest in the G2 phase [34, 35]. Simultaneous treatment with vorinostat was shown to downregulate CHK1 and thus promote the premature mitotic entry caused by WEE1 suppression, resulting in synthetic lethal of cancer cells [36, 37]. We observed only a negligible reduction, if any, of CHK1 phosphorylation by ATR in cells with entinostat treatment, and activation of mitotic signaling was not evidenced (Fig. 6E and F). In contrast, the CHK1 phosphorylation was remarkably decreased when cells treated with MPT0L184. While it remains unclear whether this effect is associated with the pronounced inhibitory activity of MPT0L184 on HDAC1/2, our data showed that at least a mechanism independent of HDAC inhibition contributes to MPT0L184-mediated CHK1 downregulation (Fig. 6F). It is thus plausible that MPT0L184 substantially perturbs ATR/CHK1 functions through various mechanisms, which synergically exacerbates WEE1 deficiency to a degree capable of enhancing CDK1 activity and therefore perturbs the timely control of mitosis onset.

Many HDACis can elicit cell-cycle arrest in the G1 phase through de-repressing expression of the CDK inhibitor p21, which is thought to
contribute to drug resistance [7,38]. p21 knockout enables HCT116 cells exempt from the G1 arrest caused by the class I HDACi romidepsin and increases the sensitivity to romidepsin treatment [39]. We did detect a strong induction of p21 in MPT0L184-treated cells (Fig. 5C), but the G1 arrest was only evidenced when cells were treated immediately after mitosis exit (Supplementary Fig. 2). Induction of p21 by MPT0L184 is unlikely a negative-feedback regulation for buffering the elevated mitotic CDK1 activity resulted from the deficiency of checkpoint activities, as p21 depletion did not further enhance mitotic signaling (data not shown). Given that CDK inhibition attenuated the toxicity of MPT0L184 (Fig. 7C), our data suggest that MPT0L184 may, through enhancing CDK activity, facilitate cell-cycle progression and thus relieve mitotic CDK1 activity resulted from the deficiency of checkpoint activ-

gates mitosis progression, which will provide important insights into cancer development and therapeutics.

Supplementary materials

Supplementary material associated with this article can be found, in the online version of this article at Biomedicine & Pharmacotherapy.

Data sharing

The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information files, or are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

T.-Y. Chang: Formal analysis, Investigation, Visualization, Writing - original draft. K. Nepali: Resources, Writing - review & editing. Y.-Y. Chen: Formal analysis, Investigation. Y.-C.S.H. Yang: Investigation. K.-C. Hsu: Investigation. Y. Yen: Conceptualization, Project administration, Supervision, Writing - review & editing. S.-L. Pan: Supervision, Visualization, Writing - review & editing. J.-P. Liou: Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing. S.-B. Lee: Conceptualization, Funding acquisition, Supervision, Writing - original draft and editing.

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Conflict of interest statement

The authors declare no potential conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopharm.2021.111485.

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