Centmitor-1, a Novel Acridinyl-Acetohydrazide, Possesses Similar Molecular Interaction Field and Antimitotic Cellular Phenotype as Rigosertib, ON 01910.Na


Abstract
Mitosis is an attractive target for the development of new anticancer drugs. In a search for novel mitotic inhibitors, we virtually screened for low molecular weight compounds that would possess similar steric and electrostatic features, but different chemical structure than rigosertib (ON 01910.Na), a putative inhibitor of phosphoinositide 3-kinase (PI3K) and polo-like kinase 1 (Plk1) pathways. Highest scoring hit compounds were tested in cell-based assays for their ability to induce mitotic arrest. We identified a novel acridinyl-acetohydrazide, here named as Centmitor-1 (Cent-1), that possesses highly similar molecular interaction field as rigosertib. In cells, Cent-1 phenocopied the cellular effects of rigosertib and caused mitotic arrest characterized by chromosome alignment defects, multipolar spindles, centrosome fragmentation, and activated spindle assembly checkpoint. We compared the effects of Cent-1 and rigosertib on microtubules and found that both compounds modulated microtubule plus-ends and reduced microtubule dynamics. Also, mitotic spindle forces were affected by the compounds as tension across sister kinetochores was reduced in mitotic cells. Our results showed that both Cent-1 and rigosertib target processes that occur during mitosis as they had immediate antimitotic effects when added to cells during mitosis. Analysis of Plk1 activity in cells using a Förster resonance energy transfer (FRET)-based assay indicated that neither compound affected the activity of the kinase. Taken together, these findings suggest that Cent-1 and rigosertib elicit their antimitotic effects by targeting mitotic processes without impairment of Plk1 kinase activity. Mol Cancer Ther; 13(5); 1054–66. ©2014 AACR.

Introduction
Mitosis has been a target of anticancer therapies for decades. The earliest mitosis-perturbing drugs were antitubulin agents such as taxanes and vinca alkaloids, and their derivatives (1, 2). These drugs inhibit microtubule assembly or disassembly dynamics by targeting tubulin subunits, the building blocks of microtubules. Microtubules undergo major rearrangements during mitosis: within a short period of time, interphase microtubules are disassembled and reassembled to form the mitotic spindle apparatus that is needed for ordered chromosome segregation and exit from M phase. Tubulin-targeting drugs interfere with these processes and impair normal spindle function and chromosome alignment at the metaphase plate (3, 4). This leads to persistent activity of the spindle assembly checkpoint (SAC; ref. 5), typically resulting in a long-lasting mitotic arrest and ultimately cell death, a phenomenon that possesses therapeutic value. Since the discovery of tubulin-targeting agents, several novel antimitotic compounds have been developed to specifically target mitotic kinases and motor proteins (for reviews see refs. 6, 7). These agents are expected to reduce side effects, such as neurotoxicity, associated with antitubulin drugs (1, 2) and provide new therapeutic opportunities for the treatment of cancer pending on their successful clinical validation.

One interesting anticancer compound that is currently in phase II and III clinical trials for the treatment of blood malignancies and solid tumors (8–10) is rigosertib (ON 01910.Na) developed by Onconova. Rigosertib functions by inducing multipolarity, mitotic arrest, and subsequent cell death (11). The mechanism of action of rigosertib was initially reported to occur via the inhibition of polo-like kinase 1 (Plk1; ref. 11), an important mitotic regulator that is necessary for spindle assembly, chromosome alignment,
and cytokinesis (12, 13). Because Plk1 is often overexpressed in cancer cells and its inhibition lowers tumor cells’ viability (14), it is considered to be a promising drug target (15). In addition to targeting Plk1, rigosertib has been reported to show activity against phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (11, 16, 17). However, the mechanism of action of the compound is not completely understood.

Here, we designed and executed a high-throughput screen (HTS) to identify low molecular weight (LMW) compounds that possess similar steric and electrostatic features but different chemical structure as rigosertib. In this study, we characterize a novel LMW compound, an acridinyl-acetohydrazide (C_{22}H_{16}BrN_{3}O_{3}), here termed as Cent-1 (Cent-1). We analyze and compare the effects of Cent-1 and rigosertib on different aspects of mitosis and microtubule dynamics.

Materials and Methods

Chemicals

Cent-1 (ChemBridge Corporation; 5676127) was used at 5 μmol/L concentration and rigosertib (ON 01910.Na; Selleck Chemicals; S1362) at 250 nmol/L concentration unless stated otherwise. Nocodazole (Sigma; M1404) was used at 0.5 to 3 μmol/L, taxol (Paclitaxel; Sigma; T7191) at 0.1 to 0.6 μmol/L, thymidine (Sigma; T9250) at 2 mmol/L, insulin (Sigma; 19278) at 100 ng/mL, vinblastine (Sigma; V1377) at 3 μmol/L, staurosporine (Sigma; S5921) at 1 μmol/L, ZM447439 (Tocris Bioscience; 2458) at 5 μmol/L, MG132 (Sigma; C2211) at 10 to 20 μmol/L, monastrol (Sigma; M8515) at 100 μmol/L, dimethylastoron (Alexis Biochemicals; ALX-270-438) at 5 μmol/L, BI2536 (Selleck Chemicals; S1109) at 200 nmol/L, and wortmannin (Tocris Bioscience; I232) at 0.2 to 1 μmol/L concentration.

Cell culture

HeLa cells (ATCC CCL-2, obtained 2006) were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, penicillin–streptomycin (0.1 mg/mL), l-glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), HEPES (20 mmol/L), and nonessential amino acids (0.1 mmol/L). HeLa cells expressing H2B-GFP and mCherry tubulin were a kind gift from Stephan Geley’s laboratory at the University of Amsterdam, the Netherlands, described in 18, and were cultured in DMEM with GlutaMAX (Invitrogen) supplemented with 6% heat-inactivated FBS and 1% penicillin–streptomycin. No authentication of the cell lines was done by the authors.

For analyzing SAC activity by Western blot, HeLa cells were synchronized with thymidine to G_{1}–S border (cells were incubated with 2 mmol/L thymidine for 19 hours, followed by 8-hour release and second thymidine block for 16 hours) and released into 5 μmol/L Cent-1 or 0.1 μmol/L taxol for 12 hours. ZM447439 (20 μmol/L) was added to mitotic cells for 2 hours before cell harvest for Western blotting. For analyzing PI3K/AKT pathway, HeLa cells were starved in serum-free medium for 16 hours, treated with dimethyl sulfoxide (DMSO), 5 μmol/L Cent-1, 250 nmol/L rigosertib or 1 μmol/L wortmannin for a total of 3 hours and 100 ng/mL insulin for 30 minutes.

Virtual HTS

Compound structures for the virtual screen were collected from four different vendors: Chembridge (ChemBridge Corporation), Chemical Diversity, Tripos (Tripos International), and Micro Source. Altogether 65,000 compound structures were used in the virtual HTS. Brutus and Almond softwares were used for the superimposition of the compound library against the template compound rigosertib, and for similarity searches using three-dimensional (3D) structure and molecular interaction fields as search criteria. All compounds were built using the vendor-provided sdf files and Sybyl ligand-preparation utilities with default settings.

Cell-based screen

HeLa cells were plated on 384-well plates with Multidrop Combi (Thermo Fisher Scientific) and screen compounds were added to the cells 24 hours after plating using Hamilton Microlab Star robotics (Hamilton) at 0.2 or 5 μmol/L final concentrations. Eg5 inhibitor monastrol was used as a positive control compound that induces mitotic arrest, and DMSO served as a negative control. Cells were imaged live with phase contrast optics at 6 and 24 hours after treatment followed by fixation and DNA staining.

Immunofluorescence

Cells were fixed for 15 minutes with 2% paraformaldehyde in 60 mmol/L Pipes, 25 mmol/L Heps, 10 mmol/L EGTA, 4 mmol/L MgSO_{4} (PHEM) containing 0.5% Triton-X-100. For imaging microtubules, 0.2% glutaraldehyde was included in the fix. For EB1 immunofluorescence, cells were fixed in ice-cold MeOH for 10 minutes, and rehydrated in PBS. Cells were washed with 10 mmol/L MOPS, 150 mmol/L NaCl, and 0.05% Tween 20 (MBST), blocked in MBST containing 20% boiled normal goat serum for 1 hour at room temperature, and stained with antibodies for 1 hour at room temperature. Primary antibodies included mouse anti-Bub1 (Upstate; 05-899), rabbit anti-BubR1 (Proteinatlas), mouse anti-BubR1 (Abcam; ab4637), mouse anti-centpA (Abcam; ab13939), mouse anti-CETN3 (Abnova; H00001070-M01), human autoimmune serum (Crest; Antibodies Inc.), mouse-anti-EB1 EA3 (kind gift from Prof. Gary Gorbsky, Oklahoma Medical Research Foundation, Oklahoma City, OK), mouse anti-NuMA (kind gift from Prof. Markku Kallajoki, University of Helsinki, Laboratory of René Medema, the Netherlands Cancer Institute, Amsterdam, the Netherlands), described in 18, were cultured in DMEM with GlutaMAX (Invitrogen) supplemented with 6% heat-inactivated FBS and 1% penicillin–streptomycin. No authentication of the cell lines was done by the authors.
University of Jyväskylä, Jyväskylä, Finland, mouse anti-
Plk1 (Abcam; 17057), rabbit anti-pericentrin (Abcam; 
ab4448), rat anti-α-tubulin YL-1/2 (Abcam; ab6160), 
mouse anti-α-tubulin DM1A (Abcam; ab7291), and 
mouse anti-γ-tubulin (Abcam; ab11316). Secondary anti-
bodies were Alexa Fluor 488, 555, and 647 dyes against 
mouse, rabbit, rat, and human antigens (Invitrogen). DNA 
was stained with DAPI (4,6-diamidino-2-phenylindole). 
Coverslips were washed with H2O and mounted on 
microscope slides with Vectashield mounting medium 
(Vector Laboratories; H-1000).

Microscopy
Zeiss inverted 200M microscope (Zeiss GmbH) 
equipped with MetaMorph software (Molecular Devices) 
was used for analyzing 384-well plates and fixed cells on 
coverslips. Kinetochore intensities were quantified with 
Metamorph from maximum projections created from a 
Z-stack of images acquired every 0.5 μm. Zeiss Axiovert 
200M microscope equipped with spinning disk CSU22 
confocal scanner (Yokogawa) and SlideBook 5.0 software 
(Intelligent Imaging Innovations, Inc.) was used for micro-
tube dynamics measurements and for the analysis of 
kinetochore distances. ImageJ was used for image proces-
sing and quantification of pole fragmentation. An area 
was drawn around each centrosome so that all centrosome 
fragments that were seen adjacent to main centrosomes 
were included inside the region of interest. Only bipolar 
cells were chosen for the analysis. The parameters mea-
sured were area, aspect ratio (AR = major axis divided by 
minor axis), and roundness (formulas available at http:// 
rsbweb.nih.gov/ij/docs/menus/analyze.html).

In vitro tubulin polymerization assay
Fluorescence-based tubulin polymerization assay (Cyto-
skeleton Inc.; BK011P) was used to determine the effects of 
Cent-1 on tubulin polymerization in vitro. The assay was 
performed as described by the manufacturer. DMSO, taxol, 
and vinblastine were used as controls. Tubulin polymeri-
zation was recorded every minute for 60 minutes by Victor 
1420 Multilabel HTS Counter (PerkinElmer).

Determination of microtubule dynamicity
Microtubule dynamicity measurements were performed 
in live A549 cells stably expressing EGFP-α-tubulin 
(BD Biosciences; Clontech) using the spinning disk 
confocal microscope with 100× oil objective. Measure-
ments were performed 1 to 2 hours after compound 
addition on cells. Z-stacks containing four focus levels 
with 0.3-μm step size were acquired every 10 seconds 
during a filming session that lasted for 250 seconds. The 
plus-ends of microtubules were digitally marked in the 
recorded time-lapse movies to allow measurement of their 
dynamicity. Dynamicity refers here to the combined 
growth and shrinkage velocities of the microtubules, 
which were determined as a measurement of the distance 
that the microtubule plus-end traveled in a specific period 
of time. Ten to 20 microtubules per cell and 5 to 8 cells per 
sample were analyzed in each of three independent 
experiments. Microtubule dynamics data were analyzed 
using SlideBook 5.0 and Graph Pad Prism 4 (GraphPad 
Software, Inc.).

FRET for analysis of Plk1 kinase activity
U2OS cells stably expressing a Plk1-responsible FRET-
based probe (18) were monitored on a DeltaVision Spec-
tris Imaging system (Applied Precision), using a NA 0.75 
20/0 oil objective. Cells were imaged at 37°C in Liebowitz-
15 medium supplemented with 6% heat-inactivated FBS 
and 1% penicillin-streptomycin. Images were acquired 
every 20 minutes and processed using ImageJ (http://rsb. 
info.nih.gov/ij/). Imaging and quantification of FRET 
ratio was performed as described previously (19).

Results
Discovery of Cent-1, an antimitotic LMW compound
To identify small-molecule analogs of rigosertib 
(Fig. 1A), we performed a ligand-based virtual HTS, in

Figure 1. Comparison of rigosertib and Cent-1 structures. A, structure of the template compound rigosertib (ON 01910.Na). B, hit compound N-(3-bromo-4- 
hydroxybenzylidine)-2-(9-oxo-10(9H)-acridinyl)acetohydrazide named Cent-1. C, Brutus-aligned structures of rigosertib (blue ball-and-stick model) and 
Cent-1 (gray/turquoise stick model) are shown. Brutus score of 1.62 indicates exceptionally high level of similarity in 3D structure and electrostatic potential 
between the two compounds. Oxygens are colored red and sulfur yellow.
which 65,000 LMW compounds were screened for those that have similar interaction fields and 3D structures as the template compound. Virtual screening was conducted using Almond and Brutus (20–22), field-based molecular alignment, and virtual screening tools that take into account both steric and electrostatic features of the molecules. One criterion was that the compounds should not have similar chemical structures as the template compound. Therefore, compounds having similar backbone structures as rigosertib were omitted from the hit list. The 200 top-ranking compounds from each hit list were purchased and subjected to secondary cell-based screens, in which HeLa cells were treated with the compounds and analyzed for mitotic phenotypes. In total, 4 hit compounds resulted in a notable increase in mitotic index and were investigated further.

One of the highest scoring compounds was an acridinyl-acetohydrazide (C_{22}H_{16}BrN_{3}O_{3}) that we named as Cent-1 (Fig. 1B) due to its effects on centrosome morphology. The field-based similarity score of Cent-1 in comparison with rigosertib was 1.62, which is very high (the maximum is 2.0), but less than 0.01% of random compound pairs will reach a value higher than 1.5 according to in-house tests), indicating that these molecules closely resemble one another in their 3D structure and charge distribution. The superimposition image of Cent-1 and rigosertib is shown in Fig. 1C. Besides the high similarity of Cent-1 to rigosertib in 3D comparisons, the compound induced a strong antimitotic phenotype that resembled the cellular effects of rigosertib. For these reasons, Cent-1 was selected for more detailed analyses.

**Cent-1 induces a transient mitotic arrest followed by abnormal exit from M phase or cell death**

To analyze the impact of Cent-1 on cell-cycle progression, the compound (5 μmol/L) was applied on cycling HeLa cells that were subsequently filmed with IncuCyte live-cell imager using 1-hour image capture interval (Fig. 2A). The fates of 60 individual mitotic cells were determined for each treatment condition (Fig. 2B). DMSO served as a negative control, and nocodazole and taxol as mitotic arrest inducing positive controls. DMSO-treated cells progressed normally through mitosis (the average duration of mitosis was 1.5 ± 1.8 hours). All nocodazole- and taxol-treated cells arrested in mitosis for several hours before they underwent cell death (average time spent in mitosis before cell death was 14.3 ± 5.4 and 16.7 ± 5.9 hours, respectively). Almost half of Cent-1–treated cells (48.3%) exhibited a long M-phase arrest followed by cell death (average time spent in mitosis before cell death was 14.3 ± 5.9 hours). Rest of the cells exhibited a mitotic delay before they exited mitosis with (38.3%) or without (13.3%) cytokinesis. Cells often died postmitotically soon after the aberrant exit from mitosis (result not shown). For comparison, we determined the fate of rigosertib–treated cells. The majority of rigosertib-treated HeLa cells died in mitosis (76.7%), whereas a smaller proportion exited M phase with (16.7%) or without (6.7%) cytokinesis (Supplementary Fig. S1A). We also determined mitotic and cell death indices from the same samples at 0-, 12-, 24-, and 36-hour time points after exposing the cells to the compounds (Fig. 2C). In Cent-1–treated populations, mitotic index peaked at 24 hours (49.4%) and cell death index was the highest at 36 hours (50.8%). Rigosertib-treated cells caused a similar accumulation of cells into mitosis followed by increase in cell death index (Supplementary Fig. S1B).

As rigosertib was earlier shown to cause apoptosis (11), we analyzed the potential apoptotic effects of Cent-1 on HeLa cells using Annexin V-FITC and propidium iodide staining followed by flow cytometry, and Western blotting with an antibody recognizing cleaved PARP at 12-, 24-, and 48-hour time points after adding the compound. Cent-1 caused the accumulation of cells to G2–M-phase and an increase in the sub-G1 cell fraction in comparison with DMSO control (Supplementary Fig. S2A). After 48-hour Cent-1 treatment, 32.8% of cells were in early apoptosis and 14.3% in late apoptosis or necrosis measured by Annexin V-FITC and propidium iodide staining. In comparison, after DMSO treatment, 10.4% of cells were early apoptotic and 8.6% late apoptotic or necrotic (Supplementary Fig. S2B). Furthermore, Cent-1 caused a slight increase in the level of cleaved PARP (Supplementary Fig. S2C).

**Cent-1 induces multipolarity and centrosome fragmentation**

Next, the mitotic effects of Cent-1 were investigated using a HeLa cell line stably expressing H2B-GFP and mCherry-tubulin. Cells were imaged live immediately after Cent-1 or DMSO was added to the culture medium (Supplementary Movies S1 and S2). All Cent-1–treated cells exhibited a long prometaphase delay that was followed by death or abnormal exit from mitosis. Typically, cells assembled multipolar spindles immediately upon nuclear envelope breakdown (NEB) and underwent multipolar anaphase after spending several hours in prometaphase (Supplementary Movie S1). Alternatively, a number of cells formed bipolar spindles through fusion of multiple poles during the prometaphase delay (Supplementary Movie S1) or established a bipolar spindle upon entry into mitosis. Many chromosomes in these cells congressed to the metaphase plate, indicating that spindle fibers and kinetochores were capable of establishing functional connections. However, cells always exhibited a number of chromosomes that remained unaligned near the spindle poles (Supplementary Movie S1). Control cells established a bipolar spindle and divided normally (Supplementary Movie S2).

To confirm the live-cell imaging findings, DMSO- or Cent-1–treated HeLa cells were fixed and immunostained with various antibodies. Cent-1 treatment caused heterogeneous anomalies that ranged from cells having bipolar spindles with a few unaligned chromosomes to cells having multipolar spindles and fully misaligned chromosomes (Fig. 3A). The proportion of multipolar cells was...
28.5% after 8-hour treatment with the drug (Fig. 3B) and increased during longer incubation times (37.5% at 24 hours and 44% at 48 hours). Similarly, rigosertib treatment resulted in a mixture of bipolar and multipolar cells (Supplementary Fig. S3).

Rigosertib was earlier shown to reduce the centrosome-associated pool of \( \gamma \)-tubulin and cause centrosome fragmentation (11). To evaluate the possible effects of Cent-1 on centrosomes, cells were stained with antibodies against \( \gamma \)-tubulin, pericentrin, and centrin after 6-hour Cent-1 treatment. In these cells, \( \gamma \)-tubulin was greatly diminished at spindle poles (Fig. 3C). Moreover, based on pericentrin staining, the centrosomes were disintegrated (Fig. 3D). Using morphometric analysis, we detected significant differences \(( P < 0.01—P < 0.001)\) in the following centrosome parameters: area, roundness, and AR between Cent-1 and DMSO, and between rigosertib and DMSO treatments (Fig. 3E). Furthermore, Cent-1–induced multipolar cells exhibited \( \alpha \)-tubulin–positive foci that were often negative for pericentrin and centrin, suggesting that these foci lack normal centrosome constituents (Fig. 3F). The same observation was made in rigosertib–induced multipolar cells (data not shown). In addition to having centrosome defects, Cent-1– and rigosertib-treated cells exhibited shorter spindle length compared with control (Fig. 3G). In control cells treated with DMSO, the average
spindle length was 10.4 ± 0.4 μm, whereas Cent-1 reduced the spindle length to 8.9 ± 1.4 μm (P < 0.001) and rigosertib to 8.0 ± 0.9 μm (P < 0.001).

One mitotic protein contributing to the formation of spindle poles and maintenance of normal spindle architecture is the nuclear mitotic apparatus protein 1 (NuMA; ref. 23). Interestingly, depletion of NuMA causes defects in chromosome alignment (24), resembling the phenotype caused by Cent-1 and rigosertib. Treatment of cells with Cent-1 or rigosertib caused a prominent redistribution of
NuMA from the spindle poles to several new foci throughout the cytosol in comparison with control (Fig. 3H). Collectively, these data show that Cent-1 and rigosertib induce multipolarity and cause the disintegration of the centrosomes.

Mitotic checkpoint is active in Cent-1–treated cells

To investigate why cells arrest in mitosis after Cent-1 treatment, we analyzed the levels of proteins involved in SAC signaling. The kinetochore occupancy of SAC proteins BubR1 and Bub1 at the unaligned chromosomes is a common marker for an active SAC (25, 26). To analyze SAC activity, HeLa cells were incubated in the presence of Cent-1, or control compounds (taxol or DMSO) for 6 hours. After fixation and immunofluorescence staining, BubR1 and Bub1 levels were quantified at the kinetochores of unaligned chromosomes near the spindle poles. Only bipolar cells were chosen for the analysis, because it was more feasible to categorize aligned/unaligned chromosomes in comparison with multipolar cells. Similarly to control metaphase cells, BubR1 and Bub1 were mostly undetectable at the kinetochores of metaphase-aligned chromosomes in Cent-1–treated cells (Fig. 4A and B). However, in the same cells, the levels of both proteins remained elevated at the kinetochores of unaligned chromosomes, indicating continued SAC activity (Fig. 4C and D). Also in rigosertib-treated cells the kinetochores of unaligned chromosomes were positive for BubR1 and Bub1 (Supplementary Fig. S4).

In another approach, we used an Aurora B kinase inhibitor ZM447439 that is known to cause premature SAC inactivation, leading to forced exit from M phase (27). Hela cells were accumulated into mitosis by treatment with Cent-1 or taxol, collected and further incubated with or without ZM447439 for 2 hours in the continued presence of Cent-1 or taxol. If mitotic arrest is Aurora B and SAC-mediated, cells should exit mitosis within the 2-hour incubation period. This was the case for both Cent-1 and taxol; the levels of mitotic markers cyclin B1 and phosphohistone H3 were greatly reduced upon ZM447439 treatment (Fig. 4E).

Cent-1 and rigosertib modulate microtubule dynamics and microtubule plus-ends

It is well established that interference with microtubules leads to defects in spindle assembly, resulting in a SAC-mediated mitotic arrest (28). To test whether Cent-1 impairs microtubule functions, we first investigated whether the compound directly affects tubulin polymerization in vitro. The polymerization rate of tubulin was monitored in the presence of various concentrations of Cent-1. Vinblastine that induces tubulin depolymerization and taxol that increases polymerization rate served as controls. Both reference drugs affected the rate of tubulin polymerization as expected, whereas the addition of Cent-1 at 1 or 5 \( \mu \text{mol/L} \) concentrations had no effect on the reaction (Fig. 5A). Higher concentrations (10 and 20 \( \mu \text{mol/L} \)) of Cent-1 resulted in a slight increase in tubulin polymerization rate (Fig. 5A). It was previously reported that rigosertib does not affect the rate of tubulin polymerization in vitro although only one concentration (5 \( \mu \text{mol/L} \)) was tested (11).

Next, we investigated the effects of Cent-1, rigosertib, and control drugs nocodazole and taxol on the microtubule network of interphase cells using immunofluorescence (Fig. 5B). HeLa cells were treated with the compounds for 6 hours, fixed and immunostained with an antibody against \( \alpha \)-tubulin to visualize microtubules. As expected, nocodazole disrupted the microtubule network in comparison with DMSO, whereas taxol caused microtubule bundling (Fig. 5B). Cent-1 treatment caused very minor, if any, changes compared with the DMSO control; the mass and shape of interphase cell microtubules seemed normal (Fig. 5B). Only slight curving of microtubules was observed in some cells. Similarly, rigosertib incubation induced slight changes to the microtubule network of interphase cells at 250 \( \mu \text{mol/L} \) concentration (Fig. 5B). It was previously published that at high concentration (2.5 \( \mu \text{mol/L} \)), rigosertib depolymerizes microtubules (29) and our experiments confirmed this finding, most microtubules were lost after 6-hour incubation with 2.5 \( \mu \text{mol/L} \) rigosertib (Supplementary Fig. S5).

Next, we fixed and immunostained Cent-1– or rigosertib–treated cells with an antibody against end-binding protein 1 (EB1), which is a highly conserved microtubule plus-end protein that is needed for normal microtubule dynamics (30, 31). In DMSO-treated control cells, EB1 showed a characteristic comet-like staining pattern at the tips of growing microtubule plus-ends (Fig. 5C). Although Cent-1 and rigosertib did not have major effects on the microtubule filaments in cells at low concentrations (Fig. 5B), the same treatments caused notable changes in the localization of EB1 in interphase cells; EB1 comets were much shorter and seemed fragmented (Fig. 5C).

This prompted us to investigate whether microtubule dynamics was affected by the compounds in cells. The model used was a human A549 lung carcinoma cell line stably expressing EGFP-\( \alpha \)-tubulin that we have earlier validated for the measurement of microtubule dynamics (32). In A549 EGFP-\( \alpha \)-tubulin cells, 6-hour treatment with 5 \( \mu \text{mol/L} \) Cent-1 caused a similar phenotype as in HeLa cells; the cells showed chromosome misalignment and multipolarity, and exhibited a long mitotic delay (Supplementary Fig. S6A and S6B). Moreover, 5 \( \mu \text{mol/L} \) Cent-1 had only minor effects on interphase cell microtubules (Supplementary Fig. S6C). Interestingly, these cells were much more sensitive to rigosertib treatment than HeLa cells, and treatment with 250 \( \mu \text{mol/L} \) concentration of rigosertib completely abolished microtubules (data not shown), whereas 50 \( \mu \text{mol/L} \) concentration did not eliminate microtubules but was sufficient to cause mitotic arrest (Supplementary Fig. S6A–S6C). For determining microtubule dynamics, A549 EGFP-\( \alpha \)-tubulin cells were treated with DMSO, 5 \( \mu \text{mol/L} \) Cent-1, or 50 \( \mu \text{mol/L} \) rigosertib for 1 to 2 hours. The cells were then imaged...
live and microtubule plus-ends were tracked manually to determine average microtubule dynamicity (the distance a microtubule plus-end travels in a set time) for each treatment. In control cells (n = 21), the average microtubule dynamicity was 0.096 ± 0.036 μm/s whereas in Cent-1 (n = 20) and rigosertib-treated cells (n = 18) overall microtubule dynamicity was reduced to 0.051 ± 0.019 (P < 0.001) and 0.039 ± 0.014 (P < 0.001) μm/s, respectively (Fig. 5D). Representative time-lapse movies of Cent-1-, rigosertib-, and DMSO-treated cells are available as Supplementary material (Supplementary Movies S3–S5). On the basis of these results, Cent-1 and rigosertib modulate microtubule plus-ends and microtubule dynamicity in interphase cells.

Cent-1 and rigosertib decrease interkinetochore tension by targeting mitotic substrates other than Plk1
To extend the analysis of Cent-1 and rigosertib effects on microtubules to mitotic cells, we measured interkinetochore distances in HeLa cells using Cenp-A as an inner kinetochore marker (33). Generation of tension across sister kinetochores stabilizes microtubule–kinetochore interactions and is required for SAC inactivation and normal mitotic progression (5). Cells were treated with Cent-1, rigosertib, or controls (DMSO, nocodazole, or taxol) for 6 hours. Interkinetochore distances were measured separately for chromosomes that had aligned at the metaphase plate and chromosomes that remained unaligned near the spindle poles in Cent-1- and rigosertib-treated cells. Only bipolar cells were selected for the analysis. Results show that the average interkinetochore distance of control cells at metaphase plate was 1.51 ± 0.09 μm, whereas treatment with Cent-1 and rigosertib reduced the distance to 1.07 ± 0.09 μm (P < 0.001) and 1.06 ± 0.04 μm (P < 0.001), respectively (Fig. 6A and B). The average interkinetochore distances of unaligned chromosomes were at similar range in control prometaphase cells (0.89 ± 0.07 μm), in Cent-1–treated cells (0.85 ± 0.02 μm), and in rigosertib-treated cells (0.85 ± 0.04 μm). As...
anticipated, the shortest distances were measured for the control drugs nocodazole (0.77 ± 0.05 μm) and taxol (0.78 ± 0.05 μm) that diminish tension to minimum by interfering directly with tubulin polymerization. These results indicate that although many chromosomes were able to move to the metaphase plate in Cent-1- and rigosertib-treated cells, the kinetochores were not under normal tension, suggesting that Cent-1 and rigosertib influence mitotic spindle forces.

To study whether Cent-1 and rigosertib elicit their antimitotic effects through targeting a protein that is operating during M phase, we applied the compounds on mitotic post-NEB HeLa cells and monitored their fate using IncuCyte at 15-minute intervals for 24 hours. Proteasome inhibitor MG132 and kinesin Eg5 inhibitors monastrol (34) and dimethylenastron were used as controls. As expected, MG132 induced a mitotic arrest followed by cell death due to the inhibition of protein degradation essential for anaphase onset and ordered exit from M phase (35). In agreement with the known early mitotic function of Eg5 in centrosome separation, monastrol and dimethylenastron had no effect when applied to post-NEB mitotic cells (34, 36). In sharp contrast, both Cent-1 and rigosertib caused an immediate effect in mitotic cells; most cells arrested to M phase for several hours before they died (Fig. 6C). On the basis of these results, the target protein of Cent-1 and rigosertib is present in mitotic cells in which it contributes to microtubule-mediated processes.

Figure 5. Cent-1 and rigosertib reduce microtubule dynamicity and cause mislocalization of EB1 in cells. A, the graph showing in vitro tubulin polymerization in the presence of Cent-1 or indicated control compounds. B, representative micrographs of drug-treated interphase HeLa cells immunostained with α-tubulin antibody. Cent-1 and rigosertib induce mild effects on the microtubule network, whereas the positive control drugs induce strong microtubule depolymerizing (Noc) and stabilizing (taxol) effects. C, representative micrographs of drug-treated interphase cells stained with EB1 antibody. HeLa cells were treated with DMSO, 5 μmol/L Cent-1, 250 nmol/L rigosertib, 0.5 μmol/L nocodazole (Noc), or 0.1 μmol/L taxol for 6 hours. The morphology of the EB1 comets was notably altered by all compound treatments compared with the DMSO control. Scale bars, 10 μm. D, the graph showing changes in microtubule dynamics upon Cent-1 and rigosertib treatments compared with DMSO. Microtubule dynamicity (the distance a microtubule plus-end travels in a set time) was measured in A549 cells expressing EGFP-α-tubulin after 1-hour incubation with DMSO, 5 μmol/L Cent-1, or 50 nmol/L rigosertib. Each dot in the graph represents the average microtubule plus-end velocity per analyzed cell (total number of cells analyzed per treatment was 18–21 from three experiments). For each analyzed cell, we recorded 10 to 20 microtubules. Line, the mean microtubule dynamicity in each treatment category. Asterisks, statistically significant differences between the controls and indicated treatments (***, P < 0.001).
Figure 6. Cent-1 and rigosertib cause a reduction in interkinetochore distance, and have immediate antimitotic effects without influencing Plk1 kinase activity. 

**A**, representative micrographs of control- and drug-treated mitotic cells stained for inner kinetochore marker CenpA (red) and centromere marker Crest (green). HeLa cells were incubated with DMSO, 5 μmol/L Cent-1, 250 nmol/L rigosertib, 0.5 μmol/L nocodazole (Noc), or 0.1 μmol/L taxol for 6 hours before fixation and immunocytochemistry. Images are maximum Z-projections of 5 to 7 layers acquired at 0.5-μm step interval. Insets, higher-magnification views of selected sister kinetochore pairs. Scale bar, 10 μm. 

**B**, the graph shows quantification of interkinetochore distances from control- and drug-treated cells. Data, mean ± SD from three experiments. A total of 100 kinetochore pairs were measured from 10 cells in each experiment, except for unaligned kinetochore pairs in Cent-1 and rigosertib treatments (for these, 1–10 kinetochores were measured per cell). Asterisks, statistically significant differences between the controls and indicated treatments (***, P < 0.001). 

**C**, mitotic HeLa cells respond to Cent-1 and rigosertib treatments. Cells in mitosis (n = 22–36) were treated with 5 μmol/L Cent-1, 250 nmol/L rigosertib, 10 μmol/L MG132, 100 nmol/L monastrol (Mon), or 5 μmol/L dimethylastron (DMA) and their fate was recorded using time-lapse filming at 15-minute intervals. Graph, the percentage of mitotic cells exhibiting the indicated fates. D and E, U2OS cells expressing a FRET-based sensor for measuring Plk1 activity were treated with 200 nmol/L BI2536, 5 or 10 μmol/L Cent-1, or 500 nmol/L rigosertib. Each dot represents one cell, n = 20 cells/condition. D, duration of mitosis. Note that many of the BI2536-treated cells were still in mitosis when filming ended, and therefore the data for BI2536 underrepresent the actual duration of mitosis. E, quantification of YFP–YFP/CFP–YFP emission ratio of mitotic cells in D. Plk1 activity is absent in interphase and peaks in mitosis.
Plk1 localization at the kinetochores of individual chromosomes changes upon their congression to the cell equator; the protein accumulates to unaligned kinetochores but is removed upon chromosome alignment to the metaphase plate (37). It has been reported that failure to remove active Plk1 from the kinetochores of chromosomes at the metaphase plate leads to decreased interkinetochore tension (38). To determine whether Plk1 is affected by Cent-1 or rigosertib, we analyzed Plk1 localization in compound-treated cells. Microscopic analysis did not reveal any changes from the normal dynamic behavior of Plk1 at mitotic kinetochores (Supplementary Fig. S7A).

We next measured the effects of Cent-1 and rigosertib on Plk1 kinase activity using U2OS cells stably expressing a FRET-based probe (18). Cent-1 and rigosertib caused a mitotic delay in U2OS cells (Fig. 6D). As expected, Plk1 activity increased when cells progressed from interphase to mitosis. The mitotic increase in Plk1 activity was efficiently blocked by BI2536 (Fig. 6E), a known Plk1 inhibitor (13). However, in cells treated with Cent-1 or rigosertib, Plk1 activity stayed at the normal mitotic level (Fig. 6E). Taken together, our results suggest that Cent-1 and ribosertib do not act through Plk1 but instead target another mitotic protein(s).

Finally, we analyzed whether Cent-1 and rigosertib affect PI3K activity in cells as rigosertib has been reported to inhibit PI3K in vitro (17). HeLa cells were starved in serum-free medium and the PI3K/AKT pathway was activated by the addition of insulin (39). On the basis of the analysis of AKT phosphorylation at Ser473, a marker of PI3K activity (40), the addition of insulin activated the kinase in the presence of DMSO, Cent-1, and rigosertib, whereas a known PI3K inhibitor wortmannin completely abolished AKT phosphorylation (Supplementary Fig. S7B and S7C). This result suggests that Cent-1 and rigosertib do not function by directly inhibiting PI3K in HeLa cells.

Discussion

In this study, we described the identification and characterization of Cent-1, a novel LMW compound that possesses similar steric and electrostatic features and antimitotic phenotype as the template compound rigosertib, which is a multikinase inhibitor with claimed activity against PI3K and Plk1 pathways (11, 16, 17). The template compound has shown cytotoxic effects in human tumor cell lines and tumor growth suppression in xenograft models (11). At the moment, rigosertib is clinically evaluated for the treatment of hematopoietic malignancies and solid tumors (9, 10, 41). We analyzed the effects of Cent-1 in cells side by side with rigosertib and relevant control compounds. Cent-1 was found to cause multipolarity and chromosome misalignment (Fig. 3) that triggered a SAC-mediated M-phase arrest (Fig. 4) followed by mitotic cell death or aberrant exit from cell division (Fig. 2). Multipolarity was often associated with the formation of acentrosomal poles that were negative for pericentrin and centrin (Fig. 3). Furthermore, we detected other spindle and microtubule defects such as reduced microtubule dynamics in interphase cells (Fig. 5) and reduced interkinetochore tension in mitotic cells (Fig. 6). Treatment of cells with rigosertib induced a similar pleiotropic antimitotic phenotype.

The template compound rigosertib was originally proposed to target Plk1 via an allosteric mechanism of action (11), but that notion has been challenged by others (29, 42, 43). Our results showing that cells exhibit strong antimitotic phenotypes upon treatment with Cent-1 or rigosertib without any inhibition of Plk1 activity (Fig. 6) support the view that Plk1 inhibition is not the mechanism by which these compounds function. Moreover, whereas Cent-1 and rigosertib treatments lead to multipolarity, specific Plk1 inhibition with small molecules causes monopolarity (13). More recently, rigosertib was shown to inhibit the PI3K/AKT pathway (16, 17). In our analysis, we did not observe significant changes in the phosphorylation status of AKT Ser473, a downstream target epitope of PI3K (40) upon Cent-1 or rigosertib treatment in insulin-stimulated cells (Supplementary Fig. S7). This does not, however, exclude the possibility that the compounds could perturb downstream elements of the PI3K/AKT pathway, or that differences in the experimental setup could explain the discrepancy between the results. Importantly, because both compounds induced immediate M-phase arrest when applied to early mitotic cells, at least one of their target proteins must be present in mitotic cells.

We made three particularly interesting observations in mitotic cells that may shed some light to the mechanism of action of Cent-1 and rigosertib during M phase. First, both compounds caused centrosome fragmentation and reduced the amount of centrosome-associated γ-tubulin (Fig. 3). Second, they significantly retarded microtubule dynamics in interphase cells (Fig. 5), shortened spindle length in mitosis (Fig. 3), and decreased tension across sister kinetochores in mitotic chromosomes (Fig. 6). Third, the compounds caused delocalization of NuMA (Fig. 3) and EB1 (Fig. 5) in mitotic cells. Together, these data imply that Cent-1 and rigosertib impair microtubule-mediated processes during M phase. It is noteworthy that acentrosomal spindle poles (44), reduced interkinetochore tension (45), mislocalization of NuMA and EB1 (46–48), and chromosome misalignment (45) are all consequences of treatment of cells with low doses of microtubule drugs, which strengthens the notion that the compounds may modulate microtubule dynamics. The compounds do not apparently perturb microtubule polymerization in vitro at low-range concentrations (Fig. 5; ref. 11). Also, the current data do not allow to conclude whether their impact on microtubules in cells is direct or indirect. Cent-1 and rigosertib may impair microtubule functions in cells by having an impact on microtubule-associated proteins. At the moment, the identity of such proteins remains unknown but it is noteworthy that depletion of EB1 has been reported to slow down microtubule plus-end dynamics and reduce spindle...
length (30, 49, 50). It is therefore plausible to hypothesize that EB1 mislocalization by Cent-1 and rigosertib can contribute to the observed microtubule defects in cells.

In summary, our results provide evidence that ligand-based in silico HTS is a feasible method for the identification of novel antimitotic compounds when a known inhibitor is used as a template. Cent-1 is a new mitotic inhibitor that possesses similar steric and electrostatic features as the template compound rigosertib, which shows clinical anticancer potency. In cells, both compounds induced various defects in mitotic processes that involve dynamic microtubules. However, the precise mechanism of action of Cent-1 and rigosertib remains to be clarified.

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

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Development of methodology: J. Rehnberg, E. Narvi, P. Tiikkainen, P. Halonen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.H.E. Mäki-Jouppila, J. Rehnberg, E. Narvi, E. Hukasova, P. Halonen

References


35. Rousselet A. Inhibiting Ccm1 causes the formation of excess acenstoronal spindle poles containing NuMA and B23, but does not affect centrosome numbers. Biol Cell 2009;101:679–93.

36. Morrison EE, Wardworth BN, Askham JM, Markham AF, Meredith DM, EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle. Oncogene 1998;17:3471–7.


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