K858, a Novel Inhibitor of Mitotic Kinesin Eg5 and Antitumor Agent, Induces Cell Death in Cancer Cells

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Abstract

The aim of this study was to investigate the mechanism of inhibition of Eg5 (kinesin spindle protein), a mitotic kinesin that plays an essential role in establishing mitotic spindle bipolarity, by the novel small molecule inhibitor K858. K858 was selected in a phenotype-based forward chemical genetics screen as an antimitotic agent, and subsequently characterized as an inhibitor of Eg5. K858 blocked centrosome separation, activated the spindle checkpoint, and induced mitotic arrest in cells accompanied by the formation of monopolar spindles. Long-term continuous treatment of cancer cells with K858 resulted in antiproliferative effects through the induction of mitotic cell death, and polyploidization followed by senescence. In contrast, treatment of nontransformed cells with K858 resulted in mitotic slippage without cell death, and cell cycle arrest in G2 phase in a tetraploid state. In contrast to paclitaxel, K858 did not induce the formation of micronuclei in either cancer or nontransformed cells, suggesting that K858 has minimal effects on abnormalities in the number and structure of chromosomes. K858 exhibited potent antitumor activity in xenograft models of cancer, and induced the accumulation of mitotic cells with monopolar spindles in tumor tissues. Importantly, K858, unlike antimicrotubule agents, had no effect on microtubule polymerization in cell-free and cell-based assays, and was not neurotoxic in a motor coordination test in mice. Taken together, the Eg5 inhibitor K858 represents an important compound for further investigation as a novel anticancer therapeutic. [Cancer Res 2009;69(9):OF1–9]

Introduction

Antimitotic agents such as the taxanes and Vinca alkaloids are clinically important chemotherapeutic drugs (1). These drugs function by binding to tubulin and blocking cell cycle progression at mitosis through the disruption of microtubule dynamics and activation of the spindle checkpoint, which ultimately result in cell death (2–4). Tubulin is an essential cytoskeletal protein that is important for cell division, cell shape, motility, and intracellular transport. Therefore, antimicrotubule agents also cause peripheral neuropathy as an adverse event by interfering with microtubule-based axonal transport (5). Antimitotic agents that target components of the mitotic machinery other than microtubules have been of great interest as new generation anticancer drugs (6, 7).

Many molecules involved in the regulation of the mitotic checkpoint or mitotic progression have been described in the past 15 years. Mitotic kinesins and kinases are important regulators of mitotic progression, and are under active investigation as antitumor drug targets. In particular, Eg5, a key molecule involved in the formation of bipolar spindles (8), is one of the most attractive target enzymes in antimitotic drug discovery, and several chemotypes of small molecule Eg5 inhibitors have been reported (9–16). The Eg5 inhibitors S-trityl-l-cystein (10), CK0106023 (11), ispinesib (12), KSP-IA (13), and MK-0731 (14) were identified in a biochemical screen for inhibitors of the ATPase activity of Eg5. Monastrol (15) and HR22C16 (16) were selected using a phenotype-based screen for antimitotic agents and were subsequently identified as Eg5 inhibitors. As another example, Plk1 (17) is a serine/threonine kinase that has multiple roles in mitosis (18) and highly expressed in malignant cells. Several Plk1 inhibitors have been identified using not only enzyme-based but also cell-based assays (17). Unlike enzyme-based biochemical assays, cell-based assays have several advantages, including increased efficiency in the selection of cell-permeable small molecules and the capacity to identify small molecules with several modes of action (19, 20).

Cell cycle checkpoints prevent the transition from one phase of the cell cycle to the next until all the processes of the current phase have been properly executed. Defects in checkpoint function result in the accumulation of genetic mutations and chromosomal damage. The spindle assembly checkpoint inhibits the activity of the anaphase-promoting complex and blocks the transition from metaphase to anaphase until all of the duplicated chromosomes are aligned at the metaphase plate, and bipolar attachment to the mitotic spindle is achieved, thus ensuring the accurate segregation of sister chromatids during cell division (21). Impairment of spindle checkpoint function results in premature mitosis and missegregation of chromosomes, leading to aneuploidy (22). Recent observations have suggested that cells with spindle checkpoint abnormalities are insensitive to antimicrotubule agents (23, 24). We also showed that long-term prometaphase arrest (~10 hours) induced by spindle checkpoint activation is required for cancer cells to undergo mitotic death when treated with DNA damaging...
agents (25). Therefore, an alternative mechanism that induces metaphase arrest by spindle checkpoint activation without affecting microtubules organization might provide a new therapeutic strategy.

We report here the identification and characterization of K858, a novel Eg5 inhibitor. K858 caused mitotic arrest accompanied by monopolar spindles through the inhibition of Eg5, and induced cancer cell death in vitro and in vivo. K858 preferentially induced cell death in cancer cells compared with nontransformed cells, underscoring its potential importance as a molecule for further investigation as a novel Eg5 inhibitor.

Materials and Methods

Reagents. K858 was synthesized at Kyowa Hakko Kirin. Paclitaxel and vincristine was purchased from Sigma. Monastrol, Adriamycin, and carboplatin were purchased from ChemBridge, Wako, and Bristol-Myers Squibb, respectively.

Cells. The human colorectal carcinoma cell line HCT116 and its isogenic derivative, HCT116 p53−/−, which lacks p53, were kindly provided by Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). The human ovarian cancer cell line A2780 was kindly provided by Dr. T. Tsuruo (Japanese Foundation for Cancer Research, Tokyo, Japan). ARPE-19 nontransformed human retinal pigment epithelial cells were purchased from American Type Culture Collection.

Caspase activity assay. Cells were seeded in 96-well plates and treated with K858. Caspase-3 activity was assayed by release of 7-amino-4-trifluoromethyl-coumarin (AFC) from Acetyl-Asp-Glu-Val-Asp-AFC substrate peptide (Peptide Institute) as previously described (26).

Cell proliferation assay. Cells were seeded in 96-well plates and treated with K858. Cell viability was assessed by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT; Cell Proliferation kit II; Roche Diagnostics Basel) assay following the manufacturer’s instruction.

Figure 1. K858 induces mitotic arrest, apoptosis, and cell growth inhibition and has no effect on microtubule polymerization. A, chemical structure of K858. B, HCT116 cells were treated with K858. The mitotic index was determined by fluorescence microscopic analysis of Hoechst 33342 stained nuclear morphology (left). Caspase 3 activation was measured using the DEVDase assay (middle). Viable cells were quantified using an XTT assay (right). Points, means (n = 3); bars, SD. C, effect on microtubule polymerization in a cell-free system. The microtubule polymerization assay was carried out in the presence of GTP (left) or in the absence of GTP (right). D, effect on interphase microtubule networks. HCT116 cells treated with vehicle, paclitaxel, vincristine, or K858 for 18 h were stained for α-tubulin (red). DNA was visualized with TOTO-3 (blue).
Antitumor Activity of Eg5 Inhibitor K858

Immunofluorescence analysis. Cells were fixed with cold methanol at −20°C for 5 min, followed by permeabilization with 0.2% Triton X-100 in PBS, or fixed with 3.7% formaldehyde/PBS. After incubation with 1% fetal bovine serum in PBS, cells were incubated with anti-α-tubulin antibody (Sigma), followed by incubation with Alexa Fluor 546–conjugated anti-mouse IgG (Molecular Probes) or FITC-conjugated anti-mouse IgG (Amersham Pharmacia). For analysis of Mad2 and γ-H2AX, cells were incubated overnight with anti-Mad2 antibody (27) and anti-γH2AX antibody (Trevigen), respectively, followed by incubation with FITC-conjugated anti-rabbit IgG (Molecular Probes) or 50 μg/ml propidium iodide (Sigma). The cells were analyzed with either a LSM510 META confocal laser scanning microscope (Carl Zeiss) or a FV300 confocal laser scanning microscope (Olympus).

Senescence-associated β-galactosidase staining. Cells grown on culture dishes were washed in PBS and fixed for 5 min in a solution of 2% formaldehyde/0.2% glutaraldehyde. Fixed cells were washed with PBS and then incubated at 37°C overnight with a senescence-associated β-galactosidase staining solution, according to the manufacturer’s instructions (Cell Signaling).

In vivo tumor xenograft studies. A2780 cells (5 × 10⁶ cells) were inoculated s.c. into BALB/cAJcl-nu mice. K858 was administered orally twice daily on days 0 to 4, and 7 to 11 at 150 and 50 mg/kg. Doses and schedules were determined by the tolerability studies performed in advance. Vehicle (0.5% methylcellulose 400) was administered orally as a control twice daily on days 0 to 4, and 7 to 11. Paclitaxel was administered i.v. on day 0 at 25 mg/kg. Carboplatin was administered i.v. on day 0 at 60 mg/kg. Drug efficacy was expressed as the ratio of the mean experimental V/V₀ value to that of the control group [treated versus control (T/C) ratio], where V is the tumor volume at the day of evaluation and V₀ is the tumor volume at the day of the initial treatment with the drug. Statistical analysis was performed using the nonparametric Wilcoxon rank-sum test.

Rota-rod test. BALB/c mice were treated with paclitaxel (25 mg/kg) i.v. at day 1, K858 (100 mg/kg) orally on days 1 to 5, or vehicle as a control. Rota-rod testing was performed twice a week. The rod was set in motion at a constant speed (6 rpm), and the mice were placed onto individual sections of the apparatus. Performance was measured as the time that elapsed between the animal being placed on the rod and falling off the rotating rod, with 180 s as the cutoff. Statistical analysis was performed using the nonparametric Wilcoxon rank-sum test.

Results

Identification of small molecules that induce mitotic arrest in the absence of microtubule binding. We have developed a screening program to identify novel antimitotic drugs that do not have microtubule-binding properties. Using a morphology-based forward chemical genetic screen, we have identified several small molecule inhibitors of cell division. One of the putative antimitotic agents is K858 (Fig. 1A), a thiadiazoline derivative unlike many other previously reported antimitotic agents (28, 29). K858 induced mitotic arrest, caspase-3 activation, and cell growth inhibition in HCT116 cells (Fig. 1B). Next, we examined whether K858 had an inhibitory or stimulatory effect on microtubule polymerization using a cell-free system. In the presence of GTP, compared with vincristine, K858 had no effect on microtubule polymerization even at 100 μmol/L (Fig. 1C). Similarly, in the absence of GTP, K858 had no effect on polymerization, although paclitaxel clearly enhanced microtubule polymerization. We also evaluated the effect on the interphase microtubule network. K858, unlike paclitaxel or vincristine, had no detectable effect on microtubule formation in interphase cells even at 100 μmol/L (Fig. 1D). These results indicated that K858 has no effect on microtubule dynamics.

K858 selectively inhibits Eg5. To identify the target of K858, we investigated the mechanism of mitotic arrest induced by K858...
using immunocytochemistry to analyze the mitotic phenotype of cells. Unlike vincristine or paclitaxel, K858 blocked centrosome separation and induced the formation of a monopolar spindle with a round-shaped chromosomal alignment during mitosis (Fig. 2A). This characteristic phenotype was also obtained when cells were treated with Eg5 siRNA (Supplementary Fig. S1) or monastrol, a known inhibitor of Eg5. To determine whether K858 affected the enzymatic activity of Eg5, we analyzed microtubule-stimulated Eg5 ATPase activity. K858 inhibited the ATPase activity of Eg5 with an IC50 of 1.3 μmol/L (Fig. 2B). By comparison, monastrol inhibited Eg5 less effectively, with an IC50 of 11 μmol/L. Kinetic analysis of the inhibition of Eg5 revealed that K858, as well as monastrol, inhibit the ATPase activity of Eg5 in an ATP-uncompetitive manner (data not shown). When we examined effect of K858 on other kinesins, even at 200 μmol/L, K858 failed to inhibit the ATPase activity of the mitotic kinesins CENP-E and MKLP1, or the conventional kinesin heavy chain (Fig. 2C). These results suggested that K858 is an ATP-uncompetitive inhibitor of Eg5 that is at least 15-fold more selective for Eg5 than other members of the kinesin superfamily.

**K858 induces mitotic arrest and growth inhibition through the activation of the Mad2-mediated spindle checkpoint.** To determine the effect of K858 on the spindle checkpoint, we analyzed the subcellular distribution of Mad2, an essential component of the spindle checkpoint. Mad2 localized to the kinetochore in K858-treated cells, which suggested that K858 induces prometaphase arrest through the activation of the spindle checkpoint (Supplementary Fig. S2A). Next, we pretreated HCT116 cells with Mad2 siRNA, or luciferase siRNA as a control, and then exposed siRNA-treated cells to K858. K858-induced mitotic arrest and growth inhibition were suppressed by pretreatment with Mad2 siRNA compared with luciferase siRNA (Supplementary Fig. S2B and S2C). These results suggested that K858-induced mitotic arrest and growth inhibition are due to the activation of the Mad2-dependent spindle checkpoint.

**Figure 3.** K858 induces mitotic cell death in cancer cells but not in normal cells. A, p53+/+ HCT116 cells were treated with K858 and then analyzed by time-lapse microscopy (top). Most cells underwent cell death directly from mitosis (arrow), whereas some underwent mitotic slippage to survive without cell division (arrowhead). The indicated cell lines were treated with K858 for 48 h (bottom). Using time-lapse video recordings, the duration of mitotic arrest, defined as the length of time cells maintained a round shape, was measured (n = 20 individual cells). B, the indicated cell lines were treated with K858 for the indicated times. Cells stained with propidium iodide to label the DNA were analyzed by flow cytometry. C, the indicated cell lines were treated continuously with K858 and then analyzed by flow cytometry, as described for B, with the exception that floating cells were removed by washing before analysis, and only adherent living cells were collected (top). The indicated cell lines were treated continuously with K858 for 7 d and then subjected to senescence-associated β-galactosidase staining (bottom). D, ARPE-19 cells treated with K858 (5 μmol/L, 12 h) or Adriamycin (500 ng/mL, 18 h) were stained for γ-H2AX (green). DNA was visualized with propidium iodide (red). PI, propidium iodide.
K858 induces mitotic cell death in cancer cells but not in normal cells. We next characterized the mitotic events using time lapse differential interference contrast microscopy. K858-treated HCT116 cells underwent cell cycle arrest at metaphase, which was sustained for >10 hours in most cells (Supplementary Video S1; Fig. 3A and B). These results indicated that the spindle checkpoint is properly activated in these cells. After this period of arrest (~10 hours), a large proportion of cells underwent cell death directly, while in mitotic phase (Supplementary Video S1 and S2; Fig. 3A and C). However, a small number of cells escaped mitotic cell death and underwent mitotic slippage without cytokinesis to become tetraploid G1 cells (Supplementary Video S1 and S2; Fig. 3A). When we examined p53-null HCT116 cells, mitotic cell death after transient mitotic arrest was also observed, similar to wild-type HCT116 cells (Supplementary Video S3; Fig. 3A and B). Similarly, in HeLa cells, in which p53 is efficiently degraded by HPV-18 E6, most of the cells arrested at metaphase and then underwent mitotic death after treatment with K858 (data not shown). These results indicated that the p53 status of the cell does not affect the induction of mitotic arrest and mitotic death by K858. As shown by time lapse microscopy, K858-treated cells generally failed to divide into two daughter cells due to a failure of cytokinesis, which suggested that the growth-inhibitory activity of K858 is due to both the inhibition of cell division and induction of mitotic death. The treatment of ARPE-19 cells, which are nontumorigenic and nontransformed retinal pigment epithelial cells, with K858 rarely resulted in cell death of mitotic cells, although cells arrested at metaphase, similar to HCT116 cells (Supplementary Video S4 and S5; Fig. 3A and B). These results suggested that K858 preferentially induces mitotic cell death in cancer cells but not in nontransformed cells.

K858-treated cancer cells become polyploid after mitotic slippage and undergo senescence. Treatment of cancer cells with K858 resulted in the emergence of a small number of tetraploid cells, due to mitotic slippage after mitotic arrest. To determine the fate of these tetraploid cells, we analyzed the effect of long-term exposure to K858. In the presence of K858, within 5 days, p53-null HCT116 cells entered a new round of DNA synthesis without cell division to become octaploid and hexadecaploid cells (Fig. 3C). Interestingly, a considerable number of wild-type HCT116 cells also slowly became octaploid and hexadecaploid (Fig. 3C), which suggested that the postmitotic checkpoint that induces cell cycle arrest of tetraploid cells in G1 is impaired in these cells, even in the presence of intact p53. Upon continued K858 treatment, hexadecaploid cells stopped synthesizing DNA, and many cells underwent cell death, independent of p53 status (data not shown). The remaining viable giant cells became senescent, as detected by senescence-associated β-galactosidase staining (Fig. 3C). Thus, continuous treatment with K858 resulted in the inhibition of proliferation of cancer cells through the induction of mitotic cell death, or polyploidization followed by senescence. K858 treatment of ARPE-19 cells also resulted in mitotic slippage and cell cycle arrest in G1 phase in a tetraploid state (Fig. 3B). However, tetraploid ARPE-19 cells never progressed through the cell cycle, most likely because the postmitotic checkpoint function was intact in these cells. Upon further analysis, we observed that the cells did not move for a long period of time and, after 7 days of K858 treatment, were positive for senescence-associated β-galactosidase, which suggested that most of the cells underwent senescence with a 4N DNA content (Fig. 3C). Polyploidization is often followed by centrosome amplification, and is believed to be the cause of unequal cell division and aneuploidy. Therefore, in nontransformed cells, an intact postmitotic checkpoint and the induction of senescence play a critical role in preventing chromosomal instability. Importantly, K858 did not induce double-strand DNA breaks in ARPE-19 cells, as assessed by immunofluorescence staining with anti-γ-H2AX antibodies (Fig. 3D), which suggested that the G1 cell cycle arrest observed in K858-treated tetraploid ARPE-19 cells was not due to the activation of a DNA damage-induced checkpoint. Thus, K858 exhibited three advantageous properties in nontransformed cells: it did not induce DNA damage, and did not result in the formation of polyploid or aneuploid cells.

K858-treated cells show more normal nuclear morphology than paclitaxel-treated cells. In contrast to antimicrotubule agents, which often induce the formation of multipolar spindles and a scattered chromosomal distribution in metaphase-arrested cells (Fig. 4A), K858 treatment resulted in the formation of monopolar spindles with a round-shaped chromosomal alignment (Fig. 4A). The nuclear morphology of cells that survived K858 treatment was also strikingly different from that of paclitaxel-treated cells. Similar to previous results, paclitaxel treatment induced abnormal chromosomal segregation and micronuclei formation (Fig. 4B). However, cells treated with K858 did not exhibit fragmentation of the nuclei, and became mononuclear tetraploid cells (Fig. 4B). Thus, even in cancer cells, K858 treatment seemed to prevent chromosome gain and loss, and ploidy changes caused by incidental and unequal cell division.
K858 exhibits antitumor activity and induces mitotic arrest in a human tumor xenograft mouse model. We evaluated antitumor activity of K858 in an A2780 ovarian cancer xenograft model. Treatment of mice with 150 mg/kg K858 suppressed tumor growth, with a minimum T/C ratio (T/Cmin) of 0.052, and no overt evidence of toxicity (P > 0.05 for body weight loss compared with controls; Fig. 5A and B). Treatment with 50 mg/kg K858 resulted in significant growth delay, and a T/Cmin of 0.35. The T/Cmin ratios for paclitaxel and carboplatin were 0.21 and 0.67, respectively. In a HCT116 colon cancer xenograft model, at a dose of 100 mg/kg twice a day orally for 5 days, K858 also exhibited antitumor activity, with a T/Cmin of 0.35. By comparison, a single dose of oxaliplatin (9 mg/kg) administered i.v. resulted in a T/Cmin of 0.59 (data not shown). These results showed that under these experimental conditions, the antitumor activity of K858 in nude mice is similar to or more potent than other widely used antitumor drugs.

To investigate whether K858 inhibited Eg5 function in the A2780 xenograft model, we examined the phenotype of mitotic cells in tumor tissues. Vehicle or K858 was administered orally twice at a 7-hour interval. Analysis of tumor tissue revealed that K858 induced the accumulation of mitotic cells with monopolar spindle phenotypes in a dose-dependent manner (Fig. 5C and D). Taken together, these results indicated that K858 causes mitotic arrest accompanied by monopolar spindles through the inhibition of Eg5, and has antitumor activity in xenograft tumor models.

K858 does not display neurotoxic side effects. Neurotoxicity associated with antimitotic agents, such as paclitaxel, can be assessed by measuring motor coordination in mice using a rota-rod test (30, 31). To compare the effect on motor coordination of paclitaxel and K858, we chose doses and schedules of paclitaxel (25 mg/kg, i.v., single dose) and K858 (100 mg/kg, p.o., qdx5) based on the results of the xenograft experiments. Paclitaxel induced a significant (P < 0.01) impairment in coordination in the rota-rod test on days 7, 8, and 14, with no significant (P > 0.05) changes in body weight change (Fig. 6A). In contrast, there was no significant difference (P > 0.05) in performance between K858-treated mice and the control group (Fig. 6B). These results suggested that at the effective dose for antitumor activity in vivo, K858 does not exhibit neurotoxicity.

Discussion

The mitotic process is highly dynamic, and any compound that alters or inhibits this process is of potential interest as an
anticancer agent (6, 7). The clinical success of the taxanes clearly validates microtubules as excellent anticancer targets. However, antimicrotubule drugs cause adverse events, such as peripheral neuropathy, due to interference with microtubule-based axonal transport. Therefore, there is an urgent need for new small molecules with antimitotic activity that do not affect microtubule dynamics.

In the current study, we performed a phenotype-based screen and identified the novel Eg5 inhibitor K858. Unlike antimicrotubule drugs, K858 had no effect on microtubule polymerization. K858 selectively inhibited Eg5 ATPase activity with an IC₅₀ of 1.3 μmol/L. Like other Eg5 inhibitors, K858 induced mitotic arrest without affecting the interphase microtubule network, supporting the hypothesis that K858 is primarily active in dividing tissues, and spares nondividing cells (e.g., neurons) from adverse events. In fact, mice treated with K858 did not exhibit significant impairment in motor coordination compared with control mice in the rota-rod test, which has been widely used to assess the potential for neurotoxicity (30, 31).

Time-lapse microscopy of K858-treated cells showed that, similar to antimicrotubule drugs, K858 induces cell death in cells that are undergoing prolonged mitotic arrest. As expected, because Eg5 is required for the formation of a bipolar spindle, inhibition of Eg5 function by K858 resulted in a characteristic monoastral phenotype. The generation of a monoastral spindle results in a rosette-like distribution of condensed chromosomes and mitotic arrest, due to the activation of the spindle assembly checkpoint. There are contradictory reports regarding the role of the spindle assembly checkpoint in mediating cell death triggered by antimitotic drugs. Although most studies report that checkpoint activation is required for cell death triggered by paclitaxel or Eg5 inhibitors (2–4, 13), some suggest that cell death is checkpoint independent (32, 33). Although the precise reason for this reported variation remains poorly understood, the requirement of a functional spindle checkpoint for drug-induced cell death may be due to a variety of different factors. In the current study, K858 induced mitotic arrest and growth inhibition, both of which were dependent on the activation of Mad2-mediated spindle assembly checkpoint.

The similarities in the cellular responses to K858 and antimicrotubule drugs indicated that cell death induced by these agents is triggered mainly through mitotic arrest and checkpoint activation, and not other effects of changes in microtubule dynamics. Interestingly, we found that nontransformed ARPE-19 cells treated with K858 rarely underwent cell death, regardless of checkpoint integrity. This difference in sensitivity between tumor and nontransformed cells to K858 is likely to be due to the different levels of oxidative stress that causes cell death. Recent studies have shown that paclitaxel induces early reactive oxygen species production in cancer cells, and paclitaxel-induced cancer cell death in vitro and in vivo involves the production of hydrogen peroxide (34–36). In general, tumor cells exhibit altered basal antioxidant defense levels. It has been shown that tumor cells with higher total antioxidant capacity are more resistant to paclitaxel than those with lower total antioxidant capacity (35). Of note, we recently found that intracellular reactive oxygen species levels are elevated during mitosis, even under normal culture conditions.⁸ Therefore, one possible explanation for the current results is that prolonged mitotic arrest induced by K858 treatment results in

Figure 6. K858 does not exhibit neurotoxic side effects. A and B, effect of paclitaxel and K858 on motor coordination, as assessed by the rota-rod test, in mice. Points, mean (n = 10); bars, SD. **, P < 0.01.

⁸ In preparation.
continuous exposure to oxidative stress and cell death, which is preferentially induced in tumor cells compared with normal cells. Cells that escaped K858-induced mitotic death after mitotic arrest eventually progressed into G1 with tetraploid genomes, due to a failure to divide. In general, the replication of DNA in tetraploid cells is blocked by a p53- and pRb-dependent checkpoint, termed the postmitotic checkpoint (37). In cancer cells, checkpoint function is frequently impaired, and tetraploid cells can undergo a new round of DNA synthesis to become polyploid. In the current study, we found that to varying degrees, upon long-term incubation with K858, cancer cells entered a new round of DNA synthesis regardless of p53 status, whereas normal cells with tetraploid genomes never entered the next cell cycle (Fig. 3). Continued exposure to K858 induced senescence in both cancer cells with hyperploid genomes and normal cells with tetraploid genomes. In fact, it was previously reported that paclitaxel also suppresses tumor cell growth by induction of senescence (38). Thus, K858 seems to exert its antitumor effects through the induction of cancer cell death during mitotic arrest or senescence after mitotic exit.

K858 exhibited several advantageous properties compared with DNA-damaging agents or antimitotubule drugs. Unlike DNA-damaging agents, K858 did not induce double strand DNA breaks (Fig. 3), nor did it induce fragmentation of the nuclei in cells that survived treatment. This was quite distinct from the effects of paclitaxel treatment, which included the formation of micronuclei and multilobular nuclei (Fig. 4). Because the genomic plasticity afforded by aneuploidy could facilitate the emergence of protumorigenic changes in gene dosage and accelerate the accumulation of oncogenes and loss of tumor suppressor genes, these results are promising indicators that K858 confers a lower risk of chemotherapy-related secondary cancer development.

In summary, inhibitors of the mitotic kinesin Eg5 represent promising new alternatives to antimitotubule drugs. Similar to microtubule-targeted drugs, Eg5 inhibition leads to mitotic arrest and cell death (10, 11). The chemical structure of K858 is different from many previously identified Eg5 inhibitors (9–16), and our data indicate that it preferentially exerts antitumor effects while preserving normal cells, thus potentially inducing fewer adverse events. In support of this, K858 had no effect on microtubule organization and did not induce DNA damage, aberrant chromosome segregation, or aneuploid formation. Furthermore, similar to the HR22C16 analogue (39), K858 exhibited no cross-resistance in paclitaxel-resistant cancer cells (Supplementary Table S1). Therefore, K858 is a potentially safer and more effective new approach for cancer therapy. Given its promising preclinical attributes, derivatives of K858 could be important new drugs for clinical testing.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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