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Small molecule targeting the Hec1/Nek2 mitotic pathway suppresses tumor cell growth in culture and in animal

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Abstract

Hec1 is a conserved mitotic regulator critical for spindle checkpoint control, kinetochore functionality and cell survival. Overexpression of Hec1 has been detected in a variety of human cancers and is linked to poor prognosis of primary breast cancers. Through a chemical genetic screening, we have identified a small molecule, INH1, which specifically disrupts the Hec1/Nek2 interaction via direct Hec1 binding. Treating cells with INH1 triggered reduction of kinetochore-bound Hec1 as well as global Nek2 protein level, consequently leading to metaphase chromosome misalignment, spindle aberrancy and eventual cell death. INH1 effectively inhibited the proliferation of multiple human breast cancer cell lines in culture (GI₅₀ 10~21 μ M). Furthermore, treatment with INH1 retarded tumor growth in a nude mouse model bearing xenografts derived from the human breast cancer line MDA-MB-468, with no apparent side effects. This study suggests that the Hec1/Nek2 pathway may serve as a novel mitotic target for cancer intervention by small compounds.

Keywords

small molecule; cancer therapy; Hec1; mitosis

Introduction

Mitosis is a highly complex stage of the cell cycle that requires coordinated efforts among multiple regulators for proper spindle formation and efficient chromosome segregation. Spindle poisons represented by taxanes and Vinca alkaloids are a class of conventional anticancer chemotherapeutics that kill cells by targeting mitotic machinery (1). However, these drugs are known to elicit severe pathological side effects in part due to the importance of microtubules in normal tissues (2). Some mitotic kinases, such as Aurora A and B, Plk1 and CDK1, have been considered as alternative targets (3). Mitotic kinesins (KSP/Eg5 and CENP-E) are another class of prospective targets (4,5). The primary function of KSP is to regulate centrosome separation and spindle formation (6,7). On the other hand, CENP-E is required for both kinetochore assembly and spindle checkpoint control by sensing the attachment of K-fibers (8). Inhibition of CENP-E allows mitotically defective cancer cells to exit mitosis prematurely, thus promoting mitotic catastrophe and cell death. Inhibitors for the above mitotic

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targets have been developed and are in clinical development at present (3). However, evidence linking the above molecules to cancers remains sparse, except the oncogenic amplification of Aurora A kinase in several types of human cancers.

Hec1, a kinetochore outer layer component and spindle checkpoint regulator, is of particular interest because it clearly has association with cancer progression. Hec1 directly interacts with multiple kinetochore components including Nuf2, Spc25, and Zwint-1, and with mitotic kinases Nek2 and Aurora B (9). Regulation of Hec1 by Nek2 and Aurora B is critical for its mitotic function and cell survival (10–12). The primary functions of Hec1 are assigned to mitotic control for kinetochore assembly, K-fiber attachment, chromosome alignment and the retaining of Mad2 spindle checkpoint protein to inhibit premature anaphase entry (9). Interestingly, overexpression of Hec1 was previously observed in a variety of human cancers and a large portion of the NCI-60 cancer lines (13, & our unpublished data), which was later found to be an excellent prognosis marker for primary breast cancers and patients with multiple cancers (14,15). Targeting Hec1 by virus-mediated RNAi has proved to be effective in tumor retardation in animals (16,17). Therefore, Hec1 emerges as a potential mitotic target for cancer intervention.

In this study, we undertook a novel approach to targeting Hec1 by screening for inhibitory small compounds. Eight positive hits were identified from a compound library. One of the lead compounds, INH1, showed promising cancer inhibition activity in cells and in a tumor xenograft model in part by impairing the Hec1 function.

Materials and Methods

Chemicals, antibodies and cell lines

Compound library was purchased from Nanosyn. INH1 and INH2 were further synthesized at UC Irvine. Other chemicals were purchased from Sigma-Aldrich. Antibody sources were: mouse anti-Hec1 (Genetex), Goat or Rabbit anti-Nek2 (Santa Cruz Biotech.), human anti-ACA (Antibodies Incorporated) and tubulin antibodies (Sigma). Human breast cancer cell lines were maintained in DMEM medium (Invitrogen) with 10% FBS. MCF10A cells were cultured in DMEM/F12 (50:50) plus 5% horse serum, 0.1 µg/ml cholera toxin, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone and 20 ng/ml EGF.

Reverse yeast two-hybrid screening

The fusion protein (TetR-Hec1) of tet repressor fused to Hec1 C-terminal region (a.a. 250–618) was constructed with the backbone of the pCW200 plasmid and expressed constitutively in yeast, while the expression of activation domain-Nek2 (AD-Nek2, catalytic domain deleted) fusion from pB42AD plasmid was galactose-inducible under *GAL1* promoter. Yeasts were first grown in glucose containing medium and then inoculated (final OD₆₀₀ at 0.05) into the galactose medium containing 0.09% 5-FOA (5-fluoroorotic acid). The assay was performed on 96-well plates with 10 µM of one compound per well. Yeast growth was used as the readout for a positive hit (18).

Binding assays

Surface Plasma Resonance assays were performed at 22.5°C in HBSD buffer (10 mM Hepes, 150 mM NaCl, 0.1% DMSO, pH7.5) on Biacore 3000. 6xHis-Hec1 and GST-Nek2 were purified as described before (10). NTA sensor chip or glutathione-modified CM5 chip were used to capture His-Hec1 and GST-Nek2, respectively. The capture level was about 140–180 resonance units at the flow rate of 5 µl/min. For the binding assay, chips were sequentially treated with compounds (1 or 20 µM) and then proteins (50 µg/ml). Retained resonance units

(RU) were recorded and processed (triplicate experiments). Co-immunoprecipitation and Western blotting were done as described previously (19).

Microscopy and FACS analysis

Immunostaining, image processing, and FACS assays were done as detailed previously (20, 21).

Cytotoxicity and clonogenic survival assay

Standard MTT assays with a three-day drug treatment procedure were performed to measure the dose-dependent cytotoxicity of INH1 in cultured cells. Triplicate sets were measured and compiled for final data presentation. For clonogenic survival assay, 1000–3000 cells were seeded in a 10-cm Petri-dish (triplicates) for 24 hours and then treated with DMSO or INH1 (10 μ M) for 12 days. Cells were re-fed once every 3 days, then fixed and stained with 2% methylene blue in 50% (v/v) methanol. Viable percentages were calculated for plotting survival curve to derive compound GI₅₀ (drug dose resulting in 50% of growth inhibition). Colonies with at least 50 cells were scored.

Xenografted nude mice breast cancer model

Athymic female BALB/c-nude mice (nu/nu, 6–8 week old) were purchased from Harlan Sprague Dawley Inc (Indianapolis, IN). 5×10^6 exponentially growing MDA-MB-468 human breast cancer cells were suspended in 100 μ l of PBS and injected into the mammary fat pad of each mouse. After 10 days of tumor implantation, mice were injected (i.p., every other day/25 cycles in total) with vehicle A (15% DMSO, 20% Tween-20, 10% PEG-400, 55% saline), or INH1 formulated in vehicle A (50 or 100 mg/kg body weight). Tumor size was measured twice weekly by using a caliper, and the volume (mm^3) was calculated using the formula: $V = L \times W^2/2$, where L and W are the length and width diameters of the tumor, respectively. P-values were derived from the ANOVA test (SigmaPlot). Mice were weighed twice a week. Mice work was performed under the guidelines of the UCI Animal Research Committee.

Results

Identification of Hec1 inhibitors

Hec1 has no known enzymatic activity, thus precluding conventional screening for a potential Hec1 “enzymatic inhibitor”. Nonetheless, Hec1 is known to physically interact with Nek2 and such interaction plays a significant role in cell survival (10,22). This provides us a platform for identifying small compounds capable of disrupting the two binding partners, by either targeting Hec1 or Nek2. Of interest is Nek2, also a G2/M kinase important for mitotic control (23,24), overexpression of which was documented in various human cancers (25–27). Like Hec1, Nek2 is also thought to be a prospective anti-cancer target due to its mitotic specific function (24,25). Therefore, compounds inhibiting the Hec1/Nek2 interaction may be suitable for targeting either Hec1 or Nek2.

To search for such compounds, we adapted an inducible reverse yeast two-hybrid screening method that allows yeast growth when the candidate compound abolishes the interaction between the Hec1 and Nek2-derived probes: TetR-Hec1 and AD-Nek2 fusion proteins (Fig. 1A) (18,28). When not inhibited, the two probes assume normal binding, resulting in *URA3* (encoding orotidine 5-phosphate decarboxylase) transactivation and subsequent production of toxic metabolite 5-fluorouracil from the medium-supplemented component 5-FOA (5-fluoroorotic acid). We screened a library of ~24,000 compounds and identified eight positive hits, two of which were selected due to the similar chemical scaffolds (named INH1 and INH2 for Inhibitor of Nek2/Hec1) (Fig. 1B). In a similar screening system with a control interacting

pair (Rad51 and BRC repeat), INH1 and INH2 were inactive (data not shown), confirming the targeting specificity of INH1 and 2 on Hec1/Nek2. INH1 and INH2 share a phenyl thiazolyl benzamide scaffold, but INH2 possesses one extra methyl group and two methoxyl groups (Fig. 1B; Supplemental Fig. S1). In this study, we chose to focus on INH1 since both appeared to be very similar in cell killing assay.

Hec1 or Nek2 can be the direct target of INH compounds inhibiting the interaction between Hec1 and Nek2. To determine the primary target, we employed a surface plasma resonance (SPR) assay measuring a surface binding event (i.e., change of surface mass concentration at the sensor chip) (29). In principle, recombinant 6his-Hec1 and GST-Nek2 were purified from bacteria. A sensor chip attached with one of the purified proteins was pre-incubated with candidate compounds; then the surface binding event upon adding the partner protein was measured. If the compounds target the immobilized protein, the partner will no longer stick to the chip surface via protein-protein interaction (Fig. 2A), or otherwise it will be retained (Fig. 2A). We found that, upon the pretreatment of INH1 or INH2, chip-immobilized Hec1 showed reduced ability to bind to free Nek2 (39% and 55% reduction at 1 and 20 μ M respectively) (Fig. 2B), while the compound pretreatment did not affect the binding of immobilized Nek2 to free Hec1 (Fig. 2B). These results indicate that INH compounds directly target Hec1, rather than Nek2.

To further test whether INH1 can bind to Hec1 from cells, we adapted an affinity-pulldown target verification system (30), in which INH1 was conjugated to Affigel-10 matrix through a multi-step synthesis (Fig. 2C; Supplemental Fig. S2). Mixing the INH1-affinity matrix with the cell lysate brought down the endogenous Hec1, in contrast to the control matrix (Fig. 2C). The binding is specific because the matrix-bound Hec1 protein could be competed out by INH1, but not mock solvent. Furthermore, the INH1-affinity matrix did not bring down Nek2 (Fig. 2C), substantiating the notion that INH1 binds to Hec1 but not Nek2.

Next, we performed a co-immunoprecipitation assay to test whether INH1 treatment affects the cellular interaction between Hec1 and Nek2. In the lysate prepared from cells treated with INH1 (25 μ M, 36 hrs), Hec1 failed to co-immunoprecipitate with Nek2, indicating that the Hec1/Nek2 complex was disrupted (Fig. 2D). Taken together, these results suggest that INH1 preferentially binds to Hec1, leading to the disruption of Hec1/Nek2 interaction.

INH1 treatment resulted in reduced association of Hec1 with kinetochore and decrease of global Nek2 protein level

To further address the consequence of cellular Hec1 and Nek2 after treatment with INH1, we examined their protein levels by Western blotting analysis. Little change of Hec1 was observed in a dose-dependent kinetic study, although slight increase could be detected in a time-dependent experiment (Fig. 3A). Remarkably, the overall cellular Nek2 protein level was significantly reduced in both time and dose-dependent fashions upon INH1 treatment (80~90% decrease 24 hrs after INH1 treatment at 25 μ M). Nek2 downregulation was not due to G1-phase cell cycle arrest because there was no significant change of cyclin B level. Neither was it dependent on Hec1 expression, which remained steady after INH1 treatment (Fig. 3A). Consistently, Nek2 expression level was unchanged upon depletion of Hec1 by two distinct Hec1 siRNAs (Fig. 3B). These results suggest that Nek2 reduction in INH1-treated cells may be independent of Hec1.

Although the total Hec1 protein level was minimally affected by INH1, we found that the kinetochore-bound Hec1 pool was reduced by ~55% (Fig. 3C and D) using quantitative microscopy with a ratio-metric method (8, 21), suggesting defective Hec1 distribution to its primary functional site, kinetochores. Taken together, these results suggest that INH1-

treatment led to disruption of the Hec1/Nek2 complex, destabilization of Nek2, and defective localization of Hec1 at kinetochores.

INH1 inhibits tumor cell proliferation in culture

Inactivation of cellular Hec1 or Nek2 by antagonists (RNAi or antibody) is known to negatively impact mitosis and cell proliferation (31–38). Defective Hec1 localization and reduced Nek2 level upon INH1 treatment may presumably give rise to mitotic defects and thus hamper cancer cell growth. We are particularly interested in testing breast cancer, in which overexpression of Hec1 or Nek2 is linked to adverse clinical outcomes (14,15,25). To examine the potential cell killing activity of INH1, a panel of human breast cancer cell lines were treated with varying doses of INH1. All the breast cancer cell lines showed evident sensitivity towards INH1 treatment (GI_{50} at 10–21 μ M) (Fig. 4A). In contrast, MCF10A was relatively resistant to similar treatment (GI_{50} at 41 μ M). Results obtained from independent clonogenic survival assays further confirmed the cell killing activity of INH1 towards at least two breast cancer lines (MCF7 and MDA-MB-468) (Fig. 4B), as well as the cervical adenocarcinoma line HeLa and the colon cancer line HCT116 (data not shown). Taken together, these data suggest that INH1 inhibits the proliferation of cancer cells in culture.

INH1 treatment triggers a plethora of cellular phenotypes

Hec1 or Nek2 inactivation by RNAi or antibody-mediated depletion is known to trigger cellular phenotypes characteristic of mitotic abnormalities (31–40). To test whether INH1 treatment generates similar cellular phenotypes, we used HeLa cells ($GI_{50}^{INH1} \sim 10 \mu$ M) to perform cell biological studies since they were used extensively for Hec1 cell biology (31,32,34,39). Interestingly, the mitotic index was found to increase by ~ 2 folds, from 6% in control to 11% in INH1-treated cells for 8 hrs after drug addition (Fig. 5A). Cells underwent progressive morphological changes including features of cell death, evident after 36–48 hrs (Fig. 5A, panels c to e). By the 72-hr time point, a significant portion of cells were dead, showing condensed globular nuclear structures (Fig. 5A, panels e and g). No major cell cycle arrest was detected, except a slight G2/M increase at a low INH1 dose (5 μ M) (Fig. 5B, middle panel), reflective of a transient mitotic arrest. Nonetheless, cell death was apparent, indicated by the increased sub-G1 populations (Fig. 5B). Approximately 26% of the cells were apoptotic at 72 hr (10 μ M dose), compared to 2.6% in the mock-treated group. These results suggested that INH1-treatment elicits transient mitotic arrest followed by eventual cell death, reminiscent of the cell death phenotypes reported for HeLa cells partially or completely depleted of Hec1 (32,34).

Chromosomal and spindle abnormalities are common to cells defective in Hec1 or Nek2 (31, 32,39,41). Consistently, using live cell microscopy with a HeLa subline stably expressing H2B-GFP, chromosomal misalignment or lagging was readily observed upon INH1 treatment (Supplemental Fig. S3). The mitotic population with chromosomal misalignment increased over time (Fig. 5C). INH2 treatment also triggered a similar phenotype, albeit to a less severe extent (Fig. 5C). Aberrant spindles, such as deformed or multipolar spindles, were manifest (20–25% upon INH1 treatment versus 8% in control) (Fig. 5D). The drug-induced mitotic abnormalities are similar to those observed in Hec1 or Nek2 depleted cells, suggesting that INH1 treatment may impair the Hec1/Nek2 function for spindle and chromosome regulation.

Hec1 was reported to retain Mad2 checkpoint protein on kinetochores to prevent premature mitotic exit (31,32). Immunostaining experiments suggest that Mad2 recruitment on kinetochores was not affected by INH1 (data not shown), probably due to incomplete Hec1 inhibition (31,32,40). Nonetheless, a kinetic study suggests that the spindle checkpoint maintenance was weakened, compared to the control (Supplemental Fig. S4), which eventually led to mitotic slippage, consistent with our live cell microscopic study of H2B-GFP expressing HeLa stable clones. Spindle checkpoint activation and subsequent mitotic slippage are known

prerequisites of drug induced mitotic catastrophe (5,42). Thus, INH1-treated cells had positive spindle checkpoint response, but the mitotic slippage eventually prevailed, followed by cell death (Fig. 5B). Notably, a similar observation has been reported for a KSP/Eg5 inhibitor (5). Taken together, our results suggest that INH1-treatment elicits cell killing activity in part through impairing the Hec1/Nek2 pathway for the spindle checkpoint regulation.

INH1 inhibits tumor outgrowth in a xenografted breast cancer model in nude mice

To examine the anti-tumor efficacy of INH1, we tested the tumor inhibition activity in a nude mouse model harboring tumor xenografts derived from human breast cancer cell line MDA-MB-468, which were inoculated to the mammary fat pad to allow tumor outgrowth. Ten days later, INH1 was administered into the mice intraperitoneally at various doses (0, 50, and 100 mg/kg body weight) every other day for a total of 25 cycles. Tumor sizes were measured over the time. Remarkably, at both 50 and 100 mg/kg doses, INH1 treated mice showed significant tumor growth retardation (Fig. 6A). The side effects were minimal, if any, as indicated by the comparable body weight (Fig. 6B) and the autopsy (data not shown) between different groups. This study indicates that INH1 is effective in inhibiting tumor growth in animals.

Discussion

In this communication, we have identified a lead compound that specifically disrupts the Hec1/Nek2 interaction by directly binding to Hec1. INH1 treatment resulted in defective Hec1 localization on kinetochores and significant Nek2 reduction, correlating with mitotic abnormalities and cell death. The INH1-targeting effects are in part reminiscent of Hec1 or Nek2 depletion mediated by RNAi, suggesting that INH1 treatment impairs the Hec1/Nek2 function in cells. Although the off-target effects of INH1 cannot be excluded, the resulted mitotic phenotypes strongly support that the Hec1/Nek2 pathway is a major cellular target of INH1.

Structural similarities of INH1 and INH2 underlie a common mechanism of action, wherein the INH compounds target a site within the Hec1 C-terminal region critical for the Hec1/Nek2 interaction. The crystal structure of the Hec1 C-terminal region, however, remains to be resolved, precluding lead optimization via structure-based approaches. Instead, we have adopted a ligand-based strategy to test the structure-activity-relationship. Based on the activity of over 20 INH analogues synthesized to date (data not shown), it appears that both the amide and two distant phenyl rings are essential, since modifying the amide or one of the phenyl rings significantly affects the activity. For instance, the amide hydrogen can be replaced by methyl or bulky acyl groups without significant loss of activity (data not shown), suggesting that this domain of the molecule may be accessible to the solvent, and thus can be used for installing a “linker”, as was done for the INH1 affinity matrix. To this end we have not been able to identify an INH1 derivative serviceable as a true “negative” control. Notably, for human cells, the GI50s of INH1 are at a similar micromolar level to other known protein-protein interaction inhibitors such as those for androgen receptor, IL-2 and BCL-2 (43,44).

How is the Hec1/Nek2 pathway impaired? There are several possibilities. First, INH1 binding may trigger Hec1 conformational changes leading to impaired networking with other mitotic regulators. The region of Hec1 responsible for Nek2 interaction also binds to several important mitotic regulators including Nuf2, Zwint-1 and Hice1 (21,22,45,46). Our preliminary study suggests that the INH1 binding with Hec1 enhances, unlike the case of Nek2, the Hec1/Nuf2 interaction (data not shown). On the other hand, Hec1 is a substrate of Nek2. When the Hec1/Nek2 interaction is abolished, Hec1 may not be properly phosphorylated and hence become defective. Compound targeting effects eventually manifest themselves through defective Hec1 targeting/recruitment on kinetochores, its major functional sites.

Remarkably, INH1 treatment triggered dramatic reduction of Nek2 in cells, but not endogenous Hec1. This seemingly paradoxical observation can be in part explained by indirect drug effects of unknown nature, or that INH1-bound Hec1 may gain a dominant-negative function that somehow triggers Nek2 degradation. Clearly, loss of Nek2 was another important contributing factor to the cell killing effects of INH1. Nek2A, one of the major Nek2 splice variants, was reported to be degraded in an APC/C dependent manner starting at prometaphase (47). However, whether INH1 treatment may facilitate Nek2 degradation through the APC/C pathway remains to be addressed. Although the precise molecular mechanism remains to be explored, our results support that INH1 treatment may alter the overall Hec1/Nek2 network in ways that promote mitotic errors, ultimately leading to mitotic catastrophe.

INH1 treatment significantly inhibited tumor growth in a xenograft mouse model while no apparent general toxicity was observed. This may be attributed to the expeditious growth of tumor cells compared with normal cells spared in part due to the quiescence status or slower division. In the mean time, extensive toxicology tests remain to be performed to fully address the potential organismal toxicity. Nonetheless, our results clearly indicate that the INH1-mediated Hec1 impairment, but not complete shutdown, may effectively render cancer cells sensitive to mitotic or genotoxic stress, while sparing the surrounding normal cells, consistent with tumor therapy studies involving Hec1 RNAi (16,17). In essence, our study provides a proof-of-principle that inhibiting the Hec1/Nek2 pathway by small molecules may serve as an effective approach for cancer intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

INH1

N-(4-[2,4-dimethyl-phenyl]-thiazol-2-yl)-benzamide

INH2

N-(4-[2,4,6-trimethyl-phenyl]-thiazol-2-yl)-2,4-dimethoxybenzamide

GI₅₀

drug dose resulting in 50% of growth inhibition

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Figure 1.

Identification of small compounds that disrupt Hec1/Nek2 interaction

A, Schematic diagram of a reverse yeast-two hybrid system for high throughput screening. TetR fused with the C-terminal binding region of Hec1 (TetR-Hec1) was constitutively expressed; activation domain of GAL1 fused with Nek2 (AD-Nek2) was under the control of *GAL1* inducible promoter. If an inhibitor abolishes the Hec1/Nek2 interaction, 5-FOA (5-fluoroorotic acid) will not be metabolized, thus permitting yeast growth; otherwise, 5-FOA will be hydrolyzed into toxic metabolites and inhibits yeast growth. B, structures of two candidate compounds that promote yeast growth.

Figure 2.

INH1 compound binds to Hec1, but not Nek2.

A–B, the Surface Plasma Resonance (SPR) assays. *A*, Schematics showing that either Hec1 or Nek2 can be first immobilized on the chip. Protein-bound chip is pretreated with candidate compound or mock before the second protein is added. *B*, Histograms to show retained resonance units (RU) with Hec1-immobilized chip (*left*) or Nek2-immobilized chip (*right*). Full length Hec1 and Nek2, tagged with 6xHis and GST respectively, were purified from bacteria. *C*, (*left*) structures of INH1-conjugated and control matrix. Grey balls denote the Affigel-10 matrix; (*right*) Affinity-pulldown by using INH1 or control -conjugated matrix to incubate with HeLa cell extract. Matrix bound proteins were further competed with free INH1 of varying doses in the reaction (lanes 3–5). Resultant precipitates were resolved by SDS-PAGE and detected for Hec1 or Nek2 by Western blotting. *D*, co-immunoprecipitation/ Western blot assays with cells treated with DMSO (mock) or INH1 (25 μ M, 36 hrs).

Figure 3.

INH1 treatment triggered Nek2 reduction and defective Hec1 localization on kinetochores. *A*, Western blotting analysis of cellular Hec1, Nek2 and cyclin B after INH1 treatment. *B*, Western blotting for cellular Hec1, Nek2 and Rad50 (loading control) after Hec1 depletion by RNAi transfection. Luc, control luciferase RNAi; Hec #1 and #2 refer to two distinct RNAi sequences (31,34). *C*, quantitative immunofluorescent staining of INH1 or mock treated mitotic cells with anti-Hec1 or anti-ACA antibodies. The relative immunofluorescence intensity (Hec1 vs. ACA) was shown in the bar graph (*D*). Scale bar, 10 μ m.

Figure 4.

INH1 effectively inhibits the proliferation of human breast cancer lines. *A*, Table shows GI₅₀s (50% growth inhibition) of INH1 on a panel of human breast cancer cell lines, SV40-immortalized breast epithelial cell line HBL-100, and noncancerous breast epithelial cell line MCF10A. *B*, Colonogenic assays for MCF7 and MDA-MB-468 cells treated with various doses of INH1. Bar graph shows the colony number.

Figure 5.

Cellular phenotypic changes of INH1-treated HeLa cells. **A**, Panels a to e are representative phase-contrast images of HeLa cells treated with INH1. Nucleus/DNA of control cells (f) or INH1-treated cells (g) (10 μ M, 72 hrs) were stained with DAPI and arrows in (g) indicate condensed nuclear globules, characteristic of apoptosis. Scale bars, 20 μ m (black) or 10 μ m (white). **B**, cell cycle profiling of INH1-treated cells by FACS analysis. Sub-G1 populations were denoted by arrows. **C**, time-dependent kinetics showing the percentages of mitotic HeLa-H2B-GFP cells with metaphase misalignment after INH1 treatment (10 μ M). Representative images of mitotic chromosomes stained with DAPI after treatment with either DMSO (a–c) or INH1 (10 μ M) for 32 hrs (d–f). Arrows show misaligned (d–e) or lagging (f) chromosomes. Scale bar, 10 μ m. **D**, immunofluorescent staining with anti- α -tubulin and γ -tubulin to show the normal (row a; arrows) and abnormal (rows b & c; arrows) spindle morphology. Scale bar, 10 μ m.

Figure 6.

INH1 inhibits breast tumor growth in a xenografted mouse model. *A*, Changes of the tumor volumes following the INH1 treatment. MDA-MB-468 cells were injected into athymic nude mice under the mammary fat-pad 10 days before drug treatment; then these tumor bearing nude mice (N=7 mice for each group) were treated with control vehicle, 50 mg/kg or 100 mg/kg (body weight) of INH1 every other day for 7 weeks (I.P. injection, 25 cycles). Tumor volumes were measured over time and plotted in the graph. The p values were derived from the ANOVA test. *B*, the histogram showing the body weight of the mice treated in (*A*).