

Dual Degradation of Aurora A and B Kinases by the Histone Deacetylase Inhibitor LBH589 Induces G₂-M Arrest and Apoptosis of Renal Cancer Cells

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Abstract Purpose: This study is aimed at investigating antineoplastic efficacy of histone deacetylase inhibitor (HDACI) LBH589 on renal cell carcinoma (RCC) and elucidating the novel molecular mechanisms involved in growth arrest and apoptosis by targeting the important nonhistone molecules.

Experimental Design: We analyzed the growth-inhibitory effect of LBH589 on RCC by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay *in vitro* and antitumor efficacy by xenograft experiments *in vivo*. To verify the associated molecular mechanisms involved in LBH589-mediated cell death and cell cycle progression by Western blotting and fluorescence-activated cell sorting analysis.

Results: HDACI LBH589 induced degradation of both Aurora A and B kinases through a proteasome-mediated pathway by targeting HDAC3 and HDAC6. The dual degradation of Aurora A and B kinases mediated by LBH589 resulted in inducing G₂-M arrest and apoptosis of renal cancer cell lines and our results also showed that LBH589 potently inhibited renal cancer cell growth *in vitro* and suppressed tumor formation *in vivo*. The Aurora A and B kinases and HDAC3 are overexpressed in the human RCC tumor tissues examined, which make them perfect targets for HDACI LBH589 treatment.

Conclusions: Our *in vitro* and *in vivo* data showed that LBH589 has potent anticancer effect of renal cancer cells. LBH589 and other HDACI treatment resulted in inducing G₂-M arrest and apoptosis of renal cancer cells through degradation of Aurora A and B kinases by inhibition of HDAC3 and HDAC6. The clinical efficacy of LBH589 in the treatment of patients with metastatic RCC, especially those with high Aurora kinase and HDAC expression, is worthy of further investigation.

It has been well recognized that histone deacetylases (HDAC) are promising targets for cancer treatments aimed at reversing epigenetic alterations associated with cancer (1, 2). Many HDAC inhibitors (HDACI) have entered clinical trials to determine their therapeutic efficacy for different solid or

hematologic cancers (3, 4). Early preclinical and clinical studies have shown that HDACIs have potent anticancer activities, with remarkable tumor specificity. These encouraging results also raised important questions about the molecular mechanisms underlying their anticancer effects, including how they induced growth arrest or death of cancer cells. In general, the anticancer effects of HDACIs have mainly been linked to reactivated gene expression through histone hyperacetylation. However, in addition to histone, HDACs may have nonhistone targets responsible for the inhibitors' affects on tumor cell biology.

All HDACIs studied to date can induce cell cycle arrest at G₁-S through transcriptional activation of genes such as p21 and other cell cycle-regulated genes in a p53-independent manner (2, 4–7). Although less common, it is increasingly clear that HDACIs can also induce G₂-M cell cycle arrest in many human cancer cell lines (1, 8–10). Unlike HDACI-mediated G₁ arrest, the underlying mechanisms responsible for HDACI-mediated G₂-M arrest remain unclear. It has been shown that HDACI-induced pericentromeric histone hyperacetylation resulted in altered kinetochore assembly and mitotic arrest (11, 12). HDACIs disrupt the association of Aurora B and HDAC3 responsible for the aforementioned mitotic defects resulting from inhibition of histone H3S10 phosphorylation and

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Translational Relevance

The lack of demonstrably efficacious therapy for metastatic renal cell carcinoma (RCC) has led to a poor 5-year survival rate. Our study showed that a novel histone deacetylase (HDAC) inhibitor LBH589 potentially inhibited renal cancer cell growth *in vitro* and suppressed tumor formation *in vivo*. LBH589-mediated down-regulation of Aurora A and B and survivin induced G₂-M cell cycle arrest and apoptosis of RCC cells through inhibition of HDAC3 and HDAC6. The Aurora A and B and HDAC3 are overexpressed in the human RCC tumor tissues examined. Our results suggest that LBH589 has the potential to effectively treat metastatic RCC and is worthy of investigation in future clinical trial. Most importantly, the expression levels of HDACs and Aurora kinases in human renal cancer tissues may serve as therapeutic markers for the application of LBH589 and other HDACs in metastatic RCC treatment. Therefore, our study fulfills the purpose of translational research, from bench work to bedside clinical practice.

induction of histone acetylation (13). More recently, it was reported that HDACs induce Aurora A degradation, resulting in mitotic defect (14). Collectively, these results suggest that HDACs can interfere with the interaction between HDACs and Aurora kinases involved in alteration of the G₂-M cell cycle transition.

The Aurora A and B kinases, possessing conserved catalytic domains, show different subcellular localization and biological functions that are involved in G₂-M cell cycle progression (15–20). Deregulation of Aurora kinases has been linked to tumorigenesis. Overexpression of Aurora A transforms rodent fibroblast cell lines and gives rise to colonies in culture and tumors in nude mice (21, 22), indicating that Aurora A is a bona fide oncogene. Although exogenous overexpression of Aurora B is not sufficient to transform cells, it enhances the tumorigenicity and invasiveness to induce metastases in non-metastatic Chinese hamster embryo cells (23). As aberrant increases in Aurora kinase levels can enhance their oncogenic effects, the function of Aurora kinases could be effectively turned off by their regulated proteolysis. Therefore, for anticancer therapy, it would be ideal to destroy the Aurora kinase proteins instead of just inhibiting their catalytic activity. Aurora A and B kinases are commonly overexpressed in various cancers, including breast, colon, bladder, ovarian, and pancreatic cancers (21, 22, 24–29). Recently, Aurora A was also found to be overexpressed in renal cancers, which suggests that Aurora kinases may play an important role in the development of renal cancer (30).

Renal cell carcinoma (RCC) accounts for 3% of all adult malignancies and is the most lethal of the urologic cancers (31). Approximately 20% to 30% of patients present with metastatic disease and 20% to 40% of patients undergoing nephrectomy for clinically localized RCC eventually develop metastases (32). The lack of demonstrably efficacious chemotherapy and radiation therapy for advanced RCC has led to a poor 5-year survival rate, ranging from 5% to 10%. Current immunotherapy with interleukin-2 and INF- α , either alone or

in combination, is limited by their toxicity and generally poor overall response rates. Recent significant advances in understanding the biology of RCC has led to the identification of vascular endothelial growth factor as a potential target. However, vascular endothelial growth factor-targeted therapy has fallen short of achieving complete and durable responses in the majority of patients (33). Therefore, it is an urgent matter to identify new targets and develop more effective therapeutic agents for the treatment of advanced RCC.

In this study, we found that the HDACi LBH589 induced degradation of both Aurora A and B kinases through a proteasome-mediated pathway by targeting HDAC3 and HDAC6. The dual degradation of Aurora A and B kinases mediated by LBH589 resulted in the induction of G₂-M arrest and the apoptosis of many cancer cell lines, which is also a general phenomenon mediated by different HDACs. Our results also showed that LBH589 potentially inhibited renal cancer cell growth *in vitro* and suppressed tumor formation *in vivo*. The Aurora A and B kinases and HDAC3 are overexpressed in the examined human RCC tumor tissues. Therefore, our findings provide a novel mechanism for how different HDACs induce G₂-M arrest and apoptosis in renal cancer cells. In addition, these results indicate that LBH589 may have the potential to effectively treat advanced RCC, especially those tumors expressing high levels of Aurora kinases and HDACs. Most importantly, our study provides a rationale to further validate whether the expression levels of HDAC3, HDAC6, and Aurora kinases in human cancer tissues may serve as therapeutic markers for the application of LBH589 and other HDACs in the treatment of RCC.

Materials and Methods

Reagents. LBH589 from Novartis (Novartis Pharmaceuticals) was dissolved in DMSO. Trichostatin A, sodium butyrate, suberoyl bishydroxamic acid, and the proteasome inhibitor MG132 were purchased from Calbiochem. Suberoylanilide hydroxamic acid was from ATON Pharma. Propidium iodide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, RNase A, and protease inhibitor cocktail were purchased from Sigma-Aldrich. Cell culture medium, serum, penicillin-streptomycin, and Lipofectamine 2000 were from Invitrogen.

Cell culture. The cell lines PC3 and 293T were maintained in DMEM. T24, Caki-1, 786-O, 22RV1, TSGH8301, H1299, and MBT2 cells were cultured in RPMI 1640 and DU145, A498, ACHN, and J82 cells were maintained in modified Eagle's medium. Normal renal epithelial cells were obtained from the normal portions of renal cortex derived from nephrectomy kidney tissues. The used tissue was accessed and examined by a pathologist after completion of all diagnostic procedures. The procedure of primary renal epithelial cell culture followed the protocol from Detrisac et al. (34). All media were supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 50 units/mL penicillin, and 50 μ g/mL streptomycin, and cells were cultured at 37°C in a humidified 5% CO₂/air atmosphere.

Cell viability assays. The effect of LBH589 on RCC cell growth and survival was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, 1×10^4 cancer cells were seeded in 96-well microtiter plates overnight. Thereafter, cells were treated with different concentrations of LBH589 (0, 50, 75, 100, or 150 nmol/L) and an equal volume of DMSO as the control and then incubated for an additional 24, 48, or 72 h. After removing the medium, 1 mg/mL

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (100 μ L) was added and incubated at 37°C. After 3 h, the supernatant was removed and 100 μ L DMSO was added to dissolve deposited crystals. Absorbances were determined at 560 nm using a microplate reader (μ Quant; Bio-Tek Instruments).

Flow cytometry analysis of cell cycle and apoptosis. Renal cancer cells were treated with increasing concentrations of LBH589 and DMSO in complete medium for 24 and 48 h, respectively. After drug treatment, both DMSO- and LBH589-treated cells were harvested and fixed in 70% ice-cold ethanol for 1 h. Following ethanol removal, the cells were treated with 0.5% Triton X-100 and 0.05% RNase A in PBS for 30 min, stained with propidium iodide, and analyzed using a FACSCalibur flow cytometer (BD Biosciences) to determine the cell cycle-dependent distribution of DNA content. After drug treatment for 24 and 48 h, 5×10^5 cells were washed in PBS and resuspended in 200 μ L staining solution (containing Annexin V-fluorescein and propidium iodide in HEPES; Annexin V-FLUOS Staining Kit; BD Biosciences). Following incubation at room temperature for 15 min, cells were analyzed by flow cytometry. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with

Annexin V) and necrotic cells (stained with both Annexin V and propidium iodide).

Western blot analysis. Cells were plated in 100-mm dishes 24 h before treatment and then treated with different doses of LBH589, suberoylanilide hydroxamic acid, and suberoyl bishydroxamic acid. After 24 h, cells were lysed in ice-cold radioimmunoprecipitation assay buffer containing 50 mmol/L Tris (pH 7.4), 1% NP-40, 150 mmol/L NaCl, 40 mmol/L NaF, 1 mmol/L Na_3VO_4 , 1 mmol/L EDTA, and 10 μ L/mL protease inhibitor cocktail. For human RCC and mice bearing tumor tissues, 0.5 g tissue was lysed in 300 μ L ice-cold radioimmunoprecipitation assay buffer in a homogenizer. Cell extracts were centrifuged at $10,000 \times g$ at 4°C for 10 min, and supernatants were transferred to new tubes. Protein concentrations were determined using a bicinchoninic acid protein assay method (Pierce). Then, 40 μ g protein from each lysate was separated by SDS-PAGE, transferred to nitrocellulose membranes, incubated with primary antibodies against HDAC1, HDAC3, and HDAC6 (Cell Signaling Technology), Aurora A, p21, acetylated histone H3, phosphorylated histone H3-Ser¹⁰ (Upstate Biotechnology), Aurora B (BD Biosciences), survivin (R&D systems), GAPDH, or actin (Sigma-Aldrich), and visualized using a secondary

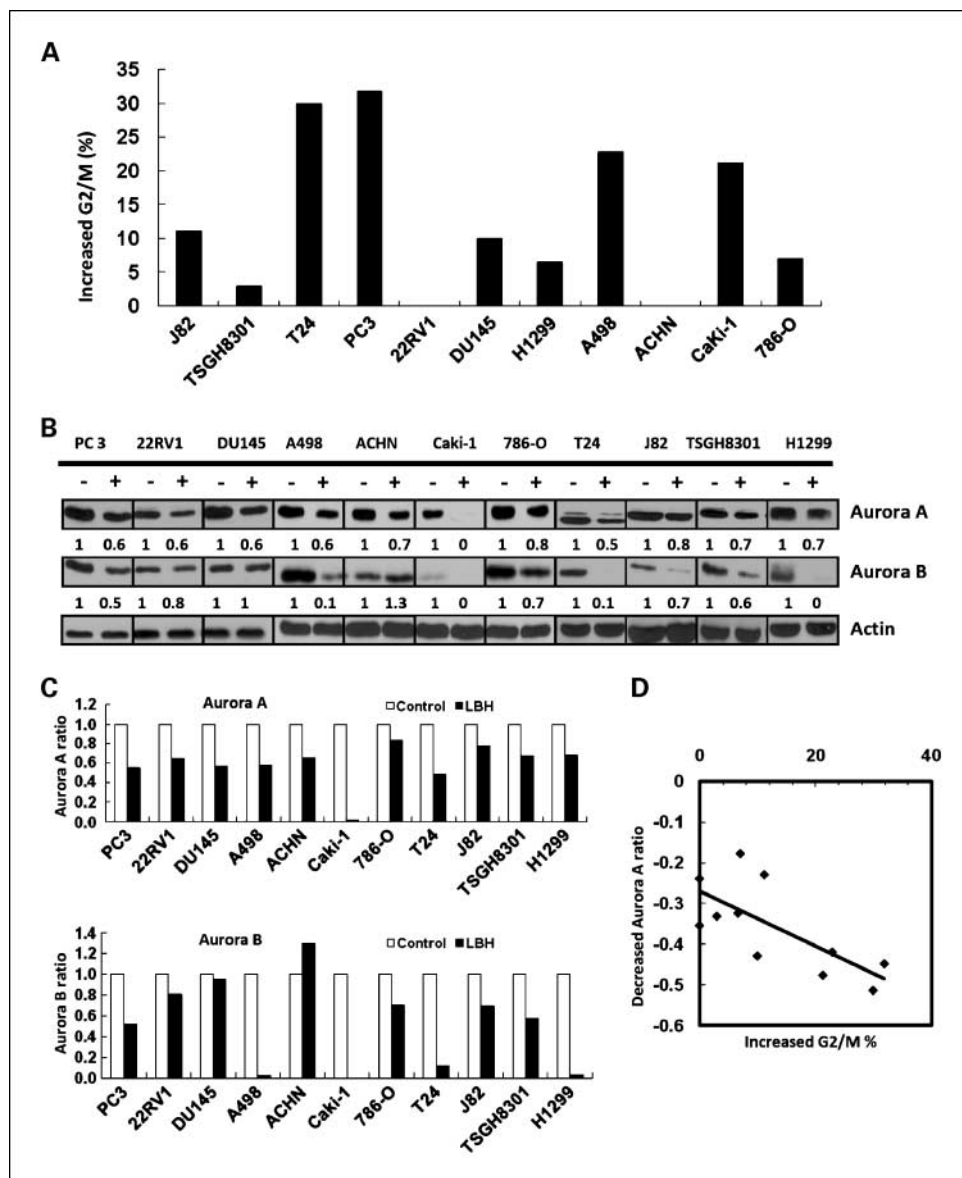
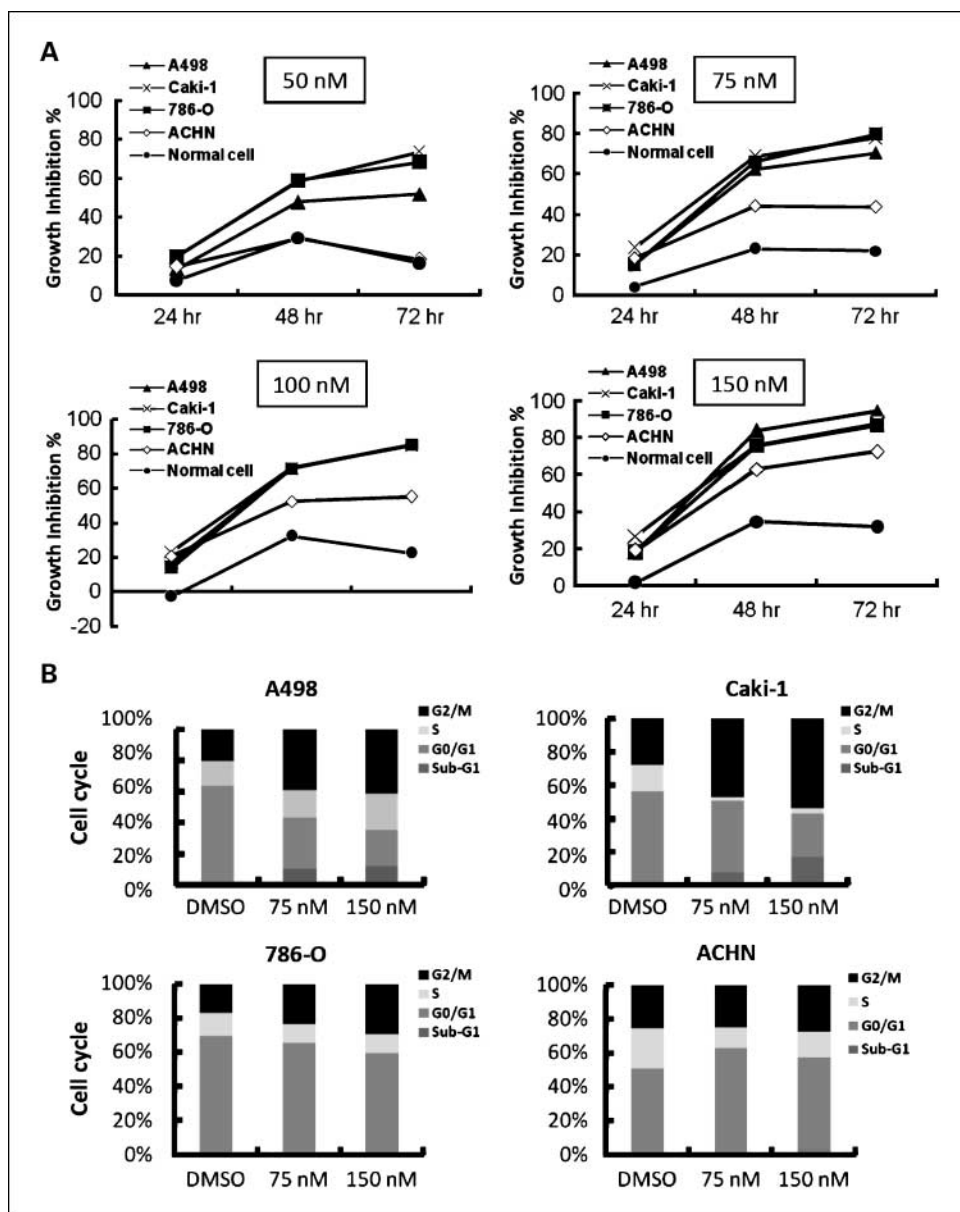


Fig. 1. Effect of LBH589 on G₂-M cell cycle arrest of a panel of cancer cells. *A*, a panel of 11 cancer cell lines, as indicated, was treated with 75 nmol/L LBH589 for 24 h. The increased percentages of cancer cells in G₂-M after LBH589 treatment were determined by flow cytometric analysis and compared with control cells (DMSO-treated defined as 0%). *B*, the same panel of cancer cell lines was treated with solvent (DMSO; -) and 75 nmol/L LBH589 (LBH; +) for 24 h. The Aurora A and B kinase protein levels were analyzed by Western blotting and quantified and plotted as the percentage of the control (DMSO) after normalization with actin. *C*, ratio of Aurora kinase compared with individual control. *D*, ratio between the reduction of Aurora A kinases and the increased percentage of G₂-M-phase cells of 11 cancer cell lines was plotted, and the positive correlation was statistically significant.

Fig. 2. Effects of LBH589 on the growth and cell cycle of renal cancer cells. **A**, renal cancer cells and normal renal epithelial cells, as indicated, were treated with various concentrations (50, 75, 100, and 150 nmol/L) of LBH589 for 24, 48, and 72 h, respectively. The increased percentages of growth inhibition after LBH589 treatment compared with control (DMSO treatment) were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **B**, renal cancer cell lines, as indicated, were treated with 75 or 150 nmol/L LBH589 for 24 h. The cell cycle profiles of cancer cells after LBH589 treatment compared with control (DMSO treatment) were determined by flow cytometric analysis.



antibody coupled to horseradish peroxidase (Jackson ImmunoResearch Labs) and a ChemiLucent Plus Western Blot Enhancing Kit (Millipore). The intensity of the protein signal was quantitated using Fuji Multi Gauge V3.0 Image software.

Transfection with HDAC small interfering RNA. HDAC1, HDAC3, and HDAC6 small interfering RNAs (siRNA) were designed to specifically target sequences of human HDAC1, HDAC3, and HDAC6 mRNAs, respectively. The siRNAs were synthesized by Dharmacon, and scrambled duplex RNA (Dharmacon) was used in the control transfection. The siRNAs were delivered to the cells over 48 h using Lipofectamine 2000 according to the manufacturer's instructions.

Reverse transcription-PCR. Total RNA was extracted from A498 cells using Trizol (Life Technologies), and the first-strand DNA was synthesized with oligo(dT) as primer using 1 μ g total RNA according to the manufacturer's instructions. The conditions for the long PCR products were 3-min denaturation step at 94°C followed by 25 to 30 amplification cycles (30 s at 94°C for denaturation, 30 s at 55°C for primer annealing, and 30 s at 72°C for primer extension) and final extension at 72°C for 7 min. Reverse transcription-PCR was done with primers encoding for Aurora A (5'-gaccgatctaagaagaac-3' and

5'-ctaagactgtttgctagc-3'), Aurora B (5'-tcaggcgacagattgaag-3' and 5'-gcccgagaaggagaactcc-3'), and β -actin (5'-gtggggcgccccaggcacca-3' and 5'-ctccttaatgtcagcagcattc-3').

Xenograft mouse model and human tumor tissues. Male BALB/c nude mice (5-8 weeks old) were used for xenograft experiment. All animal experiments were done in accordance with institutional guidelines for animal welfare. A498 (4×10^6) and 786-O (4×10^6) cells were injected subcutaneously into 5- to 6-week-old male athymic nude mice. One week after cell implantation, animals were randomized into two groups ($n = 6$ each). Each group was treated with intraperitoneal bolus injections of either the drug vehicle (DMSO) or LBH589 (10 mg/kg for A498 cells and 15 mg/kg for 786-O cells) every other day (3 days/wk). Tumors were measured with a caliper once a week, and their volumes were calculated using the formula: $A \times B \times C \times 0.5236$, where A , B , and C are the length, width, and height of the tumor, respectively. The human tissue study was approved by the ethics committee of our institution and informed consent was obtained from the patients. Normal and tumor specimens of kidney were obtained immediately after surgical resection. The tissues were frozen and stored at liquid nitrogen for further Western blotting analysis of Aurora kinases and HDAC expression.

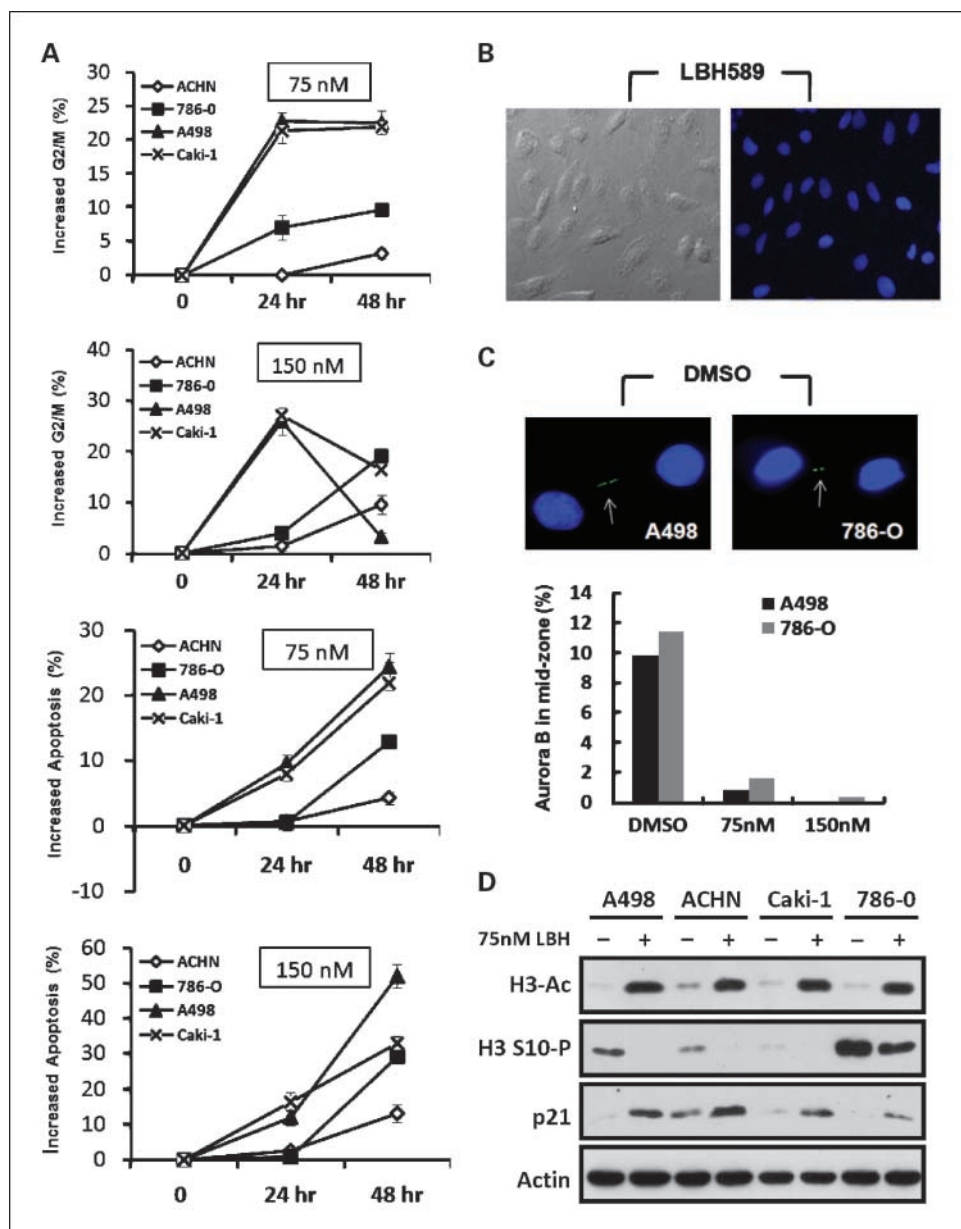


Fig. 3. LBH589 induced G₂-M cell cycle arrest and apoptosis of renal cancer cells. *A*, renal cancer cell lines, as indicated, were treated with 75 or 150 nmol/L LBH589 for 24 and 48 h. *Top two images*, increased percentages of G₂-M population of renal cancer cells after LBH589 treatment compared with control (DMSO treatment) were determined by flow cytometric analysis; *bottom two images*, increased percentages of apoptotic renal cancer cells treated with 75 or 150 nmol/L LBH589 for 24 and 48 h compared with DMSO control were measured by Annexin V assay. *B*, A498 cells were treated with 75 nmol/L LBH589 for 24 h and then fixed and stained with 4',6-diamino-2-phenylindole (*left*, DIC image; *right*, 4',6-diamino-2-phenylindole image). *C*, immunofluorescence staining of A498 and 786-O cells with anti-Aurora B (green) and 4',6-diamino-2-phenylindole (blue) using confocal microscopy. *Arrow*, two cells undergoing end-stage cytokinesis with Aurora B in midbody and the quantitative results were shown in the *bottom*. *D*, A498, 786-O, Caki-1, and ACHN renal cancer cells were treated with solvent (DMSO; -) and 75 nmol/L LBH589 (+) for 24 h. The H3 acetylation, H3-Ser¹⁰ phosphorylation, and p21 protein levels were analyzed by Western blotting.

Immunostaining. The cells were fixed with 4% paraformaldehyde/PBS for 20 min at room temperature and then permeabilized by dipping cells at 0.1% Triton X-100/PBS for 30 min. Further, the cells were washed with 0.1% Tween 20/PBS for 15 min twice. After blocking with 5% bovine serum albumin/PBS, the cells were hybridized with Aurora B antibody at 4°C overnight. Nonspecific binding was removed by washing with 0.1% Tween 20/PBS, and the slides were incubated with anti-mouse conjugated Alexa Fluor 488 antibody and 4',6-diamino-2-phenylindole at room temperature for 3 h. Finally, the cells were washed and analyzed by Zeiss Imager A1 fluorescent microscope.

Statistics. All results from the *in vitro* experiments are presented as mean ± SE. Comparisons were made using Student's *t* tests done using the SigmaPlot and SigmaStats programs.

Results

LBH589 depletes Aurora kinases and induces G₂-M cell cycle arrest of cancer cells. In an initial attempt to investigate the

effect of LBH589 on cell cycle profiles of cancer cells, 75 nmol/L LBH589 was tested in a defined cell line panel consisting of 11 tumor cell lines emanating from different tissues/organs. LBH589 treatment resulted in various degrees of G₂-M arrest in 9 of the 11 cancer cell lines (Fig. 1A). As Aurora kinase A and B are involved in the regulation of G₂-M cell cycle transition, we checked the expression levels of Aurora A and B in cancer cells before and after LBH589 treatment. Interestingly, LBH589 down-regulated the protein levels of both Aurora A and B kinases, but only down-regulation of Aurora A was highly correlated with LBH589-induced G₂-M arrest among the different cancer cell lines (Fig. 1B-D).

Effect of LBH589 on growth and the cell cycle of renal cancer cell lines. As LBH589 has entered into clinical trials for various types of cancer, we wanted to investigate whether LBH589 possesses growth-inhibitory effects on renal cancer cells for which no current demonstrably efficacious therapies are

available. We treated normal renal epithelial cells and four different RCC cell lines, A498, Caki-1, 786-O, and ACHN, with various concentrations of LBH589, and cell viability was measured at different time points (Fig. 2). Generally, LBH589 induced slight growth arrest in normal renal epithelial cells (Fig. 2A, compare *closed circles*). The growth-inhibitory effect of LBH589 was more significant on A498, Caki-1, and 786-O cells. ACHN cells were more resistant to low-dose LBH589 treatment. However, high-dose LBH589 (150 nmol/L) also potently suppressed ACHN cell growth (Fig. 2A). These results indicated that the lethal effects of LBH589 was in a dose- and time-dependent manner and exhibited a selective toxicity toward the renal cancer cell lines.

We further investigated the cell cycle profiles of these RCC cell lines following 75 and 150 nmol/L LBH589 treatment. The LBH589-induced G₂-M arrest was more prominent in Caki-1 and A498 cells followed by 786-O cells. ACHN cells only showed a small proportion of cells arrested in G₂-M phase after 24 h 150 nmol/L LBH589 treatment (Fig. 2B).

LBH589 induced G₂-M arrest and apoptosis of renal cancer cells. We further quantified the increased percentage of G₂-M and apoptosis of four RCC cell lines on 75 and 150 nmol/L LBH589 treatment for 24 and 48 h, respectively (Fig. 3A). Longer periods of 150 nmol/L LBH589 treatment increased 10% G₂-M arrest in resistant ACHN cells and triggered a loss of G₂-M arrest accompanied by a moderate to significant increase

of apoptosis in the A498 and Caki-1 cells (Fig. 3A). The cell morphology and 4',6-diamino-2-phenylindole nuclear staining of A498 and 786-O cells showed that cells under LBH589 treatment retained intact nuclear envelope without any multinucleated cells (Fig. 3B). In normal situation, ~10% to 15% cells undergoing end-stage cytokinesis were stained with Aurora B in midbody and the staining signals were eliminated in A498 and 786-O cells under LBH589 treatment (Fig. 3C). In parallel experiments, LBH589 treatment resulted in increase of histone H3 acetylation and p21 expression of all four RCC cell lines. In addition, the mitotic marker histone H3-Ser¹⁰ phosphorylation levels were decreased in all RCC cells on LBH589 treatment (Fig. 3D). All these results showed that LBH589 treatment resulted in G₂-M cell cycle arrest (most likely arrested in late G₂ phase) of four RCC cell lines.

LBH589 down-regulates Aurora A and B and survivin of renal cancer cells in a dose- and time-dependent manner. As LBH589 treatment resulted in significant G₂-M arrest and apoptosis in Caki-1 and A498 cells, we then checked the dynamic effects of LBH589 on Aurora kinase expression in these two RCC cell lines. We treated Caki-1 and A498 RCC cells with 75 nmol/L LBH589 for indicated periods and found that LBH589 efficiently depleted Aurora kinase A and B in a time-dependent manner (Fig. 4A). We further compared the expression of Aurora A and B kinases in four RCC cell lines under LBH589 treatment. Consistent with cell cycle profiling findings,

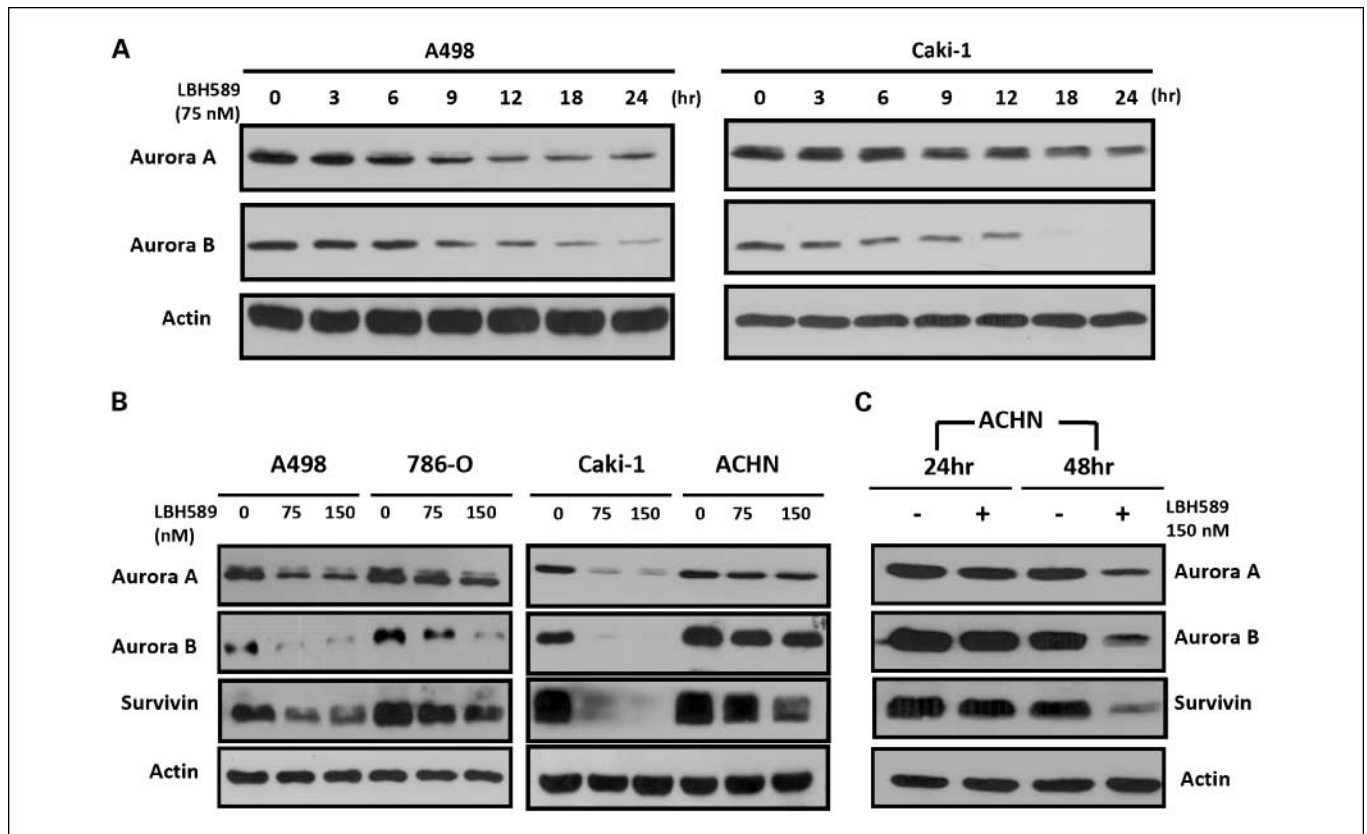


Fig. 4. LBH589 down-regulated Aurora A and B and survivin in renal cancer cells. *A*, A498 and Caki-1 renal cancer cells were treated with 75 nmol/L LBH589 for 0, 3, 6, 9, 12, 18, and 24 h. The Aurora A and B protein levels were analyzed by Western blotting. *B*, A498, 786-O, Caki-1, and ACHN renal cancer cells were treated with solvent (DMSO; 0) and 75 or 150 nmol/L LBH589 (75 and 150, respectively) for 24 h. The Aurora A and B and survivin protein levels were analyzed by Western blotting. *C*, ACHN cells were treated with 150 nmol/L LBH589 for 24 and 48 h. The protein levels, as indicated, were measured by immunoblotting.

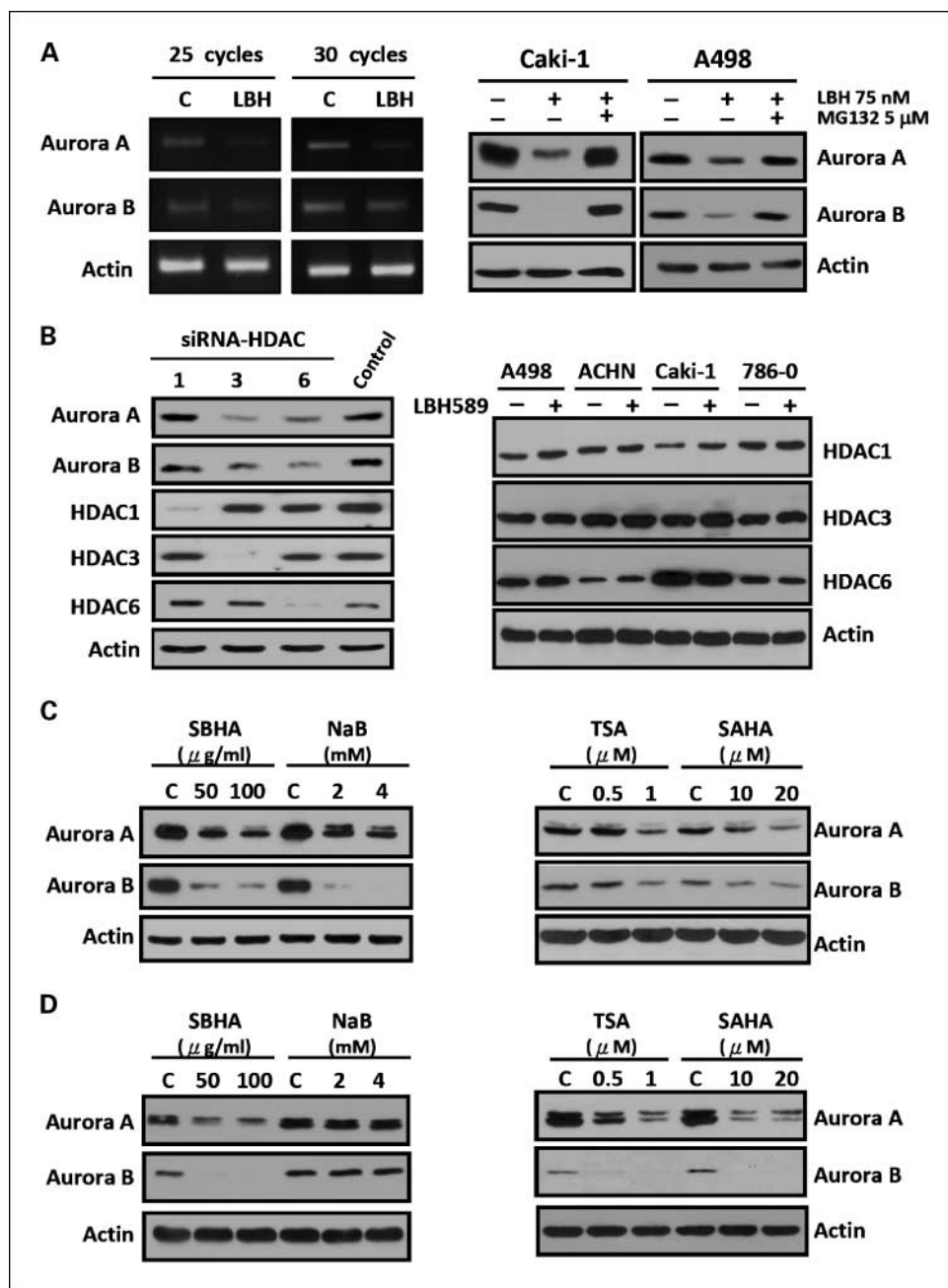


Fig. 5. HDACs suppressed Aurora kinase transcription and induced protein degradation. *A, left*, Aurora A and B mRNA expression in A498 cells was analyzed by reverse transcription-PCR after treatment with solvent (DMSO; C) or 75 nmol/L LBH589 solvent (LBH) for 24 h. The actin expression was measured as internal control. *Right*, A498 and Caki-1 cells were treated with 75 nmol/L LBH589 alone or combined with 5 μmol/L MG132 for 24 h. Cells treated with DMSO were used as control. Aurora A and B protein levels were measured by Western blot analysis. *B, left*, Western blot analysis of HDAC1, HDAC3, HDAC6, and Aurora kinases in 786-O cells transfected with control or indicated HDAC siRNA for 48 h; *right*, A498, 786-O, Caki-1, and ACHN renal cancer cells were treated with solvent (DMSO; -) and 75 nmol/L LBH589 (+) for 24 h. The HDAC1, HDAC3, HDAC6, and Aurora kinases in 786-O cells were treated with the indicated concentrations of sodium butyrate, suberoylanilide hydroxamic acid, and suberoyl bishydroxamic acid for 24 h. Aurora A and B protein levels were measured by Western blot analysis. Equivalent protein loading was confirmed using an antibody for actin.

LBH589-induced down-regulation of Aurora A and B was more prominent in Caki-1 and A498 cells, followed by 786-O cells, but was barely seen in ACHN cells after 24 h (Fig. 4B). As LBH589 treatment also induced apoptosis of RCC cells, we also examined the survival factor survivin in RCC cells treated with LBH589. Indeed, LBH589 treatment resulted in down-regulation of survivin, with the same pattern as seen for Aurora kinases in RCC cells (Fig. 4B). Most importantly, longer exposures to LBH589 treatment also down-regulated Aurora A and B and survivin in ACHN cells, which was consistent with the results showing G₂-M arrest of ACHN cells induced by longer exposures to LBH589 treatment seen in the cell cycle profile experiments (Fig. 4C).

LBH589 inhibits HDAC3 and HDAC6 to suppress the protein stability of Aurora kinases. To investigate the molecular

mechanisms involved in LBH589-mediated Aurora kinase protein depletion, we first investigated whether LBH589 had an effect on Aurora kinase mRNA expression. We analyzed Aurora kinase mRNA levels by reverse transcription-PCR in A498 cells treated with 75 nmol/L LBH589 for 24 h and found that LBH589 slightly reduced Aurora A and B kinases mRNA to 83% and 92% of control levels, respectively (Fig. 5A, left). We then further examined the effects of LBH589 on Aurora kinase protein stability. Treatment with the proteasome inhibitor MG132 resulted in marked suppression of LBH589-induced Aurora A and B proteins depletion in both Caki-1 and A498 cells (Fig. 5A, right, compare lanes 2 and 3). These results indicate that LBH589-mediated down-regulation of Aurora A and B proteins was mainly through a proteasome-mediated protein degradation pathway. LBH589 has been shown

previously to potently inhibit HDAC1, HDAC3, and HDAC6 activities (35). To examine whether inhibition of HDACs by LBH589 plays a role in the regulation of Aurora kinase depletion, we used siRNA to knock down the HDAC levels and then checked the Aurora A and B expression in 786-O cells. Knockdown of HDAC1 by siRNA did not affect Aurora A and B protein levels, whereas knockdown of HDAC3 and HDAC6 significantly down-regulated Aurora A and B protein levels (Fig. 5B, left). In addition, we noted that HDAC6 was expressed at greater levels in A498 and Caki-1 cells than in 786-O and ACHN cells (Fig. 5B, right), which may be responsible for the more dramatic Aurora protein depletion and G₂-M arrest induced by its inhibition on HDAC1 treatment. We further investigated whether other types of HDACs have the similar effects on degradation of Aurora proteins; four different HDACs, including suberoylanilide hydroxamic acid, sodium butyrate, trichostatin A, and suberoyl bishydroxamic acid, were

examined. The down-regulation of Aurora A and B was also observed by four different HDACs with various potencies in A498 and Caki-1 cells (Fig. 5C and D).

LBH589 suppresses A498 and 786-O tumor growth in a mouse xenograft model. The above results established a novel molecular mechanism of how LBH589 may down-regulate Aurora proteins through the inhibition of HDAC3 and HDAC6 deacetylase activity and inhibit RCC cell growth in an *in vitro* cell culture system. To investigate further the *in vivo* antitumor activity of LBH589, we chose A498 and 786-O xenografts as an animal model. LBH589 showed significant antitumor activity in mice bearing A498 or 786-O xenografts at the relatively low doses of 10 and 15 mg/kg, respectively (Fig. 6A). In addition, we also examined the *in vivo* effect of LBH589 on down-regulation of Aurora kinases in mice bearing A498 tumors treated with three times intraperitoneal injection of either DMSO or 10 mg/kg LBH589, respectively. Western blotting

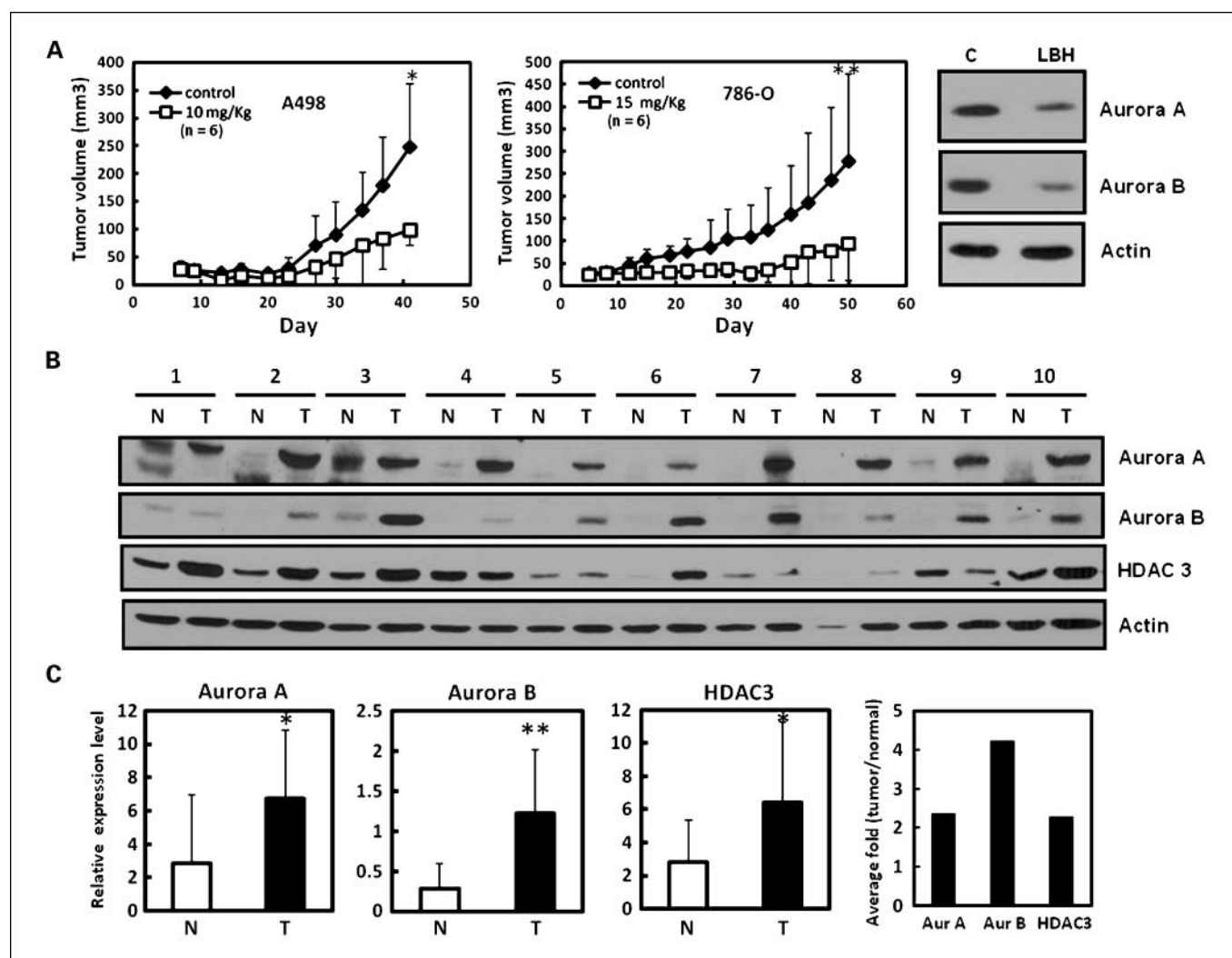


Fig. 6. LBH589 suppresses renal tumor growth *in vivo* and Aurora kinases and HDAC3 overexpress tumor growth in human RCC tumor tissues. **A**, *in vivo* antitumor activity of LBH589 was tested on nude mice bearing A498 (left) and 786-O (middle) xenografts receiving intraperitoneal injection of 10 and 15 mg/kg LBH589 or DMSO every other day, respectively. The tumor volumes of mice treated with LBH589 or DMSO were measured (*, $P < 0.01$; **, $P < 0.001$). **Right**, Aurora A and B protein levels of A498 tumors harvested from mice treated three times with DMSO or LBH589 were determined by Western blotting. **C**, DMSO treatment; **LBH**, 10 mg/kg LBH589 treatment. **B**, Western blotting analysis of Aurora A and B and HDAC3 expression levels in 10 pairs of normal and tumor tissues obtained from patients with RCC. **N**, normal tissue; **T**, RCC tissue. **C**, Aurora A and B and HDAC3 protein levels were quantified using Fuji Multi Gauge V3.0 Image software and normalized with actin in normal and RCC tissues. The average increased folds of Aurora A and B and HDAC3 in RCC tissues compared with normal counterparts (*, $P < 0.05$; **, $P < 0.005$).

analysis of fresh tumors harvested from aforementioned experiment showed that LBH589 efficiently down-regulated Aurora A and B kinases *in vivo* (Fig. 6A, right). These results showed that LBH589 possesses potent antitumor effects on these two RCC cell lines *in vivo*. As LBH589 can efficiently inhibit HDAC3 and HDAC6 to deplete Aurora A and B proteins in RCC cells, we then investigated the expression of Aurora A and B and HDAC3 proteins in 10 pairs of normal kidney and RCC tumor tissues in the Western blot analysis (Fig. 6B). We further quantified the protein levels of Aurora A and B and HDAC3 between renal cancer and normal tissues, which showed that protein expression of Aurora A and B and HDAC3 was increased by ~2-, 4-, and 2-fold in the tumor samples compared with normal tissues, respectively (Fig. 6C). These results indicate that LBH589 may have the potential to effectively treat advanced RCCs, especially those expressing high levels of Aurora proteins and HDACs.

Discussion

HDACI-induced G₁-S cell cycle arrest of cancer cells is a common event that is proposed to result from direct effects on chromatin architecture at specific loci leading to changes in gene expression (2). In contrast, G₂-M cell cycle arrest, although less common and poorly understood, can be observed in various types of cancer cells on treatment with diverse types of HDACIs. In addition to transcriptional regulation, there is growing evidence for nonhistone targets regulating diverse cellular biological processes that may be responsible for HDACI-mediated G₂-M cell cycle arrest. Recently, Li et al. showed that the HDAC3 complex with AKAP95/HA95 deacetylates histone H3 and provides a hypoacetylated H3 tail that is the preferred substrate for Aurora B, suggesting that Aurora B protein is one of the targets for HDAC-mediated G₂-M cell cycle arrest (13). However, this mechanism cannot explain why some cancer cells under HDACI treatment associated with strong H3 hyperacetylation and hypophosphorylation did not arrest in G₂-M phase.

In this study, we showed that a novel HDACI, LBH589, and other HDACIs could target the nonhistone proteins Aurora A and B kinase and induce various degrees of G₂-M cell cycle arrest in different RCC cell lines irrespective of their hyperacetylation and hypophosphorylation states. Meanwhile, we could not find remarkable changes in the expression levels of cdc2, cyclin B, and their activities (data not shown). The mechanism underlying the induction of G₂-M arrest by LBH589 seems to be independent of transcription (data not shown). LBH589 treatment resulted in down-regulation of Aurora A and B protein levels in different RCC cell lines that were highly correlated with the severity of G₂-M arrest. The depletion of Aurora A and B by LBH589 can also be seen in the more resistant cell line ACHN as long as treatment continued for a longer period. These results show that prolonged LBH589 exposure efficiently depletes Aurora kinases and inhibits cell growth in more resistant RCC cells, which suggests that LBH589 has an advantage for application in clinical practice for RCC treatment. Meanwhile, LBH589 also down-regulated Aurora kinases and induced G₂-M cell cycle arrest in other cancer cell lines. The decreased levels of Aurora kinases induced by LBH589 have a tendency to correlate with the severity of G₂-M cell cycle arrest in different cancer cell lines. Interestingly, we

did observe LBH589-mediated G₂-M arrest of some cancer cell lines without inducing Aurora protein depletion.⁷ HDACI-induced G₂-M cell cycle arrest of cancer cells through Aurora protein depletion-independent pathway may exist and needs further elucidation. All these results suggest that HDACI-induced G₂-M arrest through down-regulation of Aurora proteins is a common but still cell type-specific phenomenon.

LBH589 depleted Aurora kinases through a proteasome-mediated pathway in a dose- and time-dependent manner. A recent study has shown that HDACIs LAQ824 and SK-7068 can induce Aurora A degradation in several cancer cell lines, but not in nontransformed epithelial cells, which is consistent with our findings. However, they did not observe Aurora B degradation under these HDACI treatments (14). It is plausible that different types of HDACIs may preferentially target different HDACs that are expressed at various levels in cancer cells and are responsible for the selectivity and specificity of Aurora protein depletion. Overexpression of Aurora A and B kinases has been found in many types of cancer, and their activities are responsible for tumorigenicity, which make them perfect targets for cancer therapy. Aurora kinase inhibitors have been developed and are under preclinical and clinical investigation (36–42). Our findings show that LBH589 can induce Aurora protein degradation and then promote G₂-M cell cycle arrest and apoptosis of RCC cells in a short period. These results indicate that LBH589 may have superiority in inhibiting cancer growth, especially cancers with high expression levels of Aurora proteins. Previous studies have shown that Aurora A kinase is overexpressed in RCC (30, 43). Indeed, we compared a subset of paired fresh normal and RCC tumor tissues, which showed increased expression of Aurora A and B and HDAC3 proteins in the cancer tissues. Meanwhile, our *in vivo* data showed that LBH589 can efficiently suppress RCC tumor growth in low-dose ranges, which suggests that LBH589 may have the potential for clinical application in the treatment of RCC. In addition, the expression levels of HDAC3, HDAC6, and Aurora proteins in tumor tissues are worthy further investigating whether they can serve as therapeutic markers for the application of LBH589 or other HDACIs to give more specific and effective targeted therapies for metastatic RCC or other types of cancer.

HDACs have traditionally been linked to gene repression through deacetylation of core histone. However, the nonhistone substrates of HDACs have been rapidly discovered to be involved in many important cellular functions. Deacetylation of heat shock protein 90 by HDAC6 is responsible for stabilization of many client proteins such as Bcr-Abl, ErbB2, Raf, and Akt (8, 44–46). Park et al. have shown that knockdown of HDAC6 induces Aurora A degradation. They also showed that the HDACIs LAQ824 and SK-7068 induce heat shock protein 90 acetylation via inhibition of HDAC6 and subsequently enhance the association of heat shock protein 70 with Aurora A, leading to proteasome degradation of client Aurora A, whereas knockdown of HDAC6 induced both Aurora A and B protein degradation in our system. Meanwhile, knockdown of HDAC3 also revealed dramatic depletion of both Aurora A and B proteins. Beckers et al. recently

⁷ Our unpublished data.

investigated the isoenzyme selectivity of HDACs and showed that the hydroxamates suberoylanilide hydroxamic acid, LAQ824, and LBH589 give broad inhibition of class I and II enzymes (35). We also observed that the hydroxamate HDACs showed potent effects by inducing Aurora kinase degradation in RCC cells much greater than those of sodium butyrate and the benzamide trichostatin A. These results suggest that selective inhibition of isoenzymes by different HDACs may contribute to their various potencies in inducing Aurora protein degradation and G₂-M cell cycle arrest. Interestingly, LBH589 and LAQ824 belong to the same category of HDAC, LBH589 can induce both Aurora A and B degradation, but LAQ824 only induces degradation of Aurora A. Why they possess different capabilities to selectively deplete Aurora proteins and why the knockdown of different HDACs induced selective degradation of Aurora protein are not known, and these discrepancies are currently under investigation. Another important question worthy of further investigation is whether Aurora A and B proteins can be acetylated and served as direct substrates for HDACs.

In conclusion, our findings show that LBH589 and other HDACs can induce G₂-M cell cycle arrest and apoptosis in RCC cells through degradation of Aurora A and B kinases by inhibition of HDAC3 and HDAC6. The clinical efficacy of LBH589 in the treatment of patients with metastatic RCC, especially those with high Aurora and HDAC protein expression, is worthy of further investigation. The current elucidation of the mechanisms of how HDACs mediate G₂-M cell cycle arrest and apoptosis of cancer cells also provides a new paradigm to further design more ideal HDACs for targeted cancer therapy in the near future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Correction: Article on HDACIs and Aurora Kinases

In the article by Cha and colleagues in the February 1, 2009, issue of *Clinical Cancer Research*, the authors' affiliations footnote listed an incorrect country name. The correct affiliation footnote is as follows: **Authors' Affiliations:** ¹Graduate Institute of Life Sciences, ²Division of Urology, Department of Surgery, Tri-Service General Hospital, and ³Department of Biochemistry, National Defense Medical Center; ⁴Agricultural Biotechnology Research Center, Academia Sinica; ⁵Taipei City Hospital, Taipei, Taiwan, Republic of China and ⁶Division of Urology, Department of Surgery, Chi Mei Medical Center, Tainan, Taiwan, Republic of China.

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