

Inhibition of p38-MK2 pathway enhances the efficacy of microtubule inhibitors in breast cancer cells

Reviewed Preprint

v2 • May 14, 2025

Revised by authors

Reviewed Preprint

v1 • December 4, 2024

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eLife Assessment

This study provides **valuable** findings that MK2 inhibitor CMPD1 can inhibit the growth, migration and invasion of breast cancer cells both in vitro and in vivo. The evidence supporting the claims of the authors is **solid**, although the detailed molecular mechanism and additional animal experiments would strengthen the paper. This study will be of interest to the breast cancer field.

<https://doi.org/10.7554/eLife.104859.2.sa4>

Abstract

Microtubule-targeting agents (MTAs) have been successfully translated from basic research into clinical therapies and have been widely used as first- and second-line chemotherapy drugs for various cancers. However, current MTAs exhibit positive responses only in subsets of patients and are often accompanied by side effects due to their impact on normal cells. This underscores an urgent need to develop novel therapeutic strategies that enhance MTA efficacy while minimizing toxicity to normal tissues. In this study, we demonstrate that inhibition of the p38-MK2 (MAP kinase-activated protein kinase 2) pathway sensitizes cancer cells to MTA treatment. We utilize CMPD1, a dual-target inhibitor, to concurrently suppress the p38-MK2 pathway and microtubule dynamicity. In addition to its established role as an MK2 inhibitor, we find that CMPD1 rapidly induces microtubule depolymerization, preferentially at the microtubule plus-end, leading to the inhibition of tumor growth and cancer cell invasion in both *in vitro* and *in vivo* models. Notably, 10 nM CMPD1 is sufficient to induce irreversible mitotic defects in cancer cells, but not in non-transformed normal cells, highlighting its high specificity to cancer cells. We further validate that a specific p38-MK2 inhibitor significantly potentiates the efficacy of sub-clinical concentrations of MTA. In summary, our findings suggest that the p38-MK2 pathway presents a promising therapeutic target in combination with MTAs in cancer treatment.

Introduction

Cancer, a disease characterized by uncontrolled cell growth, results from cells that proliferate indefinitely without external growth signals^{1,2}. Consequently, targeting cell cycle progression is a powerful therapeutic strategy for cancer treatment^{3,4}. Microtubule-targeting agents (MTAs) disrupt spindle microtubule assembly during mitosis, making them widely-used chemotherapy drugs for various tumors^{5–7}. By impairing microtubule dynamics and functions, MTAs activate the spindle assembly checkpoint (SAC)^{8–10}, leading to mitotic arrest, mitotic defects, and apoptotic cell death^{5,11}. MTAs are functionally categorized into two groups: microtubule stabilizers (e.g., taxanes)^{12,13} and microtubule depolymerizers (e.g., eribulin)^{14,15}. Paclitaxel (PTX), a taxane, has been a highly successful anti-cancer drug in clinical use for over 30 years^{12,16}. However, several limitations hinder its therapeutic efficacy. Firstly, only a subset of breast and ovarian cancer patients exhibit a favorable response to PTX^{17–19}. Secondly, PTX lacks tumor specificity, leading to off-target effects, including neutropenia, gastrointestinal disorders, and peripheral neuropathy^{20–24}. Thirdly, cancer cells can acquire resistance to PTX through mechanisms such as the upregulation of drug efflux proteins (e.g., P-glycoprotein) or class III β -tubulin, which reduces PTX binding affinity^{25–27}. In contrast, eribulin, a second-line chemotherapeutic agent, has a lower incidence of peripheral neuropathy, one of the most troublesome side effect of MTAs²⁸. Due to its distinct mechanism of action, eribulin is effective against taxane-resistant tumors^{29–32}. However, clinical studies have demonstrated that fewer than 20% of metastatic breast cancer patients, previously treated with chemotherapy, respond positively to eribulin^{33,34}. MTAs remain central to the treatment of breast and ovarian cancers, including metastatic cases. Therefore, developing new strategies to overcome the limitations associated with MTAs is critical for optimizing therapeutic outcomes in cancer treatment.

The p38 mitogen-activated protein kinase (MAPK) signaling pathway, activated by a variety of environmental and intracellular stimuli^{35–38}, plays a crucial role in numerous biological processes, including DNA repair, inflammation, cell differentiation, and cell death^{39–44}. MAPK-activated protein kinase 2 (MK2) is a major downstream substrate of p38 MAPK⁴⁵. Previous studies have shown that phosphorylated MK2 localizes to mitotic spindles, and that MK2 depletion leads to abnormal spindle formation, defects in chromosome alignment, and mitotic arrest in both human cells and mouse oocytes, indicating a vital role of MK2 in mitotic progression^{46,47}. CMPD1 was originally developed as a selective inhibitor targeting the p38-dependent phosphorylation of MK2 (**Fig. 1A**)^{48–51}. Subsequent studies revealed that CMPD1 could induce G2/M arrest in glioblastoma and gastric cancer cells, as observed by flow cytometry^{52,53}, and suggested its potential role in inhibiting microtubule polymerization *in vitro*⁵³. However, the precise and dynamic nature of CMPD1's effects on microtubule dynamics and cancer cell proliferation have yet to be fully elucidated. In this study, we demonstrate that CMPD1 preferentially induces severe mitotic defects in breast cancer cells and effectively inhibits cancer cell growth, migration, and invasion in both *in vitro* and *in vivo* models. Notably, CMPD1 uniquely triggers rapid microtubule depolymerization at the plus-end *in vitro*. These results indicate that the inhibition of p38-MK2 pathway could enhance the therapeutic efficacy of MTAs. We validate this hypothesis by demonstrating that a specific MK2 inhibitor, MK2-IN-3, in combination with vinblastine, a clinically approved microtubule destabilizer^{54,55}, exhibits significantly increased efficacy in inducing mitotic defects. Thus, our results suggest that the p38-MK2 pathway may serve as a promising therapeutic target in combination with MTAs in cancer treatment.

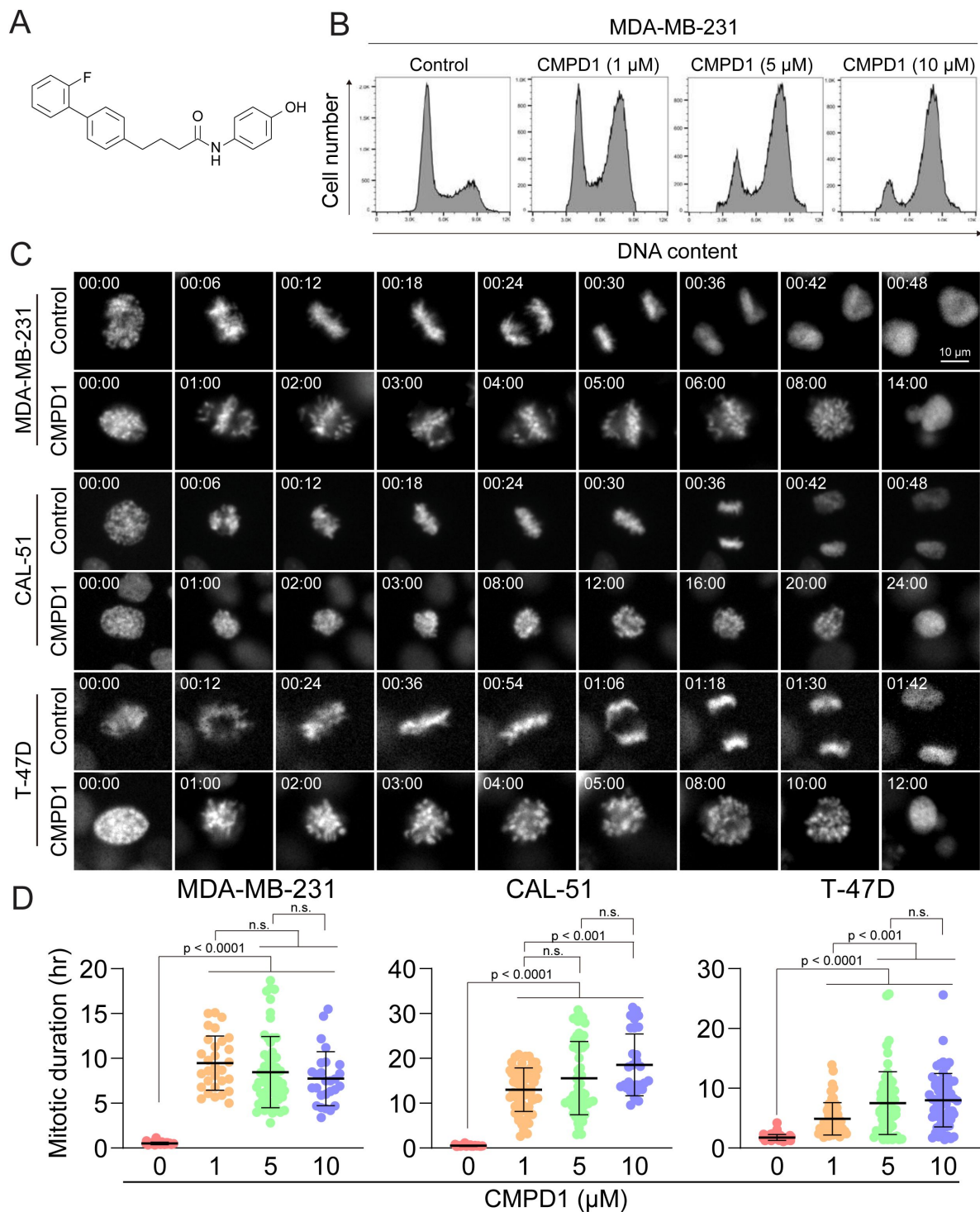


Figure 1.

CMPD1 induced severe mitotic arrest in multiple cancer cell lines

(A) Chemical structure of CMPD1. (B) FACS analysis of MDA-MB-231 cells treated with DMSO or 1, 5, or 10 μ M CMPD1 for 24 hours. (C) Representative time-lapse images of MDA-MB-231, CAL-51, and T-47D cells treated with either DMSO or 5 μ M CMPD1. Time is indicated in minutes post-NEBD. (D) Quantification of mitotic duration of MDA-MB-231, CAL-51, and T-47D treated with DMSO, 1, 5, or 10 μ M CMPD1. $n = 30$ -60 cells pooled from two independent replicates. Error bars are mean $\pm$ s.d. Asterisk above each dot plot indicates the significant difference ($p < 0.0001$, Tukey's multiple comparison test) between the indicated drug-treated condition and the control.

Results

CMPD1 induces robust prometaphase arrest in breast cancer cell lines

Cell cycle inhibitors, particularly MTAs, are standard chemotherapy drugs for breast cancer⁵⁴. To investigate whether CMPD1 induces G2/M arrest in breast cancer cells, we treated MDA-MB-231 cells, a triple-negative breast cancer (TNBC) cell line, with varying concentrations of CMPD1 and analyzed its effects on cell cycle progression using flow cytometry. The results demonstrated that CMPD1 effectively arrested MDA-MB-231 cells in the G2/M phase at concentrations ranging from 1 to 10 μ M (**Fig. 1B**), consistent with previous observations in glioblastoma cells⁵³. Given that flow cytometry is unable to distinguish between G2 phase and mitosis, we performed high-temporal resolution live-cell imaging to further dissect the impact of CMPD1 on cell cycle progression across various breast cancer cell lines, including MDA-MB-231 (TNBC), CAL-51 (TNBC), and T-47D (luminal A)^{56,57}. Our findings revealed that CMPD1 induced a severe prometaphase arrest across all tested breast cancer cell lines, regardless of subtypes, with most cells remaining arrested in prometaphase for over 10 hours, while control cells divided within 30-60 min (**Fig. 1C-D** and Supplementary Movies 1-2). Since CAL-51 cells harbor wild-type TP53, whereas the other two cell lines do not^{58,59}, these results suggest that CMPD1 induces a robust prometaphase arrest in breast cancer cells through a p53-independent mechanism.

Breast cancer cells exhibit heightened sensitivity to CMPD1 treatment compared to normal cells

We demonstrated that concentrations exceeding 1 μ M of CMPD1 effectively induced a robust prometaphase arrest in multiple breast cancer cell lines (**Fig. 1D**). In contrast, recent studies on MTA treatment for breast cancer have identified that the clinically relevant concentration of PTX in tissue culture cells ranges from 5 to 50 nM⁶⁰. Within this range of concentration, PTX does not induce severe mitotic arrest as observed at higher concentrations ($> 1 \mu$ M), but it significantly increases the incidence of mitotic errors, thereby promoting chromosomal instability (CIN) and ultimately leading to cancer cell death⁶⁰. Given these findings, we next explored whether sub- μ M concentrations of CMPD1 could induce CIN in breast cancer cells. To this end, we treated MDA-MB-231 and CAL-51 cells, along with two non-transformed cell lines (breast epithelial MCF10A and retinal pigment epithelial RPE1 cells), which served as a normal cell control, with either 10 or 50 nM CMPD1. We then compared the impact of CMPD1 on chromosome segregation using live-cell imaging. In the absence of CMPD1, no significant differences in mitotic duration were observed among MCF10A, RPE1, MDA-MB-231, and CAL-51 cells (**Fig. 2A-C** and Fig.2-Supplement 1A-C). However, upon treatment with 10 or 50 nM CMPD1, all cell lines were arrested in prometaphase, except for MCF10A (**Fig. 2A-C**). Notably, MDA-MB-231 and CAL-51 cells displayed significantly prolonged mitotic duration compared to MCF10A and RPE1 cells (**Fig. 2A-C** and Fig.2-Supplement 1A-C), indicating greater sensitivity of breast cancer cells to CMPD1 treatment relative to normal cells. Given that mitotic errors are a direct cause of CIN, we next assessed the frequency of mitotic errors including misaligned chromosome, chromosome bridge, lagging chromosome, and multipolar division under these conditions (**Fig. 2D**). Both MDA-MB-231 and CAL-51 cells exhibited a significantly higher rate of mitotic errors when exposed to 10 or 50 nM CMPD1 (**Fig. 2E**). For instance, approximately 90% and 100% of CAL-51 cells experienced mitotic errors in the presence of 10 and 50 nM CMPD1, respectively (**Fig. 2E**). Conversely, 90~100% of MCF10A and RPE1 cells underwent faithful cell division even at the same concentrations of CMPD1 (**Fig. 2E** and Fig.2-Supplement 1A-C). To determine whether CMPD1 selectively targets cancer cells compared to other MTAs, we evaluated mitotic error rates in the same cell lines treated with a clinically relevant concentration of PTX (10 nM). Our results demonstrated that both RPE1 and

breast cancer cell lines displayed comparably high rates of mitotic errors when treated with 10 nM PTX treatment (**Fig. 2F**), consistent with previous observations⁶⁰. In summary, unlike PTX, CMPD1 induces CIN with selective toxicity toward breast cancer cells.

To further investigate the selectivity of CMPD1 in breast cancer cells, we conducted a CMPD1 washout assay using the above cell lines, aiming to recapitulate the clinical condition where the concentration of chemotherapy drugs in patients is diluted due to the “drug holiday” between regular treatments. In this assay, we treated the cells with 2 μ M CMPD1 for 12 hours, followed by a washout before imaging. We observed that the mitotic duration was significantly prolonged in both MDA-MB-231 and CAL-51 cells upon CMPD1 washout (85 min and 151 min, respectively) compared to RPE1 cells (14 min) (**Fig. 2G-H** and Fig. 2-Supplement 2A-B). This suggests that breast cancer cells struggle to recover from CMPD1-induced mitotic arrest, whereas normal cells can immediately resume progression to proper anaphase. Notably, 90% and 81% of mitotic cells in MDA-MB-231 and CAL-51 cells lines, respectively, exhibited mitotic errors, whereas 50% of RPE1 cells displayed normal chromosome segregation (**Fig. 2G, I**). These findings reinforce the hypothesis that breast cancer cells are more sensitive to CMPD1 treatment under clinically relevant conditions. Interestingly, approximately 60% of MDA-MB-231 cells that entered mitosis after CMPD1 washout still exhibited mitotic errors, while only ~10% of RPE1 cells showed errors. This suggests a prolonged impact of CMPD1 on mitosis in breast cancer cells (Fig. 2-Supplement 2C). Consistent with the increased sensitivity in breast cancer cells to CMPD1, MDA-MB-231 cells exhibited a significantly higher frequency of apoptotic cell death during or shortly after mitosis within 24 of CMPD1 washout, compared to RPE1 cells (Fig. 2-Supplement 2D-E).

To further validate the heightened sensitivity of breast cancer cells to CMPD1, we measured its IC₅₀ in MDA-MB-231, CAL-51, and MCF10A cells. Consistent with our live-cell imaging results, both MDA-MB-231 and CAL-51 cells exhibited significantly lower IC₅₀ (0.21 μ M and 0.74 μ M, respectively), compared to MCF10A cells (1.16 μ M) (Fig. 2-Supplement 3A-C). Moreover, IC₅₀ of p53 knockout CAL-51 cells⁶¹ (0.88 μ M) was comparable to that of wild-type CAL-51 cells (0.74 μ M) (Fig. 2-Supplement 3D), further supporting the notion that CMPD1 induces a robust prometaphase arrest in breast cancer cells via a p53-independent mechanism. Collectively, these results underscore that breast cancer cells are more sensitive to CMPD1 treatment than normal MCF10A and RPE1 cells, particularly in a clinically relevant context.

CMPD1 inhibits anchorage independent growth and tumor growth in mouse xenograft

We subsequently evaluated the efficacy of CMPD1 in inhibiting anchorage-independent growth, a critical hallmark of tumorigenesis, in MDA-MB-231 cells. CMPD1 demonstrated a potent dose-dependent inhibition, with 500 nM being sufficient to completely suppress colony formation (**Fig. 3A-B** and Fig. 3-Supplement 1A-B). Remarkably, even at a relatively lower concentration (250 nM), CMPD1 significantly reduced colony formation, surpassing the inhibitory effects of 10 μ M PTX. These findings suggest that CMPD1 is more effective than PTX in inhibiting the anchorage-independent growth of MDA-MB-231 cells.

We demonstrated that CMPD1 effectively inhibits cancer cell growth in a tissue culture model. Next, we investigated whether CMPD1 could also inhibit tumor growth *in vivo*. To this end, we employed MDA-MB-231 and CAL-51 xenograft mouse models and found that CMPD1 significantly suppressed the tumor growth (**Fig. 3C-G** and Fig. 3-Supplement 1C-D). Notably, while both PTX and CMPD1 suppressed tumor growth in mice, CMPD1 achieved comparable efficacy at concentrations 10-100 times lower than PTX (**Fig. 3D-G**). Furthermore, mice in the CMPD1 treatment group continued to gain weight during the entire treatment regimen, comparable to the control group (vehicle-treated mice), with no observed mortality (Fig. 3-Supplement 2A-B). In contrast, the PTX-treated group exhibited a significant reduction in body weight and an increased number of deaths of individuals (Fig. 3-Supplement 2A-B). Additionally, anatomical examination

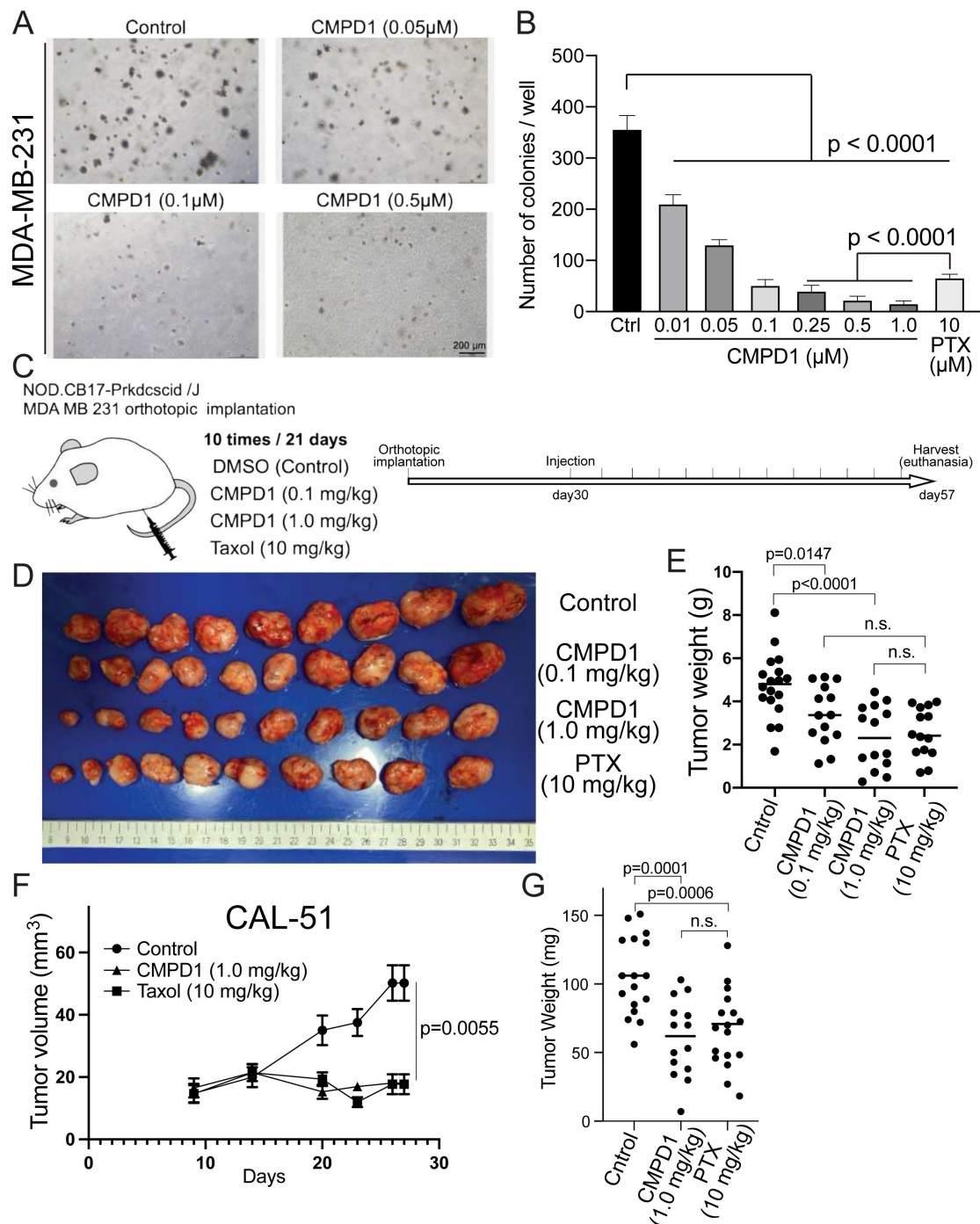


Figure 3.

CMPD1 inhibits both anchorage independent growth and tumor growth in mice

(A) Representative images of anchorage independent growth assay using MDA-MB-231 cells treated with DMSO, CMPD1 at different conditions (0.01, 0.05, 0.1, 0.25, 0.5, and 1 μM), or 10 μM PTX. (B) The normalized number of cell colonies formed in each condition. Error bars are mean $\pm$ s.d. Three independent experiments were performed for each condition. (C) Schematic diagram of the mouse xenograft experiment using MDA-MB-231 and the drug treatment schedule. (D) The photo of tumors in each condition at the time of necropsy. The tumors were arranged in order based on their size. (E) Quantification of the gross weight of tumors in the mouse xenograft experiment using MDA-MB-231 cells at the time of necropsy. (F) Quantification of the tumor volume during the treatment regimen in the mouse xenograft experiment using CAL-51 cells. (G) Quantification of the gross weight of tumors in the mouse xenograft experiment using CAL-51 cells at the time of necropsy.

showed no apparent abnormalities in kidneys or livers of CMPD1-treated mice, and blood marker analysis confirmed no significant impairment in these organ functions in CMPD1-treated mice compared to control (Fig. 3-Supplement 3A-B). Notably, the numbers of white blood cells (WBC) were comparable between CMPD1-treated and control groups, while PTX-treated group showed a marked decrease in WBC levels (Fig. 3-Supplement 3C-D). These findings suggest that CMPD1 is not only effective in inhibiting tumor growth in both *in vitro* and *in vivo* models but also appears to be more potent and safer than PTX, a standard chemotherapeutic agent for breast cancer treatment.

CMPD1 exhibits a preferential depolymerizing effect on the microtubule plus-end

We demonstrated that at least ≥ 1 μ M CMPD1 induced a robust prometaphase arrest in breast cancer cell lines (Fig. 1D). A previous study suggests that CMPD1 may possess the ability to inhibit tubulin polymerization, as indicated by *in vitro* tubulin polymerization assay⁵³. To elucidate the mechanism underlying the CMPD1-induced prometaphase arrest, we performed high spatiotemporal resolution live-cell imaging to monitor microtubule dynamics in metaphase CAL-51 cells (see **Methods**). In these cells, α -tubulin and histone H2B genes were endogenously labeled with mNeonGreen and mScarlet, respectively, using CRISPR-Cas9 technology⁶⁰. Our results revealed that the signal intensity of mitotic spindles dramatically decreased to less than 10% within 8 min following CMPD1 treatment (Fig. 4A-B and Supplementary Movies 3-4). Concurrently, the formation of multipolar spindle poles was observed (Fig. 4A). The decrease in spindle signal intensity correlated with a gradual disruption of the metaphase plate, likely due to the loss of kinetochore-microtubule attachments (Fig. 4A). Moreover, we observed that CMPD1-treated cells exited G2 phase and underwent nuclear envelope breakdown (NEBD), even though these cells were unable to assemble mitotic spindles (Fig. 4C). This finding further supports that CMPD1 specifically arrests cells in prometaphase rather than G2 phase. To determine whether CMPD1 induces rapid microtubule depolymerization or the degradation of tubulin proteins, we pre-treated the cells with MG132, a proteasome inhibitor, for 1 hour prior to CMPD1 treatment (Fig. 4A-B and Supplementary movie 5). We found that proteasome inhibition did not prevent the CMPD1-mediated reduction in spindle signals (Fig. 4A-B), suggesting that CMPD1 directly induces microtubule depolymerization in breast cancer cells.

To further investigate the mechanism underlying CMPD1-induced microtubule depolymerization, we conducted TIRFM (Total Internal Reflection Fluorescence Microscopy) experiments to observe microtubule behaviors in response to CMPD1 treatment. The conventional TIRFM assay utilizing PTX-stabilized immobilized microtubules is widely used due to its ease of use. However, this approach lacks the dynamic nature of microtubules as seen in living cells, limiting the study of CMPD1's effects under more physiological conditions. To overcome this limitation, we utilized GMPCPP-stabilized microtubule seeds combined with rhodamine-labeled tubulin, which allows for the observation of microtubule growth and shrinkage at both ends, thereby better mimicking the dynamic behavior of microtubules in mitotic cells. As expected, microtubules exhibited repeated cycles of growth and shrinkage at both plus and minus ends, with a higher stability at minus ends (Fig. 4D). Upon treatment with CMPD1, there was a marked reduction in the proportion of microtubules with plus-end extensions within just 1 min (Fig. 4D-F), along with an increased frequency of microtubule catastrophes, reduced growth rates, and decreased maximum lengths of plus-end extensions (Fig. 4G-H). Interestingly, CMPD1's effects on the minus ends of microtubules were notably different. Neither the fraction of microtubules with minus end extensions nor the catastrophe frequency at minus ends were significantly different from control (Fig. 4D-H). However, minor reductions were observed in the average maximum length of minus-end extensions and their growth rates (Fig. 4H). In contrast, vinblastine, a well-characterized microtubule destabilizer, rapidly depolymerized microtubules from both ends (Fig. 4D-E), highlighting that CMPD1 uniquely and preferentially depolymerizes microtubules from the plus ends. Collectively, our cell biology and biochemical data demonstrate that CMPD1 alone can depolymerize microtubules, with a pronounced preference for plus-end depolymerization.

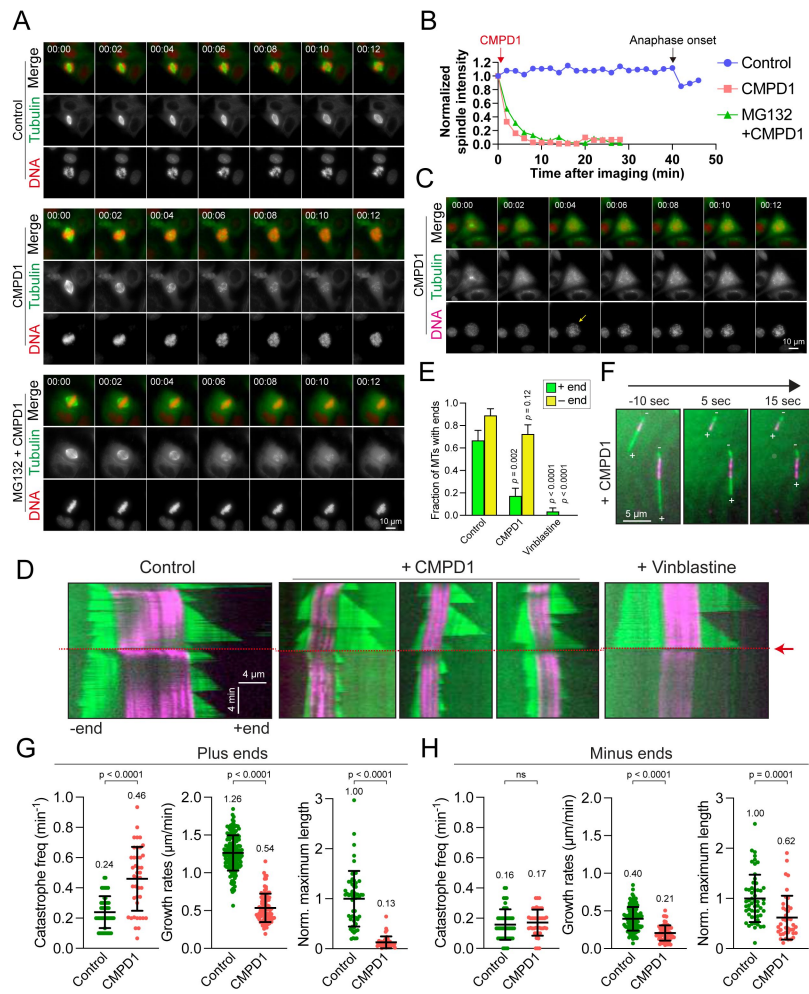


Figure 4.

CMPD1 induces microtubule depolymerization

(A) Representative time-lapse images of CAL-51 cells expressing α -Tubulin-mNeonGreen and H2B-mScalret upon the treatment with DMSO, 2 μ M CMPD1, or 10 μ M MG132 along with 2 μ M CMPD1. CMPD1 was added into the cell culture media immediately after images at the first time point were acquired. Time is indicated in minutes. (B) The quantification of the signal levels of mitotic spindles over time in each condition shown in (A). (C) Representative time-lapse images (interval: 2 min) of a G2 phase CAL-51 cell expressing α -Tubulin-mNeonGreen and H2B-mScalret in the presence of 2 μ M CMPD1. Note that the two bright dots in the Tubulin channel indicate the two clustered centrosomes before NEBD. The arrow in the DNA channel indicates the time of NEBD. Time is indicated in minutes. (D) Representative kymographs depicting microtubule plus and minus end dynamics before and after the addition of polymerization mix supplement with 15 μ M tubulin alone, or 15 μ M tubulin supplemented with either 20 μ M CMPD1 or 5 μ M vinblastine, as indicated (GMPCPP-stabilized microtubule seeds, magenta; microtubules polymerized from seeds, green; see Methods). Plus ends are positioned to the right, and minus ends are positioned to the left of the seeds in all kymographs. Red arrow and dashed line indicate the time of addition of tubulin alone, or tubulin plus drug. (E) Plot depicting fraction of microtubules with detectable plus or minus ends 1 minute after addition of drug, or tubulin alone ($n = 27, 29$, and 30 microtubules from left to right). Error bars indicate mean $\pm$ s.d. (F) Representative images from a time-lapse sequence showing microtubule plus and minus end lengths 10 seconds prior to, and 5 and 15 seconds after addition of polymerization mix supplemented with 20 μ M CMPD1. (G) Plots depicting plus end catastrophe frequencies ($n = 51$ and 41 microtubules from left to right), plus end growth rates ($n = 172$ and 91 events from left to right), and normalized maximum length ($n = 55$ and 41 microtubules from left to right) achieved over the imaging period. (H) Plots depicting minus end catastrophe frequencies ($n = 51$ and 41 microtubules from left to right), minus end growth rates ($n = 129$ and 63 events from left to right), and normalized maximum length ($n = 55$ and 40 microtubules from left to right) achieved over the imaging period. Error bars indicate mean $\pm$ s.d. Data were pooled from at least 2-4 independent replicates (G-H).

CMPD1 inhibits cell migration and invasion

Since proper microtubule dynamics is essential for regulating cell locomotion^{62–64}, we hypothesized that the improper microtubule dynamics induced by CMPD1 might inhibit cell migration. To investigate this, we evaluated the migratory capacity of CAL-51 cells using a wound healing assay following treatment with CMPD1. Our findings revealed that CMPD1, at concentrations ranging from 100 nM to 10 μ M, significantly inhibited wound closure compared to the DMSO-treated control (**Fig. 5A–B**), demonstrating its potential to suppress cancer cell migration and invasion. To further examine this hypothesis, we conducted a transwell-invasion assay using MDA-MB-231 cells. Treatment with CMPD1 at concentrations greater than 100 nM significantly suppressed breast cancer cell invasion, and at 1 μ M, CMPD1 completely abolished this cancer invasion (**Fig. 5C–D**).

To determine whether CMPD1 inhibits cancer cell invasion *in vivo*, we assessed the frequency of invasion of cancer cells into blood vessels by examining the tissue sections of mice with cancer cell-derived xenografts described in **Fig. 3C**. CMPD1-treated mice displayed a significantly reduced frequency of cancer cell-infiltrated vessels compared to vehicle-treated mice, suggesting that CMPD1 significantly inhibits metastasis *in vivo* (**Fig. 5E**). Together, these results show that CMPD1 suppresses cancer cell migration and invasion *in vitro* and *in vivo*.

Inhibiting the p38-MK2 pathway significantly enhances the efficacy of microtubule depolymerizing agents

CMPD1's distinct capacity to inhibit tumor growth may stem from its dual inhibitory effects on both the p38-MK2 signaling pathway and microtubule dynamics. To rigorously test the hypothesis that inhibition of the p38-MK2 pathway could potentiate the efficacy of MTAs, we assessed the combinatorial effects of MK2-IN-3 (hereafter referred to as MK2i), a selective MK2 inhibitor⁶⁵, and vinblastine at clinically relevant concentrations (1 or 5 nM) on mitotic progression in CAL-51 cells. We first confirmed that MK2i effectively inhibits MK2 activity in CAL-51 cells, as both 1 and 10 μ M concentrations significantly reduced phosphorylation levels of Hsp27, a key downstream phosphorylation substrate of MK2^{66,67}, following H₂O₂-induced oxidative stress (**Fig. 6-Supplement 1A–B**). In contrast, treatment with 10 μ M MK2i alone had no detectable impact on cell proliferation of CAL-51 cells over a 4-day period (**Fig. 6-Supplement 1C**). Consistent with these findings, treatment with either 10 μ M MK2i or 1 nM vinblastine alone did not significantly alter the frequency of mitotic slippage or mitotic cell death compared to the control (**Fig. 6A**). However, both individual treatments modestly prolonged mitotic duration and increased the frequency of mitotic errors (approximately 2 to 3-fold compared to control) (**Fig. 6A**). Strikingly, the combination of 10 μ M MK2i with 1 nM vinblastine resulted in a profound synergistic effect, extending mitotic duration by approximately 13-fold and markedly increasing the frequency of mitotic errors and cell death (**Fig. 6A**, **Condition 4**). A similar synergistic effect was also observed when cells were simultaneously treated with 10 μ M MK2i and 5 nM vinblastine, although the 5 nM vinblastine alone induced a more pronounced mitotic arrest compared to the control and 1 nM vinblastine (**Fig. 6A**, **Condition 6**). Importantly, similar synergistic effects, characterized by elevated mitotic index and increased errors during metaphase and anaphase, were also observed upon MK2 depletion by siRNA in cells treated with 1 nM vinblastine, further reinforcing the notion that suppression of the MK2 signaling pathway can enhance MTA efficacy (**Fig. 6-Supplement 2A–C**). To investigate whether this synergy extends beyond mitosis, we next assessed the effects of MK2i and vinblastine co-treatment on TNBC cell migration. As expected, both 10 μ M MK2i and 1 nM vinblastine individually impaired cell migration in CAL-51 cells, however, the combination treatment resulted in significantly greater inhibition compared to individual treatment group or the control group (**Fig. 6-Supplement 3A–B**).

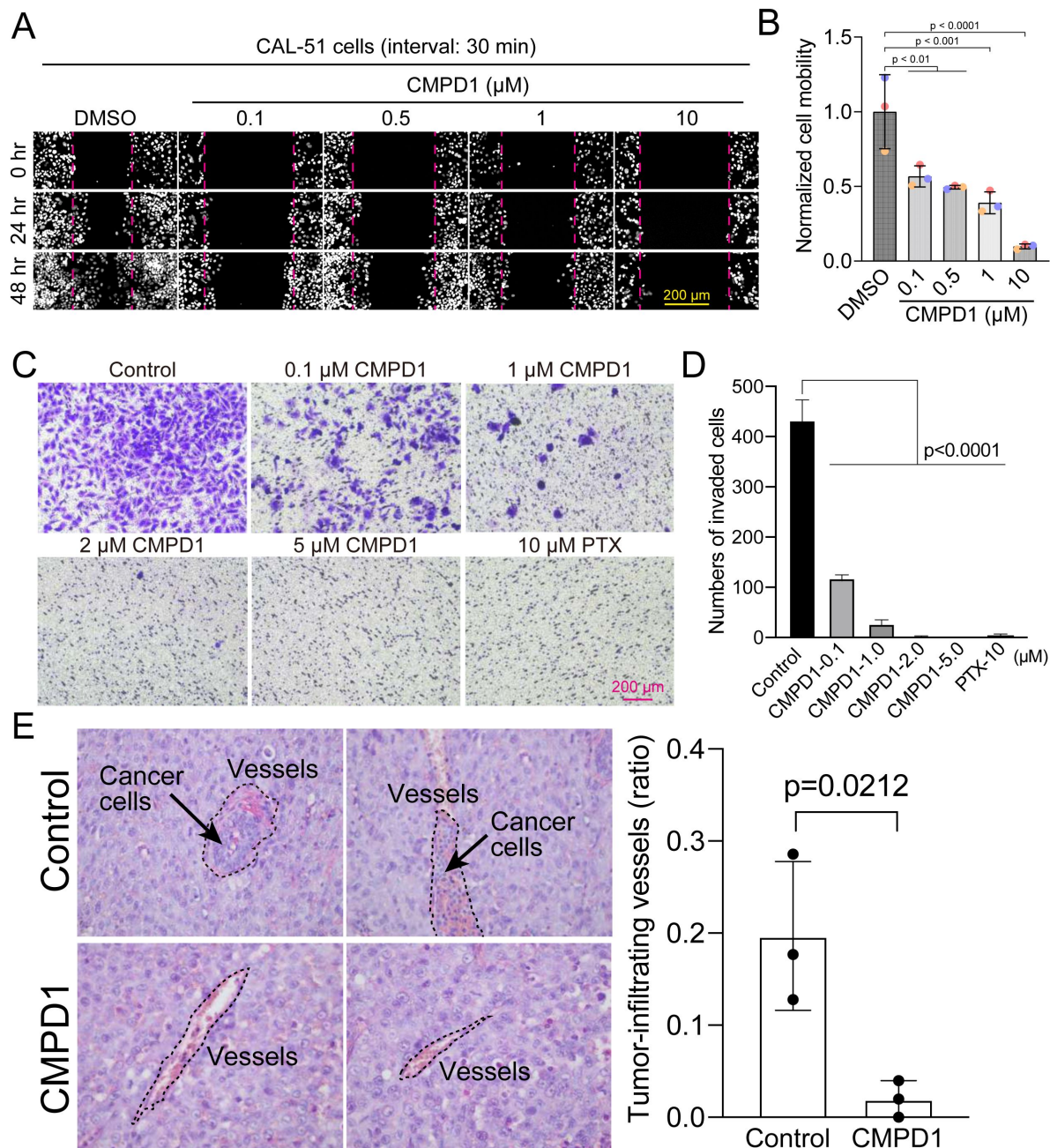


Figure 5.

CMPD1 inhibits cancer cell migration and invasion

(A) Representative images of H2B-mScarlet CAL-51 cells treated with DMSO, or CMPD1 at different concentrations (0, 0.1, 0.5, 1, 10 μM) at three different time points (0, 24, and 48 hr post-treatment). A wound (cell-free zone) was created using a tip, followed by the addition of indicated drugs and live-cell imaging (interval: 30 min). (B) Quantification of cell migration speed when cells were treated with indicated drugs. Each condition was normalized to the speed of DMSO-treated cells. The distance between the edge of the wound was measured using imageJ macro. Results are the mean ± s.d. N = 3 biological replicates. (C) Representative images of trans-well invasion assay using MDA-MB-231 cells treated with DMSO, CMPD1 (0.1, 1, 2, 5 μM), or 10 μM PTX. (D) The quantification of the average number of invaded cells in each condition shown in (C). (E) Left: example images of blood vessels in xenograft tumors derived from mice treated with DMSO or 1.0 mg/kg CMPD1 as shown in Fig. 3C. Two sets of example images acquired from different tumors were shown. Right: the quantification of the percentage of blood vessels infiltrated with cancer cells. Error bars are mean ± s.d.

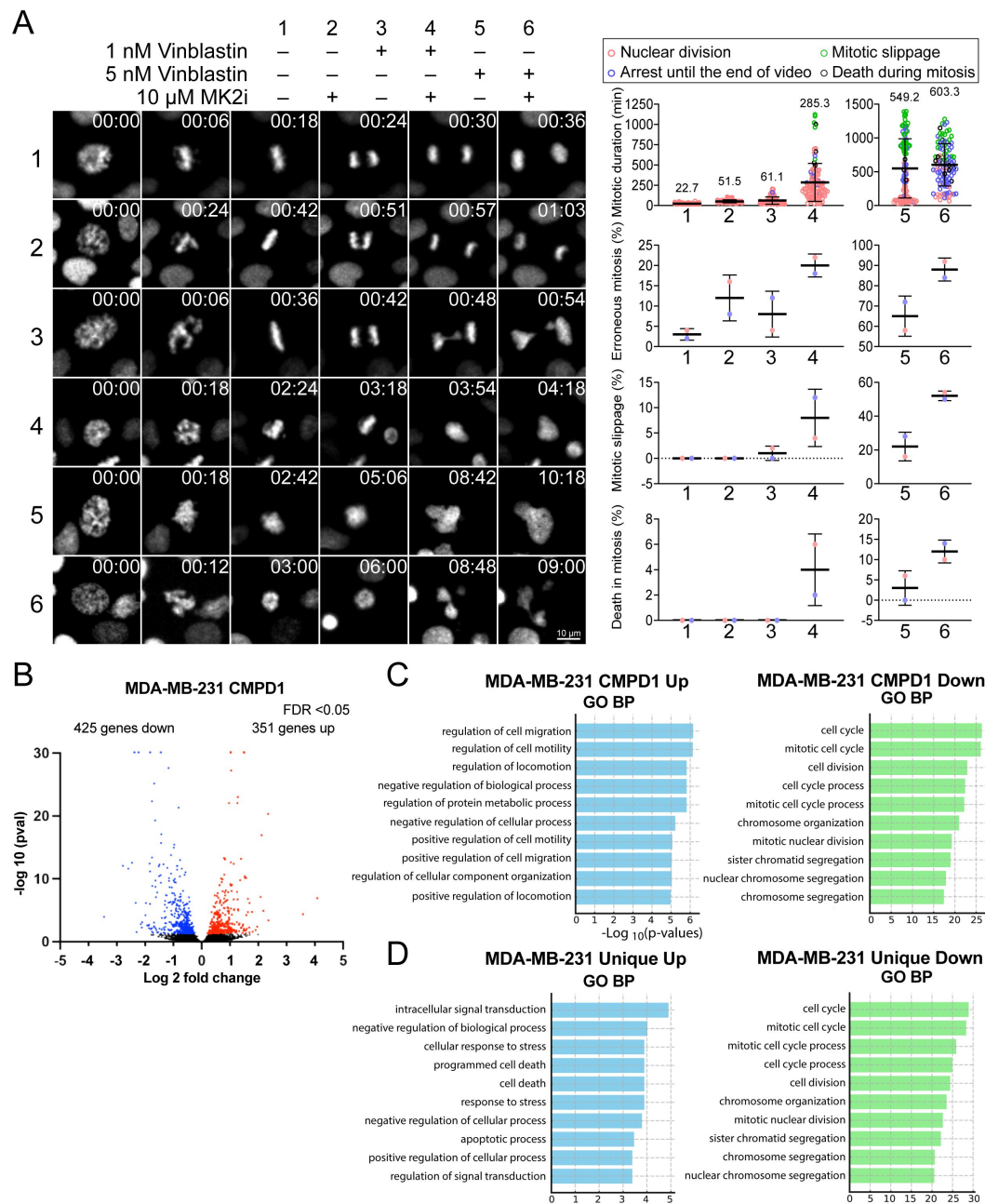


Figure 6.

MK2 inhibition enhances the efficacy of microtubule inhibitors in cancer cells

(A) Left: representative time-lapse images of mitotic CAL-51 cells treated with indicated combinations of drugs (10 μ M MK2i, 1 nM vinblastine, 5 nM vinblastine). Right: quantification of the mitotic duration, mitotic error rate, mitotic slippage rate, and the frequency of death in mitosis. The fate of mitotic cells was color-coded as indicated above the quantification plots. N = 100 cells pooled from two biological replicates for each condition. Time shown on the upper right corner of the representative images is indicated in minutes after NEBD. (B) Volcano plot displaying changes in gene expression following CMPD1 treatment in MDA-MB-231 cells. RNA-seq was conducted with three biological replicates. Differentially expressed genes (DEGs) are highlighted in red (up-regulated) and blue (down-regulated). (C) Pathway enrichment analysis of differentially expressed genes using the Gene Ontology (GO) Biological Processes (BP). Enrichment analysis was performed with the DAVID online tool. (D) Comparison of GO Biological Process enrichment analysis of DEGs unique to MDA-MB-231 cells relative to RPE1 cells. Genes uniquely up- or down-regulated in MDA-MB-231 cells, but not in RPE1 cells, were subjected to GO BP enrichment analysis.

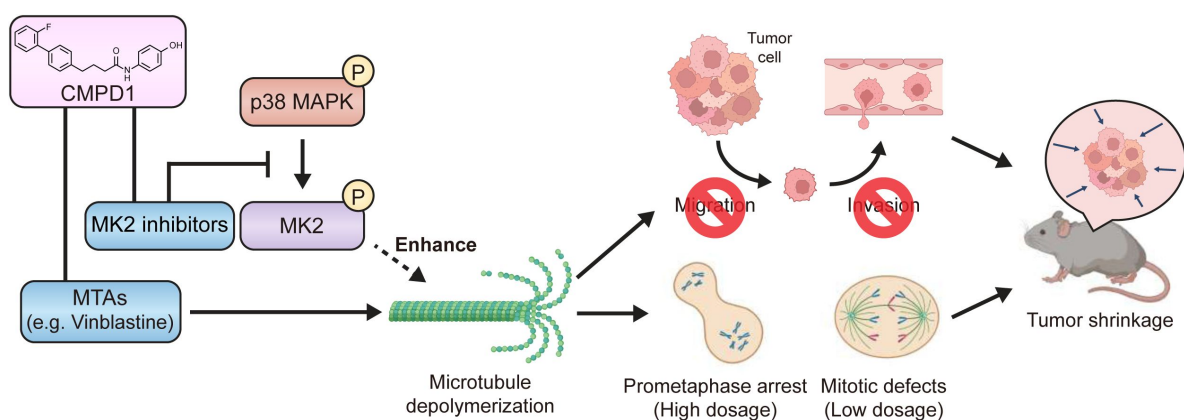


Figure 7.

The model of CMPD1-mediated cytotoxicity effects on cancer cells.

CMPD1 exhibits its tumor-specific cytotoxicity likely via two pathways. First, CMPD1 acts as a kinase inhibitor which prevents p38 MAPK-dependent phosphorylation of MK2, leading to the disruption of proper actin remodeling and spindle formation during mitosis. Second, CMPD1 serves as a MTA which can specifically induce depolymerization at the plus ends of microtubules. Both impaired actin reorganization and attenuated microtubule filament formation inhibit cancer cell migration and invasion, thereby preventing metastasis. On the other hand, failure of spindle assembly during mitosis causes the extended prometaphase arrest and the diminished mitotic fidelity, resulting in apoptosis of tumor cells and tumor shrinkage.

To better understand the impact of CMPD1 treatment on cancer cells at the gene expression level, we conducted RNA-seq analysis in MDA-MB-231 cells. When an FDR < 0.05 was applied using DESeq2 for differential gene expression analysis, 351 genes were found to be up-regulated, and 425 genes were down-regulated 24 hours after treatment with 10 μ M CMPD1 (**Fig. 6B** [↗](#), Fig. 6-Supplement Figure 4, and Fig. 6-Data file 1). Gene ontology (GO) biological process (BP) pathway enrichment analysis revealed that the most significantly enriched pathways in up-regulated genes relate to cell migration, while down-regulated genes are predominantly associated with mitosis and chromosome segregation (**Fig. 6C** [↗](#) and Fig. 6-Supplement Figure 4). To explore whether unique pathways were up- or down-regulated specifically in cancer cells, RNA-seq analysis was also performed on RPE1 cells. Comparing differentially expressed genes between MDA-MB-231 cells and RPE1 cells, cell death and apoptosis pathways were significantly enriched in genes uniquely up-regulated in MDA-MB-231 cells (**Fig. 6D** [↗](#) and Fig. 6-Data file 2). Genes specifically down-regulated in MDA-MB-231 cells were again enriched in pathways related to mitosis and chromosome segregation (**Fig. 6D** [↗](#)), consistent to the results that cancer cells are more sensitive to CMPD1 treatment (**Fig. 2** [↗](#)). Collectively, CMPD1 up-regulates cell death pathways while selectively down-regulates mitotic genes in cancer cells, highlighting its potent cancer cell specificity. The pivotal role of the p38-MK2 signaling pathway in enhancing the efficacy of microtubule destabilizers likely contributes to these observed alterations in gene expression.

Discussion

MTAs have been widely utilized as first- or second-line chemotherapy agents for various cancer; however, MTA-based treatment is often compromised by several limitations, including severe adverse effects and the development of drug resistance^{20–24} [↗](#). Consequently, the development of novel therapeutic strategies to enhance the effectiveness of MTAs remains critical for improving clinical outcomes in cancer patients. In this study, we propose that inhibiting the p38-MK2 pathway can synergize with MTAs to significantly enhance their therapeutic efficacy. We demonstrate this synergistic effect by using a combination of an MK2-specific inhibitor with the microtubule depolymerizer vinblastine, alongside CMPD1, a dual-target inhibitor that simultaneously disrupts both the MK2 signaling pathway and microtubule dynamics (**Fig. 6A** [↗](#)). Our findings reveal that CMPD1 exhibits a unique ability to depolymerize microtubules specifically at their plus-ends, as well as selectively inducing mitotic defects and altering gene expression profiles in cancer cells (**Fig. 2** [↗](#), **4D-H** [↗](#), and **6B-D** [↗](#)). Furthermore, CMPD1 exhibited potency comparable to or greater than PTX in inhibiting tumor growth both *in vitro* and *in vivo* (**Fig. 3** [↗](#)). In terms of tumor specificity, PTX at clinically relevant concentrations lacks cancer selectivity⁶⁰ [↗](#), whereas CMPD1, at similar low concentrations, specifically induces mitotic defects in breast cancer cells without affecting mitotic fidelity in non-transformed cells (**Fig. 2** [↗](#)). These findings suggest that p38-MK2 inhibition has the potential to enhance MTA efficacy, allowing for the improved tumor growth inhibition at a lower MTA concentration, thereby reducing the risk of side effects.

To gain a deeper understanding of CMPD1's cancer cell selectivity, we conducted RNA-seq to compare global gene expression profiles between MDA-MB-231 and RPE1 cell lines, both with or without CMPD1 treatment (**Fig. 6B** [↗](#)). CMPD1 treatment selectively up-regulated gene pathways associated with cell death, aligning with its observed higher cytotoxicity to cancer cells. The cancer-specific selectivity of CMPD1 may also be attributed to differential expression levels of p38 and MK2. Previous studies have reported that MK2 is overexpressed in multiple myeloma (MM) and has a potential to serve as a marker of poor prognosis^{68,69} [↗](#). Since MK2 facilitates the proliferation of MM cells by activating the Akt signaling pathway, depletion or inhibition of MK2 significantly impairs the growth of MM cells^{68,69} [↗](#). Furthermore, p38, MK2, and phospho-MK2 are markedly upregulated in primary tumors of various cancers and exhibit an inverse correlation with overall survival rates^{70–74} [↗](#). Therefore, cancer cells with elevated MK2 or phospho-MK2 expression are likely more sensitive to treatments involving MK2 inhibitors.

However, further studies are required to elucidate the detailed molecular mechanisms by which MK2 inhibitors enhance the efficacy of MTAs. Our study has demonstrated synergistic effects between MK2 inhibitors and MTAs, suggesting that the p38-MK2 pathway represents a promising therapeutic target in combination with MTAs for cancer chemotherapy.

Materials and Methods

Cell Culture

RPE1, MCF10A, CAL-51, MDA-MB-231, T47-D, and HeLa cells were originally obtained from ATCC. These cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco), DMEM/F12 (Gibco), Mammary Epithelial Cell Growth Medium (MEBM; Lonza) with MEGM kit (CC-4136, Lonza) and 100 ng/ml cholera toxin (Sigma, C8052), supplemented with 10% FBS (Sigma), 100U/ml penicillin and 100 mg/ml streptomycin (Gibco) at 37 °C in a humid atmosphere with 5% CO₂. CMPD1 (Abcam), Taxol (Sigma), MG132 (Sigma), and MPS1 inhibitor (SIGMA, AZ3146) were dissolved in DMSO (Sigma). For washout assay, cells were incubated with 2 μM CMPD1 for 4 hours, then washed by pre-warmed complete media for four times before starting live cell imaging. For the siRNA experiment shown in Figure 6-Supplement Figure 2, RNAiMAX (Invitrogen) and ON-TARGET plus SMART pool siRNA targeting MK2 (a mixture of four siRNAs; Horizon Discovery) were used according to the manufacturer's protocols⁷⁵.

Imaging

For time lapse image acquisition, 3D stacked images were obtained sequentially with 1 μm or 3 μm z steps for 12~15 μm total to cover entire mitotic cells using Nikon Elements software and a high-resolution Nikon Ti-2 inverted microscope equipped with a high-resolution Hamamatsu Flash V2 CMOS camera. The following objectives were used with above scope; 20x (NA 0.75 air), 40x (NA 1.25 silicon, for microtubule depolymerization in a cell), 60x (NA 1.4 oil), 100x (NA 1.4 oil). Images were takes by every 2 or 3 min interval for 24~72 hrs. Cells were grown on glass bottom dish with #1.5 coverslips and incubated in a Tokai Hit STX stage top incubator with PureBox Shiraito clean box (Tokai Hit). Feed-back control of Tokai Hit stage top incubator was used to stably maintain 37 °C at growth medium. All imaging was replicated at least three times. For fixed immunofluorescence experiments, cells were grown on high-precision #1.5 coverslips and imaged by a Nikon Ti-2 equipped with a Yokogawa SCU-W1-SoRa spinning disc confocal, Uniformizer, and a Hamamatsu Flash V3 CMOS camera. Quantitative image analysis was performed with Nikon Element (Nikon), MetaMorph (Molecular Devices), and Imaris (Bitplane) (See details in Image analysis section). No post-image processing was performed in all images. All imaging was replicated at least two to four times.

Image analysis

Mitotic durations were measured time between NEBD and anaphase onset or mitotic exit if cells did not exhibit anaphase onset such as CMPD1 treated cells. Histone H2B-EGFP or -mScarlet/RFP was used for determination of mitotic stages. Those images were analyzed using Nikon Element. Tubulin signal intensities on the spindle were measured using intensities in the custom region of interest (ROI). ROI was drawn onto entire cells in tubulin images, then additional ROI was drawn in cytoplasmic, which is avoided spindles. Second ROI was used for background correction. Similar equation was used in our previous study. Briefly, the integrated intensities from both ROIs were obtained, then the intensity of second ROI was divided by area and times the area of entire cells (first ROI). This value was used for corrections of background for the first ROI. For kinetochore signal measurement in **Figure 2B**⁷⁶, Integrated fluorescence intensity (minus local background (BG)) measurements were obtained for kinetochores as described previously⁷⁶. A 13 x 13 pixel region was centered on a fluorescent kinetochore to obtain integrated fluorescence, whereas a 15 x 15 pixel region centered on the 13 x 13 pixel region was used to obtain surrounding BG intensity.

Measured values were calculated by: F_i (integrated fluorescence intensity minus BG) = integrated intensity for 13 x 13 region – (integrated intensity for the 15 x 15 – integrated intensity for 13 x 13) x pixel area of the 13 x 13 / (pixel area of the 15 x 15 region – pixel area of a 13 x 13 region). Measurements were made with Metamorph 7.7 software (Molecular Devices) using Region Measurements⁷⁶. At least two or three independent replicates of measurements were performed. For cell migration and mobility analysis in **Figure 5A-B**, we obtained the center of nucleus by particle detection module in Imaris software (Oxford Instrument). The mobility or migration was tracked overtime and then the total length, displacements, and speed were also obtained by Imaris software.

***In vitro* microtubule dynamics assays**

Microtubules nucleated from GMPCPP-stabilized seeds were prepared and imaged as follows. A microtubule seed mixture was assembled from 50 μ M tubulin (7:1:2, unlabeled:488-labeled: biotin-labeled) in BRB80 (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.9), clarified by centrifugation at 90K rpm for 10 min, divided into 2 μ l aliquots, snap-frozen in liquid nitrogen, and stored at $-80^\circ C$. On the day of imaging, one of these aliquots was thawed, and to this, 0.4 μ l 10 mM GMPCPP (Jena Biosciences; 1 mM final) and 1.6 μ l BRB80 were added to bring the tubulin to a final concentration of 20 μ M. The reaction was incubated at $37^\circ C$ for 10 minutes (to promote polymerization) and stored at room temperature for up to 1 day. To prepare for an imaging session, 1 μ l of the polymerized biotinylated-seed mixture was diluted 100X (to 200 nM final) into 79 μ l BRB80 and 20 μ l 0.75% methylcellulose (dissolved in BRB80), both at room temperature (the mixture was pipetted up and down to shear seeds into smaller sizes). Flow chambers ($\sim$ 4-6 μ l in volume) were assembled by adhering plasma-cleaned and biotin/PEGylated glass coverslips (as described in a previous study⁷⁷) to glass slides using double-stick tape. Streptavidin (0.1 mg/ml; in BRB80) was introduced into the imaging chamber, after which the chamber was incubated at room temperature for 2-10 minutes. The chamber was washed with 10 μ l BRB80 supplemented with 1% Pluronic and incubated for $\sim$ 10-30 seconds, after which 10 μ l of the diluted biotinylated-seed mixture was introduced. The imaging chamber was then immediately placed on a microscope stage prewarmed to $37^\circ C$ to monitor seed density in the chamber (we did our best to ensure each chamber had a similar density of seeds). Chambers were washed with 10 μ l BRB80 supplemented with 1% Pluronic.

For the polymerization mix, a stock solution of 50 μ M tubulin (12.5:1, unlabeled: rhodamine-labeled tubulin) in BRB80 was prepared, clarified by centrifugation at 90K rpm for 10 min, divided into 20 μ l aliquots, snap frozen in liquid nitrogen, and stored at $-80^\circ C$. Immediately prior to imaging, one of these aliquots was thawed and placed on ice. A 10 μ l polymerization mix (final tubulin concentration, 15 μ M) was assembled using the following: 3 μ l 50 μ M tubulin (from above), 0.5 μ l 20 mM GTP, 0.5 μ l 20 mM β -mercaptoethanol, 2 μ l 0.75% methylcellulose, 0.5 μ l glucose oxidase/catalase mix (1.2 mg glucose oxidase + 28.1 μ l catalase + 99.1 μ l BRB80), 0.5 μ l 30% glucose, 1 μ l casein (from a 5% stock of Alkali-soluble Casein), and either 2 μ l BRB80 or 2 μ l of appropriate drug solution (20 μ M CMPD1, or 5 μ M vinblastine, each of which was prepared in BRB80). Note that all reagents were prepared or diluted in BRB80. The solution was gently mixed, warmed slightly between gloved fingertips for 10-20 sec, and then immediately added to the flow chamber with the adhered GMPCPP-stabilized microtubule seeds (as described above). Chambers were then incubated for 15 minutes on the microscope state at $37^\circ C$ (to approach steady-state), after which a freshly prepared polymerization mix with either tubulin only, or tubulin and a drug was added to the chamber and imaged for 15-20 minutes. For experiments in which a drug was added mid-way through the imaging period (see kymographs in **Figure 4D**), we did the following: after the initial 15-minute incubation with tubulin only, a fresh polymerization mix without drug was added, and a 20-minute movie was initiated. After 10 minutes, the movie was briefly paused during which another freshly prepared polymerization mix with drug (or tubulin only) was added to the chamber, and the acquisition was then continued.

Microtubule dynamics were imaged using total internal reflection fluorescence (TIRF) microscopy on an inverted Nikon Ti-E using a 1.49 NA 100X objective equipped with a Ti-S-E motorized stage (Nikon), piezo Z-control (Physik Instrumente), a motorized filter cube turret, a stage-top incubation system (LiveCell, Pathology Devices), and an iXon X3 DU897 cooled EM-CCD camera (Andor). We used 488 nm and 561 nm lasers (Nikon), a multi-pass quad filter cube set (C-TIRF for 405 nm/488 nm/561 nm/638 nm; Chroma), and emission filters mounted in a filter wheel (525 nm/50 nm, 600 nm/50 nm; Chroma). Images were acquired every 10 seconds. The microscope system was controlled by NIS-Elements software (Nikon), and images were analyzed using FIJI/ImageJ (NIH). Microtubule plus and minus ends were identified by exhibiting dynamics parameters distinct to each end (see kymographs in [Figure 4D](#) [↗](#) for examples).

Anchorage independent growth assay

6-well dish (Eppendorf) was used for anchorage independent growth assay. 2ml of autoclaved 1% agarose was used for bottom layer. After bottom layer became solid, 24×10^3 cells (MDA-MB-231 cells) were mixed into 2 ml of 0.8% agarose / media with or without CMPD1 or Taxol. After top layer gel became solid, cells were incubated in a humid atmosphere with 5% CO₂ for three weeks. 3 drops of complete media were supplied in each well every 3 days to avoid drying out. Cells were stained by 100 µg/ml of iodinitrotetrazolium chloride solution (Sigma) prior to taking photos.

Wound healing assay

H2B-mscarlet CAL-51 cells were plated in a 8-well chamber slide (ibidi, 80807). 24 hours after cell seeding, a stripe of cell-free zone (the wound) was manually created on a highly confluent monolayer of cells using a sterile pipette tip. The cell culture media was then immediately replaced with a new one containing the indicated drugs (DMSO, 0.1, 0.5, 1, and 10 µM CMPD1). Then, the cell locomotion dynamics was monitored with live-cell imaging at an interval of 30 min. To quantify the cell migration efficiency in each condition, we used an ImageJ macro to measure the distance between two boundaries of the wound. Briefly, the two boundaries of the wound were drawn and defined manually. Then, 40 lines perpendicular to the two boundaries were drawn automatically and the length of each line was calculated and shown. The distance of cell movement was calculated by subtracting the distance at 20 hr post drug treatment from the original distance (0 hr). Each condition was normalized to the control.

Trans-well migration assay

CytoSelect 24-well cell invasion assay kits (Cell Biolabs) or VitroGel Invasion assay kits (TheWell Bioscience) utilizing basement membrane-coated inserts were used according to the manufacturer's protocol. Following the overnight starvation of MDA-MB-231 or CAL-51 cells, the cells were seeded at 3.0×10^4 cells/well in the upper chamber and incubated with the media containing DMSO (control), CMPD1, vinblastine, MK2i, or vinblastine plus MK2i in the lower chamber for 24 or 48 hours. The invasive cells passing through the basement membrane layer were stained, and the absorbance of each well was measured at 560 nm after extraction.

Blood Invasion evaluation

All slides from mouse tumor with or without CMPD1 treatments were stained with HE and were screened for vascular invasion using strict criteria based on previous reports [78](#), [79](#) [↗](#). All slides were blindly evaluated by two investigators (MT and HY). All these vascular invasions were adopted only if they were picked up on HE staining. Vascular invasion was identified by tumor cells within vessels. The cases were categorized as blood vessel invasion-positive or negative. Typical histologic pictures of blood vessel invasion positive and negative by HE staining are shown in [Figure 5E](#) [↗](#).

RNA-seq

24 hours after 10 μ M CMPD1 treatment, RNAs were purified by the RNeasy Mini Kit (Qiagen). For MDA-MB-231 cells, RNA-seq library preparation was conducted with the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (LEXOGEN), and the libraries were sequenced on a NextSeq500 at Kazusa DNA Research Institute. In the case of RPE1 cells, sequencing libraries were prepared using the TruSeq Stranded mRNA Kit and sequenced on a NovaSeq 6000 at MacroGen Japan. Adapter sequences were removed from the raw sequencing data, and after adapter trimming, reads were mapped to the human reference genome (GRCh38) using the STAR aligner⁸⁰. Read counts for each gene were collected by featureCounts (version v1.6.4)⁸¹. Differentially expressed genes were identified with DESeq2⁸² using filtering thresholds of FDR < 0.05. Pathway enrichment analysis was performed by DAVID⁸³.

Tumor xenograft

CB17-Prkdc^{scid}/Jcl mice were used for establishment of orthotopic breast cancer model and therapy. This mice strain was purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were maintained under specific pathogen-free conditions at the Chiba University. All experimental procedures were performed in strict accordance with the National Institute of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Chiba University. For in vivo experiments, samples sizes were determined on the basis of knowledge of inter-tumor growth rate variability, gained from previous model-specific experience. MDA-MB-231 and CAL-51 cells (1×10^6 cells / 0.1 ml) in 1:1 PBS: Matrigel were subcutaneously injected into the mammary fat pad of female mice. The experiment was performed twice. The first experiment used MDA-MB-231 cells and the second experiment used both MDA-MB-231 and CAL-51 cells. In the first experiment, tumors were allowed to develop for 30 days, after which mice were randomly assigned to each treatment group, ensuring that baseline tumor volumes were balanced between treatment arms. The mice were treated with CMPD1 (i.p., 15 μ g/mouse/injection, 10 times for 3 weeks) or PTX (i.p., 5 mg/kg/injection, 10 times for 3 weeks). In the second experiment, treatment was started on the 10th day after transplantation, and the treatment drug was administered five times over 16 days until the 26th day. During the second experiment, weight was measured, blood was collected at the end of the study, and the liver and kidneys were also removed for further analysis. Both maximum and minimum diameters of the resulting tumors were measured every other day using a slide caliper. Tumor volumes were calculated as previously described. Mice were euthanized via CO₂ inhalation. The maximum tumor diameter permitted under the relevant animal protocols is 25 mm, and this limit was not exceeded in any experiment.

Biochemical analysis of animal models

Whole blood samples were collected from all mice and blood biochemistry determinations were performed with an Automatic Analyzer Model 7070 (Hitachi Co., Ltd., Tokyo, Japan). Parameters were aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transpeptidase (GGT), blood urea nitrogen (BUN) and creatinine (CRE) (Oriental Yeast Co., Ltd., Tokyo, Japan).

Hematoxylin and Eosin (HE) Staining

HE staining was performed using tissue samples obtained from the xenograft model. Tissue samples were thin sliced to a thickness of 4 μ m. For staining, slides were first stained with hematoxylin for 5 minutes, followed by eosin staining for 2 minutes. The slides were then dehydrated through a series of ethanol solutions, cleared in xylene, and mounted. The staining was evaluated based on the tissue morphology.

Giemsa Staining

Slides were fixed in absolute methanol for 30 seconds to facilitate cell attachment and preserve optimal staining characteristics, then allowed to air dry. The slides were immersed in Wright-Giemsa stain (container 1) for 60 seconds without agitation. For the Rapid Wright's One-Step Stain reagent, the staining duration was reduced to 15-30 seconds. Excess stain was removed by draining or blotting the edges of the slides, avoiding direct contact with the smear. The slides were then immersed in buffer solution (container 2) for 60 seconds (15-45 seconds for the Rapid Wright's reagent), followed by draining to remove excess buffer. Subsequently, the slides were dipped in rinse solution (container 3) for 2-10 seconds, with the Rapid Wright's reagent requiring quick dips for 25 seconds. Excessive buffering and rinsing were avoided to prevent decolorization. The slides were air-dried in a vertical position on a paper towel. For microscopic analysis, leukocytes were counted per field at low magnification, ensuring that fields were selected uniformly across specimens.

FACS

MDA-MB-231 cells were incubated with present or absent of CMPD1 for 24 hours. Cells were then twice washed by PBS. Approximately 2×10^6 cells were fixed by ice cold Ethanol for 16 hours. Samples were washed by cold PBS, and stained by PI (Sigma) containing Triton-X (Sigma), and DNase-free RNase A (Sigma) in PBS for 30 min. Then samples were measured by a flow cytometer.

Statistics

Statistical significance was determined using two tailed unpaired student's t-test for comparison between two independent groups or one way ANOVA for multipled comparison. Two-tailed T-test was performed for [Fig. 1C](#), [1G](#), [2A-B](#), [4A-B](#), [4E](#), and supplementary figs 4A, 4E-F, 6B-C, 6E, 7C-E. One way ANOVA (multiple comparison) was performed for [figs 1D](#), [2H-I](#), [3B](#), [3D](#), [3G](#), [4D](#), [5B](#), [5F-G](#), and Fig 3-Supplemen 2A. For TIRF experiments in [Figure 2 G-I](#), [p](#) values were calculated from Z scores (2G; as previously described in [Marzo et al., 2019](#); PMID 31364990), Mann-Whitney tests (2H-I, left and right), or by unpaired two-tailed Welch's t-tests (2H-I, middle). The latter two tests were selected as follows: the unpaired two-tailed Welch's t-test was used when the data sets were determined to be normal (by the D'Agostino and Pearson test for normality; $p > 0.05$). In the case where only one (or neither) was determined to be normal ($p < 0.05$), the Mann-Whitney test was used. For significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ were considered statistically significant. All quantifications were replicated at least two independent experiments.

Data availability

RNA-seq data generated in this study are available at Gene Expression Omnibus under Accession Number: GSE224462:

Go to <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE224462>

Acknowledgements

We thank Drs. Most S. Parvin, Ainslie Homan, Ozge Ali, Will Rossman, Terry Juang, Albert Wang, Masaharu Kasuya, Wang Junchao, Randy Owen, and Alex Li for help with data and image analysis. CAL-51 and CAL-51 p53 KO cells were kindly gifted from Drs. Beth Weaver, Mark Burkard, and Jennifer A. Pietenpol. Part of this work was supported by Wisconsin Partnership Program, Research Forward from the Office of the Vice Chancellor for Research (OVCR) and Wisconsin Alumni Research Foundation (WARF), start-up funding from University of Wisconsin-Madison

SMPH, UW Carbone Cancer Center, and McArdle Laboratory for Cancer Research, and NIH grant R35GM147525 and U54AI170660 (to A.S.), NIH P20GM104360 and UND School of Medicine & Health Sciences Dean's fund (to M. Takaku), JSPS KAKENHI 21K08638 (to M. Takada), R35GM130365 and NSF NSF1518083 (to J. G. DeLuca), and R35GM139483 and NSF1518083 (to S. Markus).

Additional information

Author Contributions

Y. Chen. performed most *in vitro* cell biology experiments and data analysis with guidance by A. Suzuki. M. Takada, M. Yu, H. Yamada, T. Nagashima, and M. Ohtsuka performed FACS, part of anchorage independent growth assay, mouse xenograft experiments, invasion assay, blood Invasion evaluation and related data analysis. M. Takada, A. Nagornyuk. and M. Takaku performed RNAseq experiments and data analysis. J. G. DeLuca and S.M. Markus performed *in vitro* microtubule dynamics experiments and data analysis. A. Suzuki conceptualized and supervised the entire project. M. Suzuki and Y. Chen drafted the manuscript, and M. Takada, S.M. Markus, J. G. DeLuca, and M. Takaku edited manuscript. All authors revised and contributed to the manuscript.

Additional files

Supplementary Figures [↗](#)

Supplementary Figure Legends [↗](#)

Supplementary Movie 1 [↗](#)

Supplementary Movie 2 [↗](#)

Supplementary Movie 3 [↗](#)

Supplementary Movie 4 [↗](#)

Supplementary Movie 5 [↗](#)

Figure 6 - Source data 1 [↗](#)

Figure 6 - Source data 2 [↗](#)

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Reviewer #1 (Public review):

In this paper, the authors reveal that the MK2 inhibitor CMPD1 can inhibit the growth, migration and invasion of breast cancer cells both in vitro and in vivo by inducing microtubule depolymerization, preferentially at the microtubule plus-end, leading to cell division arrest, mitotic defects, and apoptotic cell death. They also showed that CMPD1 treatment upregulates genes associated with cell migration and cell death, and downregulates genes related to mitosis and chromosome segregation in breast cancer cells, suggesting a potential mechanism of CMPD1 inhibition in breast cancer. Besides, they used the combination of an MK2-specific inhibitor, MK2-IN-3, with the microtubule depolymerizer vinblastine to simultaneously disrupt both the MK2 signaling pathway and microtubule dynamics, and they claim that inhibiting the p38-MK2 pathway may help to enhance the efficacy of MTAs in the treatment of breast cancer.

<https://doi.org/10.7554/eLife.104859.2.sa3>

Reviewer #2 (Public review):

Summary:

This study explores the potential of inhibiting the p38-MK2 signaling pathway to enhance the efficacy of microtubule-targeting agents (MTAs) in breast cancer treatment using a dual-target inhibitor.

Strengths:

The study identifies the p38-MK2 pathway as a promising target to enhance the efficacy of microtubule-targeting agents (MTAs), offering a novel therapeutic strategy for breast cancer treatment. The study also employs a wide range of techniques, especially live-cell imaging, to assess the microtubule dynamics in TNBC cells. The revised manuscript added new in vitro and in vivo evidence that further supported the conclusions.

Comments on revisions:

The authors have appropriately addressed all of my comments and concerns. Specifically, they performed additional in vitro experiments using MCF10A cells and p53 knockout cells to determine the IC50 of CMPD1. They also repeated the in vivo treatment experiment and evaluated the toxicity of the drug treatment in the CAL-51 model. Furthermore, they provided genetic evidence for the combination treatment. I'm satisfied with the revision and have no further major comments. Minor comment: make sure the name of the chemo drug shown in Fig. 3 is consistent.

<https://doi.org/10.7554/eLife.104859.2.sa2>

Reviewer #3 (Public review):

Summary:

The authors demonstrated MK2i could enhance the therapeutic efficacy of MTAs. With the tumour xenograft and migration assay, the author suggested that the p38-MK2 pathway may serve as a promising therapeutic target in combination with MTAs in cancer treatment.

Strengths:

The authors provided a potential treatment for breast cancer.

Comments on revisions:

A xenograft experiment should be included to evaluate the synergistic effect of MK2i and vinblastine.

<https://doi.org/10.7554/eLife.104859.2.sa1>

Author response:

The following is the authors' response to the original reviews

Public Reviews:

Reviewer #1 (Public review):

In this paper, the authors reveal that the MK2 inhibitor CMPD1 can inhibit the growth, migration, and invasion of breast cancer cells both in vitro and in vivo by inducing microtubule depolymerization, preferentially at the microtubule plus-end, leading to cell division arrest, mitotic defects, and apoptotic cell death. They also showed that CMPD1 treatment upregulates genes associated with cell migration and cell death, and

downregulates genes related to mitosis and chromosome segregation in breast cancer cells, suggesting a potential mechanism of CMPD1 inhibition in breast cancer. Besides, they used the combination of an MK2-specific inhibitor, MK2-IN-3, with the microtubule depolymerizer vinblastine to simultaneously disrupt both the MK2 signaling pathway and microtubule dynamics, and they claim that inhibiting the p38-MK2 pathway may help to enhance the efficacy of MTAs in the treatment of breast cancer. However, there are a few concerns, including:

(1) What is the effect of CMPD1 on breast cancer metastasis?

In this study, we hypothesized that the MK2 signaling pathway could synergize with microtubule-targeting agents (MTAs) to enhance anti-cancer efficacy. We utilized CMPD1 as a potent dual-function inhibitor, targeting both MK2 and microtubule dynamics. By simultaneously inhibiting these pathways, CMPD1 not only shows the therapeutic impact of MTAs, but also significantly suppresses breast cancer cell migration and invasion. Therefore, we propose that CMPD1, through its dual inhibition of MK2 activity and microtubule dynamics, may offer enhanced specificity and efficacy in preventing breast cancer metastasis and limiting tumor progression.

(2) The mechanism is lacking as to how MK2 inhibitors enhance the efficacy of MTAs.

Thank you for the valuable suggestion. We agree that our current findings do not fully elucidate the underlying mechanism by which MK2 inhibition synergistically enhances the efficacy of MTAs. We recognize this as an important area for further investigation and are committed to exploring the molecular interplay between MK2 signaling and microtubule dynamics in future studies. A deeper mechanistic understanding will be critical to establishing a strong rationale for the potential co-treatment of MK2 inhibitors and MTAs in clinical breast cancer therapy.

Reviewer #2 (Public review):

Summary:

This study explores the potential of inhibiting the p38-MK2 signaling pathway to enhance the efficacy of microtubule-targeting agents (MTAs) in breast cancer treatment using a dual-target inhibitor.

Strengths:

The study identifies the p38-MK2 pathway as a promising target to enhance the efficacy of microtubule-targeting agents (MTAs), offering a novel therapeutic strategy for breast cancer treatment. In addition, the study employs a wide range of techniques, especially live-cell imaging, to assess the microtubule dynamics in TNBC cells.

We sincerely appreciate your recognition of the significance and impact of our work.

Weaknesses:

The study primarily uses RPE1 cells as the control for normal cells, which may not fully capture the response of normal mammary epithelial cells. While CMPD1 is shown to be effective in suppressing tumor growth in MDA-MB-231 xenograft, the study lacks detailed toxicity data to confirm its safety profile in vivo.

Thank you for your valuable suggestions. In the revised manuscript, we have included CMPD1 treatment in MCF10A cells, a more appropriate non-transformed control line commonly used in breast cancer research. Notably, MCF10A cells exhibited results similar to

those observed in RPE1 cells, further reinforcing our conclusion that breast cancer cells display increased sensitivity to CMPD1 treatment. These new findings are presented in Figure 2-Supplement 1A-C. Additionally, we performed further xenograft experiments using CAL-51 and MDA-MB-231 cells. We collected data on tumor growth, mouse body weight, survival rates, and other relevant parameters to comprehensively assess toxicity. The newly obtained results are presented in Figure 3F-G and Figure 3-Supplement 1-3.

Reviewer #3 (Public review):

Summary:

The authors demonstrated MK2i could enhance the therapeutic efficacy of MTAs. With Tumor xenograft and migration assay, the author suggested that the p38-MK2 pathway may serve as a promising therapeutic target in combination with MTAs in cancer treatment.

Strengths:

The authors provided a potential treatment for breast cancer.

Thank you for recognizing the importance and significance of our work.

Weaknesses:

(1) In Figure 2, the authors used a human retinal pigment epithelial-1 (RPE1) cell line to show that breast cancer cells are more sensitive to CMPD1 treatment. MCF10A cells would be suggested here as a suitable control. Besides, to compare the sensitivity, IC50 indifferent cell lines should be measured.

In the revised manuscript, we have addressed these points by determining the IC50 values for CMPD1 in MDA-MB-231, CAL-51, MCF10A, and CAL-51 p53 knockout cells. These new results are presented in Figure 2-Supplement Figure 3.

(2) The data of MDA-MB-231 in Figure 1D is not consistent with CAL-51 and T47D, also not consistent with the data in Figures 2B-C.

In the revised manuscript, we have included all relevant statistical analyses in Figure 1D. In MDA-MB-231 cells, there are no statistically significant differences in mitotic duration between 1 μ M and 5 μ M, 5 μ M and 10 μ M, or 1 μ M and 10 μ M CMPD1 treatments. Similarly, no significant differences are observed between 1 μ M and 5 μ M or 5 μ M and 10 μ M CMPD1 treatments in CAL-51 cells, and between 5 μ M and 10 μ M in T-47D cells. These results suggest that mitotic duration does not exhibit a clear dose-dependent relationship within the 1–10 μ M range, likely because mitotic arrest has reached a near-plateau effect at these concentrations.

It is also important to note that the experimental conditions in Figures 1 and 2 are fundamentally different. Figure 1 investigates the effects of higher concentrations of CMPD1 (≥ 1 μ M), which severely disrupt microtubule organization and result in robust mitotic arrest, with cells arrested in mitosis for over 8 hours. In contrast, the conditions in Figure 2 utilize much lower concentrations of CMPD1 (10–50 nM), which are insufficient to cause complete microtubule depolymerization, but are capable of inducing a subtle yet statistically significant mitotic delay, particularly in breast cancer cell lines. These lower concentrations were chosen to mimic clinically relevant intratumoral drug levels. Previous studies have reported that paclitaxel (PTX) concentrations in patient tumors approximate ~50 nM when modeled in vitro. At these physiologically relevant levels, PTX does not induce strong mitotic arrest but instead causes moderate delays that result in division errors and chromosomal instability, ultimately contributing to cancer cell death. In this study, the conditions used in

Figure 2 emulate these clinically relevant concentrations for CMPD1. We found that, similar to PTX, low-dose CMPD1 induces a slight but significant mitotic delay without triggering a full mitotic arrest. Notably, unlike PTX, CMPD1 appears to exert this effect selectively in breast cancer cells, contributing to mitotic errors and potentially enhancing therapeutic efficacy through targeted chromosomal instability.

(3) To support the authors' conclusion in Figure 5, an additional animal experiment performed by tail vein injection would be helpful.

While current technical limitations have precluded us from conducting this suggested experiment in this study, we have performed complementary xenograft studies using CAL-51 cells treated with CMPD1. These experiments included a comprehensive toxicity analysis. Furthermore, we carried out an in vitro migration assay using CAL-51 cells under combined treatment with the MK2 inhibitor and vinblastine. These additional findings are presented in Figure 3–Supplement 1–3 and Figure 6–Supplement 3. We recognize the importance of the suggested tail vein injection approach and are actively pursuing further mechanistic studies, including this experiment, in our ongoing and future work.

(4) Page 14, to evaluate the combination result of MK2i and vinblastine, an in vivo animal assay must be performed.

We appreciate the reviewer's valuable suggestion. We are actively investigating the synergistic mechanisms between the MK2 inhibitor and microtubule-targeting agents (MTAs). In future studies, we plan to extend our findings by conducting xenograft experiments to further evaluate their therapeutic potential in vivo.

(5) The authors used RNA-seq to show some pathways affected by CMPD1. What are the key/top genes that were affected? How about the mechanism?

In the revised manuscript, we have included the top 20 upregulated and downregulated genes identified from RNA-seq analysis using MDA-MB-231 cells. This new data is presented in Figure 6-Supplement Figure 4. Gene Ontology (GO) Biological Process (BP) pathway enrichment analysis revealed that the most significantly enriched pathways among upregulated genes are associated with cell migration, whereas the downregulated genes are primarily involved in mitosis and chromosome segregation. These transcriptional changes are consistent with the phenotypic outcomes observed in our experiments, supporting the functional relevance of CMPD1 treatment. However, further investigation will be necessary to elucidate the detailed molecular mechanisms underlying these effects.

(6) Line 127, more experiments should be involved to support the conclusion.

In the revised manuscript, we have addressed this point by performing additional experiments, including determination of the IC₅₀ values of CMPD1 in MDA-MB-231, CAL-51, MCF10A, and CAL-51 p53 knockout cells. We also conducted live-cell imaging analyses using MCF10A cells. These new results further reinforce our conclusion that breast cancer cells are more sensitive to CMPD1 treatment than normal breast epithelial cells, and that this sensitivity is independent of p53 status. The new data are presented in Figure 2-Supplement Figures 1 and 3.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

(1) Figure 1D: As the concentration of CMPD1 increased, the mitotic duration of MDA-MB-231 cells decreased, why was that?

Although there appears to be a slight decrease in mitotic duration with increasing concentrations of CMPD1, our quantitative analysis reveals no statistically significant differences among the 1 to 10 μM treatment groups in MDA-MB-231 cells. In the revised manuscript, we have included all relevant statistical analyses in Figure 1D for clarity. Importantly, all CMPD1-treated groups exhibit a pronounced and statistically significant prolongation of mitosis compared to the DMSO-treated control. While the average mitotic duration in control cells is approximately 30 minutes, cells exposed to 1–10 μM CMPD1 consistently display mitotic durations exceeding 8 hours, indicating a strong and sustained mitotic arrest across this concentration range.

Reviewer #2 (Recommendations for the authors):

(1) The rationale for using RPE1 as normal cell control instead of normal mammary epithelial cells as control is unclear. Using normal mammary epithelial cells such as MCF10A for the study is recommended.

Thank you for this valuable suggestion. In the revised manuscript, we have included additional experiments using non-transformed mammary epithelial MCF10A cells. The new data, presented in Figure 2-Supplement Figures 1 and 3, include both IC₅₀ measurements and live-cell imaging analyses. These results further support our conclusion that breast cancer cells are significantly more sensitive to CMPD1 treatment compared to normal mammary epithelial cells.

(2) It is intriguing that CAL-51 cells are more sensitive to CMPD1 than MDA-MB-231 cells; examining how p53 signaling changes in these cells would be worthwhile.

We appreciate this insightful comment. In the revised manuscript, we have measured the IC₅₀ values for both CAL-51 and CAL-51 p53 knockout (p53KO) cells. The results show no significant difference in CMPD1 sensitivity between the two, suggesting that the enhanced sensitivity of CAL-51 cells is independent of p53 status. These new findings are presented in Figure 2—Supplement Figure 3.

(3) Figures S1A and B are not described and cited in the main text.

We apologize for this oversight. In the revised manuscript, we have correctly cited and described Figures S1A and B (Figure 2-Supplement Figure 2 A-B in revised manuscript) in the main text.

(4) I'm not that convinced by the conclusion made from Lines 201-204. First, Figure S2C, which is the growth of tumor volume, does not reflect the toxicity of the drug treatment. No additional data evaluating the toxicity (such as body weight change) under the regimen was shown. Second, although the tumor weight by the endpoint indicated some anti-tumor effect in the MDA-MB-231 xenograft model, the tumor volume does not show the same pattern (the dot lines do not well distinguish which group from which). I would suggest repeating the in vivo experiment using CAL-51 cells since it is more sensitive to CMPD1 according to the previous data.

Thank you for this thoughtful and constructive feedback. In the revised manuscript, we have addressed these concerns through several additional experiments. We performed new xenograft studies using CAL-51 TNBC cells, in parallel with further toxicity-focused analyses in the MDA-MB-231 model. Consistent with previous results, CMPD1 treatment significantly suppressed tumor growth in CAL-51 xenografts (Figure 3F-G), further supporting its efficacy in a more sensitive cell line. To evaluate drug-associated toxicity, we measured body weight changes throughout the course of treatment. CMPD1-treated mice maintained a comparable

weight gain to the control group, whereas mice treated with paclitaxel (PTX) showed significantly reduced body weight (Figure 3-Supplement Figure 2A). Notably, animal deaths occurred only in the PTX-treated groups in both MDA-MB-231 and CAL-51 models (Figure 3-Supplement Figure 2B). We also assessed organ toxicity, including both anatomical and functional evaluations of the kidney and liver, and observed no significant damage in CMPD1-treated mice (Figure 3-Supplement Figures 3A-B and 3D). Furthermore, white blood cell (WBC) counts remained stable in the CMPD1 group, while PTX treatment led to a significant reduction (Figure 3-Supplement Figures 3C-D). These additional data provide strong evidence for the anti-tumor efficacy and lower toxicity of CMPD1 *in vivo*.

(5) While I appreciate the combination effect of treating cells with the MK2 inhibitor with vinblastine. I would consider using genetic knockdown as a complementary approach to demonstrate that inhibiting the p38-MK2 pathway synergized with microtubule depolymerizing agents. In addition, could inhibition of the p38-MK2 pathway alone induce the cell growth inhibition observed with CMPD1 treatment?

Thank you for these important suggestions. In the revised manuscript, we have incorporated siRNA-mediated knockdown of MK2 in combination with vinblastine treatment. This genetic approach revealed synergistic effects on mitotic index and mitotic errors, closely mirroring the phenotypes observed with pharmacological co-treatment using the MK2 inhibitor and vinblastine (Figure 6-Supplement Figure 2A-C). These results further validate the role of the p38-MK2 pathway in modulating mitotic progression in the presence of MTAs. To address whether MK2 inhibition alone is sufficient to impair cell growth, we performed validation experiments using the MK2 inhibitor at 10 μ M. At this concentration, the inhibitor effectively blocked phosphorylation of Hsp27, a major downstream substrate of MK2, under H₂O₂-induced ROS stress conditions (Figure 6-Supplement Figure 1A-B), confirming MK2 signaling pathway inhibition. However, treatment with the MK2 inhibitor alone did not significantly affect cell proliferation, as shown by a 4-day growth curve analysis in CAL-51 cells (Figure 6-Supplement Figure 1C). These findings suggest that inhibition of the p38-MK2 pathway alone is not sufficient to suppress cancer cell growth, and that its synergistic interaction with MTAs, such as vinblastine, is essential for the observed anti-proliferative effects.

(6) Phenotypic studies (such as anchorage-independent growth and cell migration and invasion assay) of combining MK2 inhibitor with vinblastine in TNBC cells are recommended.

Thank you for this valuable suggestion. In the revised manuscript, we have conducted cancer cell migration assays using CAL-51 TNBC cells treated with control, MK2 inhibitor alone, vinblastine alone, or the combination of both. Our results demonstrate that the combination treatment significantly enhances the inhibition of cell migration compared to either agent alone (Figure 6-Supplement Figure 3A-C). These findings provide additional phenotypic evidence supporting the synergistic interaction between MK2 inhibition and microtubule-targeting agents in TNBC cells.

Reviewer #3 (Recommendations for the authors):

The authors can utilize diverse experiments to support their conclusions.

Thank you for this important suggestion. In the revised manuscript, we have conducted a series of additional experiments to robustly support our conclusions.

These include:

(1) Xenograft studies using CAL-51 TNBC cells, along with comprehensive toxicity evaluations.

- (2) CMPD1 sensitivity analysis in non-transformed MCF10A mammary epithelial cells.
- (3) IC50 measurements in MDA-MB-231, CAL-51, CAL-51 p53 knockout, and MCF10A cells.
- (4) Cell migration assays assessing the combination effects of MK2 inhibitor and vinblastine
- (5) siRNA-mediated genetic knockdown of MK2 to complement pharmacological findings

Collectively, these additional data sets substantially strengthen the evidence base for our conclusions and provide a more comprehensive mechanistic understanding.

<https://doi.org/10.7554/eLife.104859.2.sa0>