HDAC Inhibitors Synergize Antiproliferative Effect of Sorafenib in Renal Cell Carcinoma Cells

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Abstract. *Aim: To investigate the anticancer effect of histone deacetylase inhibitors (HDACIs) in combination with sorafenib in wild-type and mutant von Hippel-Lindau (VHL)-expressing renal cell carcinomas (RCCs). Materials and Methods: We exposed clear cell RCC cells to HDACIs (vorinostat or belinostat) or sorafenib. We performed 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays, western blotting, flow cytometry and enzyme-linked immunosorbent assays (ELISA) to evaluate mechanisms of cell death, and used CalcuSyn to analyze the potential synergism. Results: HDACIs alone inhibited the growth of clear cell RCC cell lines, increased acetylation of histone 3 and of tubulin, activated caspases-8, -9, and -3, and augmented the sub-G_1 population, independently of VHL and permeability glycoprotein (P-gp). Moreover, pre-treatment of Caki-1 (wild-type VHL) and 786-O (mutant VHL) with HDACIs followed by sorafenib reduced cell viability synergistically via activation of caspases and downregulation of the levels of myeloid leukemia cell differentiation protein (MCL1), phospho-extracellular signal-regulated kinase (ERK), and secreted vascular endothelial growth factor (VEGF). Conclusion: Sorafenib is more effective in combination with HDACIs even for clear cell RCCs harboring mutant VHL.*

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The incidence of renal cell carcinoma (RCC) has increased over the last 20 years and now accounts for 2% of all adult malignances (1-3). Seventy-five percent of RCCs are clear-cell RCCs (CCRCCs) and are associated with the loss of the von Hippel-Lindau (*VHL*) gene together with overexpression of hypoxia-inducible factors (HIFs), activation of vascular endothelial growth factor (VEGF), and with intense vascularity. Sorafenib is a small molecular multi-kinase inhibitor of VEGF receptor (VEGFR) 1, 2, and 3, platelet-derived growth factor receptor-β (PDGFRβ), RET, c-KIT, and RAF (4). It was approved as a second-line therapy, for the treatment of the patients with advanced CCRCC whose previous therapies had failed in 2005 by the US Food and Drug Administration (FDA) (5). But it led to only partial response rates (~10%) and caused quite serious adverse events (6). Although the median progression-free survival (PFS) period is prolonged to 5.5 months by sorafenib, compared to of 2.8 months in placebo-treated patients, there remains an unmet need for treatment options for patients with RCC.

One area of intense research interest in cancer is the role of epigenetic changes caused by modifications of histone proteins. Acetylation of histones lowers the affinity of histones for DNA, producing a more open DNA structure that facilitates gene expression (7). Histone deacetylases (HDACs) are overexpressed in many kinds of cancer including RCC, and repress genes involved in tumor suppression and differentiation (7, 8). HDAC inhibitors (HDACIs) are considered to be anticancer drugs. HDACIs also increase acetylation of non-histone proteins, including tubulin, DNA repair proteins (*e.g.* Ku-70), chaperone proteins [*e.g.* Heat shock protein 90 (HSP90)], and transcription factors [*e.g.* p53 and Nuclear Factor-kappa B (NF-κB)], modulating the activities of these proteins and ultimately causing cancer cell death (9, 10). The HDACI, vorinostat was approved by the US FDA in 2006 for the treatment of advanced cutaneous T-cell lymphoma, and belinostat is currently undergoing phase I and II clinical trial

for the treatment of various cancers (11). There has been little research about the anticancer effects of HDACIs in RCC cells. Moreover, the mechanisms of cytotoxicity induced by sorafenib in combination with vorinostat or belinostat in RCC cells has received little research attention.

In this study, we investigated how HDACIs alone inhibit the growth of CCRCC cells and examined the mechanisms underlying the synergistic antiproliferative effects of combined HDACI and sorafenib treatment.

Materials and Methods

Cell culture and anticancer drugs. The CCRCC cell lines A498, ACHN, Caki-1, SN12C, TK-10, RXF393, UO31, and 786-O were received from the National Cancer Institute (Fredrick, MD, USA) and were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Vorinostat and belinostat were synthesized by Crystal Genomics (Seoul, Korea) and sorafenib was provided by Bayer Healthcare Pharmaceutical (Montville, NJ, USA). Cells were treated with vorinostat, belinostat or sorafenib in 5% (v/v) FBS-containing RPMI 1640 medium. For sequential combination treatment with HDACI and sorafenib cells were exposed to the former drug for 24 h and were then exposed to the next drug for additional 48 h.

Cell proliferation assay and combination index analysis. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt (MTS) assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. Cell viability was determined from the absorbance of each well relative to the average absorbance of control wells (defined as 100% viability). The combination indexes (CIs) were computed using the CalcuSyn software (Biosoft, Cambridge, UK). A CI>1 indicates antagonism; a CI=1 indicates an additive effect; and a CI<1 indicates synergism.

Flow cytometric analysis. Cells were fixed with 70% ethanol overnight at 4˚C, and stained with 60 μg/ml propidium iodide (Sigma, St. Louis, MO, USA) containing 10 U/ml RNaseA for 30 min. The percentage of cells in sub-G_1 phase (apoptotic cells) was measured by counting 10,000 cells using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with the ModFit LT 3.0 software.

Western blotting. Cells were lysed in a buffer containing 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail tablet (Roche, Mannheim, Germany). Western blotting was performed as described previously (12). Antibodies to acetylated histone 3 (Ac-H3, Lys18); histone 3; procaspase-3, -8, -9; cleaved caspase-3, -8, -9; α-tubulin; phospho-ERK; and ERK were purchased form Cell Signaling (Beverly, MA, USA). Antibodies against β-actin, acetylated tubulin (Sigma), and myeloid cell leukemia 1 (MCL1) (Santa Cruz Biotechnology, Santa Cruz, CA) were also used.

Quantification of VEGF released by cultured cells. The amount of VEGF secreted by the cultured cells was determined using the human VEGF DuoSet enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical analysis. All data are presented as the means±SD of three individual experiments. Statistical significance ($p<0.05$) was determined by one-way ANOVA.

Results

Antiproliferative effects of HDACIs in CCRCC cell lines. Eight CCRCC cell lines were treated with the indicated concentrations of belinostat or vorinostat for 48 h, and the viability of cells was measured by the MTS assay (Figure 1A and B). These HDACIs effectively induced cell death in all cell lines. The half maximal inhibitory concentration (IC_{50}) values for inhibition of cell proliferation by HDACIs are summarized in Table I. The HDACIs inhibited the cell viability efficiently in CCRCC cells, independently of VHL status (wild-type or loss of function mutation). They also induced cell death in UO31 cells which express high levels of permeability glycoprotein (P-gp), a multi-drug resistance (ABCB1) gene product

We selected four CCRCC cell lines: two HDACI-sensitive, Caki-1 and SN12C (wild-type VHL); one relatively HDACI-resistant, 786-O (mutant VHL); and the high P-gp-expressing UO31 cells for further study. To assess the inhibitory effects of HDACIs on intracellular HDAC activity, we analyzed the levels of Ac-H3 and those of acetylated tubulin by western blot analysis in four cell lines exposed to 1 or 10 μM belinostat or vorinostat for the indicated times (Figure 2A and B). HDACIs effectively increased acetylation of H3 and tubulin in all tested cell lines, indicating that HDACIs effectively blocked HDAC activity in these cells.

Analysis of cell cycle distribution by flow cytometry in Figure 3A indicated that 1 or 10 μM HDACI increased the apoptotic sub-G_1 population in all tested cells. In addition, exposure of CCRCC cells to HDACIs reduced the levels of pro-caspase-8, which plays a role in the extrinsic apoptotic pathway, and increased those of cleaved caspase-3 and -9, which are involved in the intrinsic apoptotic pathway (Figure 3B).

Synergistic antiproliferative effect of combined treatment with HDACI and sorafenib. Because HDACIs are cytotoxic toward many CCRCC cells, we tested whether those molecules potentiate cell death induced by sorafenib in the presence or absence of VHL. Caki-1 (wild-type VHL) cells were treated with different concentrations of HDACIs for 24 h, followed by treatment with 10 μM sorafenib for 48 h additionally (Figure 4A). These combinatorial treatments significantly increased cytotoxicity in Caki-1 cells compared with HDACI and sorafenib alone (Figure 4A). All CI values were less than 1, indicating that the combination was strongly synergistic (Figure 4A). In 786-O cells (mutant VHL), HDACI pre-treatment was moderately synergistic (Figure 4B). The synergistic effect of vorinostat in Caki-1 and 786-O cells was similar to that of belinostat, although

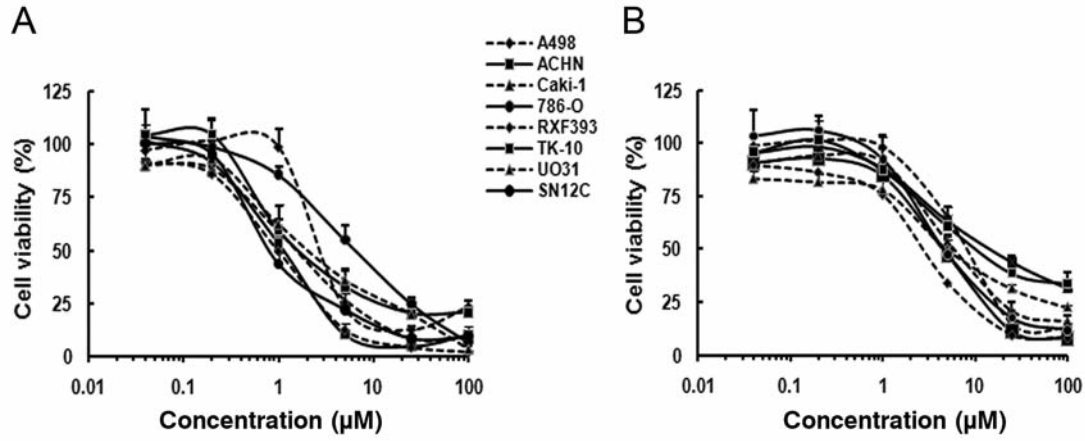

Figure 1. *The antiproliferative effects of histone deacetylase inhibitors (HDACI) on clear cell renal cell carcinoma (CCRCC) cells. A498, ACHN, Caki-1, 786-O, RXF393, TK-10, UO31, and SN12C cells were exposed to 0.04, 0.2, 1, 5, 25, and 100 μM belinostat (A) or vorinostat (B) for 48 h. Cell viability, expressed as the mean±SD (n=4), was assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt (MTS) assays.*

the concentration of vorinostat required to achieve this effect was higher than that of belinostat (Figure 4A and B).

Next, we analyzed the mechanisms of cell death in Caki-1 and 786-O cells exposed to belinostat in combination with sorafenib. Sequential exposure of these cells to belinostat, followed by sorafenib revealed activation of caspases-3, -8, and -9 compared to cells treated with belinostat, or sorafenib alone (Figure 5A). We measured the levels of MCL1 and phospho-ERK, which play roles in survival and proliferation of cells (Figure 5B and C). Combinatorial treatment reduced the levels of MCL1 and phospho-ERK compared to those of control cells, and cells treated with either drug alone. Finally, we measured the amount of VEGF in the medium, and found that combinatorial treatment significantly reduced VEGF secretion (Figure 5D). Collectively, these results suggest that combinatorial treatment with belinostat and sorafenib induces cell death by reducing the levels of MCL1, phospho-ERK, and VEGF, and by activating caspases in CCRCC cells.

Discussion

Multi-tyrosine kinase inhibitor, sorafenib has recently been introduced for the therapy of RCC (2, 13). Although the use of such drugs has improved clinical response rates, long-term results are unsatisfactory; thus, novel strategies to improve clinical outcomes are necessary. Because HDACIs have been reported to induce apoptosis, reduce tumor growth, and inhibit angiogenesis in hematological malignancies and solid tumors (10, 11, 14), in the current study we evaluated the antitumor effects of HDACIs alone, as well as in combination with sorafenib in CCRCC cells.

First, we examined the possible use of HDACIs as a monotherapy for CCRCC. HDACIs inhibited the growth of CCRCC cells in a concentration- and time-dependent manner (Figure 1A and B), and induced apoptosis *via* intrinsic and extrinsic pathways (Figure 3A and B) in four selected CCRCC cell lines, even in 786-O cells harboring mutant VHL. These results suggest the possible use of HDACIs for the therapy of patients with CCRCC, regardless of whether their tumors have functional VHL. In addition, the observation that HDACIs kill UO31 cells is consistent with reports that these drugs are not substrates of MDR proteins (15, 16). These results predict that HDACIs could be used against tumors previously treated with MDR-inducing chemotherapeutic agents.

Next, we examined whether HDACIs potentiated the antitumor effects of sorafenib, an agent widely used in patients with advanced CCRCC whose previous therapies have failed. Recently, it was reported that HDACIs potentiate the antiproliferative effects of sorafenib in hepatic, renal, and pancreatic adenocarcinoma (vorinostat) (17, 18); in

Table I. *The half maximal inhibitory concentration (IC_{50}) values (μM) of HDACIs for clear cell renal cell carcinoma cell lines.*

Cell line	Belinostat	Vorinostat	VHL
ACHN	1.3	4.5	WT
Caki-1	1.6	5.8	WT
RXF393	1.0	3.0	WT
SN12C	0.8	4.7	WT
TK-10	1.6	10.0	WT
UO31	2.0	5.0	WT
A498	2.8	8.0	MT
786-O	6.5	16.0	MT

VHL, von Hippel-Lindau; WT, wild-type; MT, mutant-type.

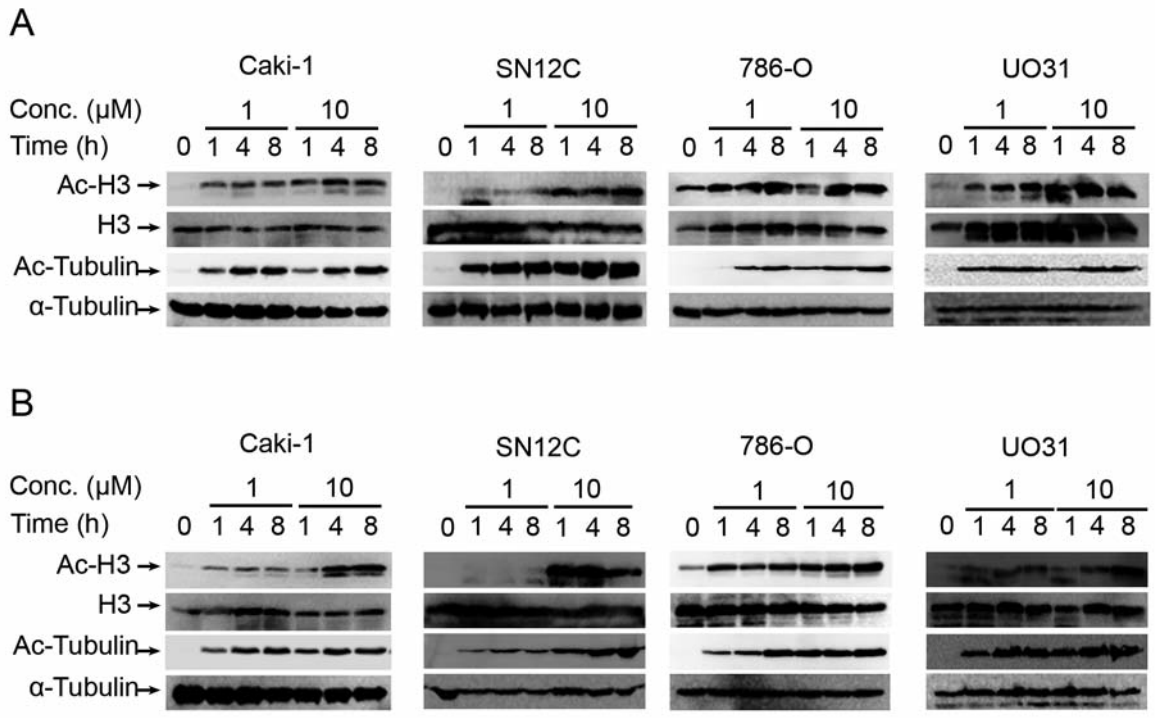

Figure 2. *The effects of histone deacetylase inhibitors (HDACI) on acetylation of histone 3 and tubulin. Western blots of acetylated histone 3 (Ac-H3), histone 3 (H3), acetylated tubulin (Ac-tubulin), and tubulin were carried out in Caki-1, SN12C, 786-O, and UO31 cells, treated with 1 or 10 μM belinostat (A) or vorinostat (B) for the indicated times. α-Tubulin was used as a loading control.*

cholangiocarcinoma (entinostat) (19); and in glioblastoma (valproic acid) (20). In accordance with these results, we found that exposure of belinostat- or vorinostat-pretreated cells to sorafenib led to a synergistic decrease of cell viability, in both wild-type VHL Caki-1 cells and in mutant VHL 786-O cells (Figure 4A and B). Because the IC_{50} and CI values showed that belinostat was more efficacient than vorinostat in RCC cells (Figure 4), we focused on belinostat and sorafenib in analysing the mechanisms by which the combination treatment induced cell death.

Given that HDACIs alone activated caspases-3, -8, and -9 (Figure 3B), we examined whether exposure of belinostat-treated cells to sorafenib also potentiated the activation of these caspases. This combination reduced the expression of pro-forms and induced active cleaved forms of all three caspases in Caki-1 and 786-O CCRCC cells, indicating activation of both extrinsic and intrinsic apoptosis pathways (Figure 5A). To elucidate the underlying signaling pathways, we first analyzed the levels of the anti-apoptotic BCL-2 family protein, MCL1, which is a short-lived protein susceptible to down-regulation by agents that disrupt its transcription, translation, and post-translational modification (17, 18). The second overlapping signaling is the RAS-RAF-MAP kinase pathway, which plays a central role in the survival of cancer cells. We found that combined treatment with belinostat and sorafenib significantly reduced the levels of MCL1 and phospho-ERK in CCRCC cells compared to those in cells treated with either drug alone, regardless of VHL status (Figure 5B and C). The third convergent target inhibited by sorafenib and HDACIs is VEGF signaling. It has been reported that HDACIs attenuate secretion of VEGF by inhibiting HIF-1α activity (21, 22), and sorafenib reduces secretion of VEGF from neuroblastoma tumor cells (23). In accordance with these reports, our data show that combination treatment reduced the secretion of VEGF in Caki-1 and 786-O cells (Figure 5D). These results indicate that belinostat and sorafenib interact in a synergistic fashion *via* down-regulation of MCL1, phospho-ERK, and VEGF in CCRCC cells. The evidence that sorafenib and belinostat impinge on multiple overlapping signaling pathways that have been implicated in tumor cell survival increases the likelihood that a combined treatment regimen might be more effective than monotherapy. Although further studies are required to establish an effective therapeutic approach for combined treatment with belinostat and sorafenib in CCRCC, our results reveal that the effects of sorafenib in CCRCC, whether VHL is functional or not, are highly potentiated by combination with HDACIs, especially belinostat. These pre-clinical findings suggest the possibility of using this drug combination as a new therapeutic modality for CCRCC.

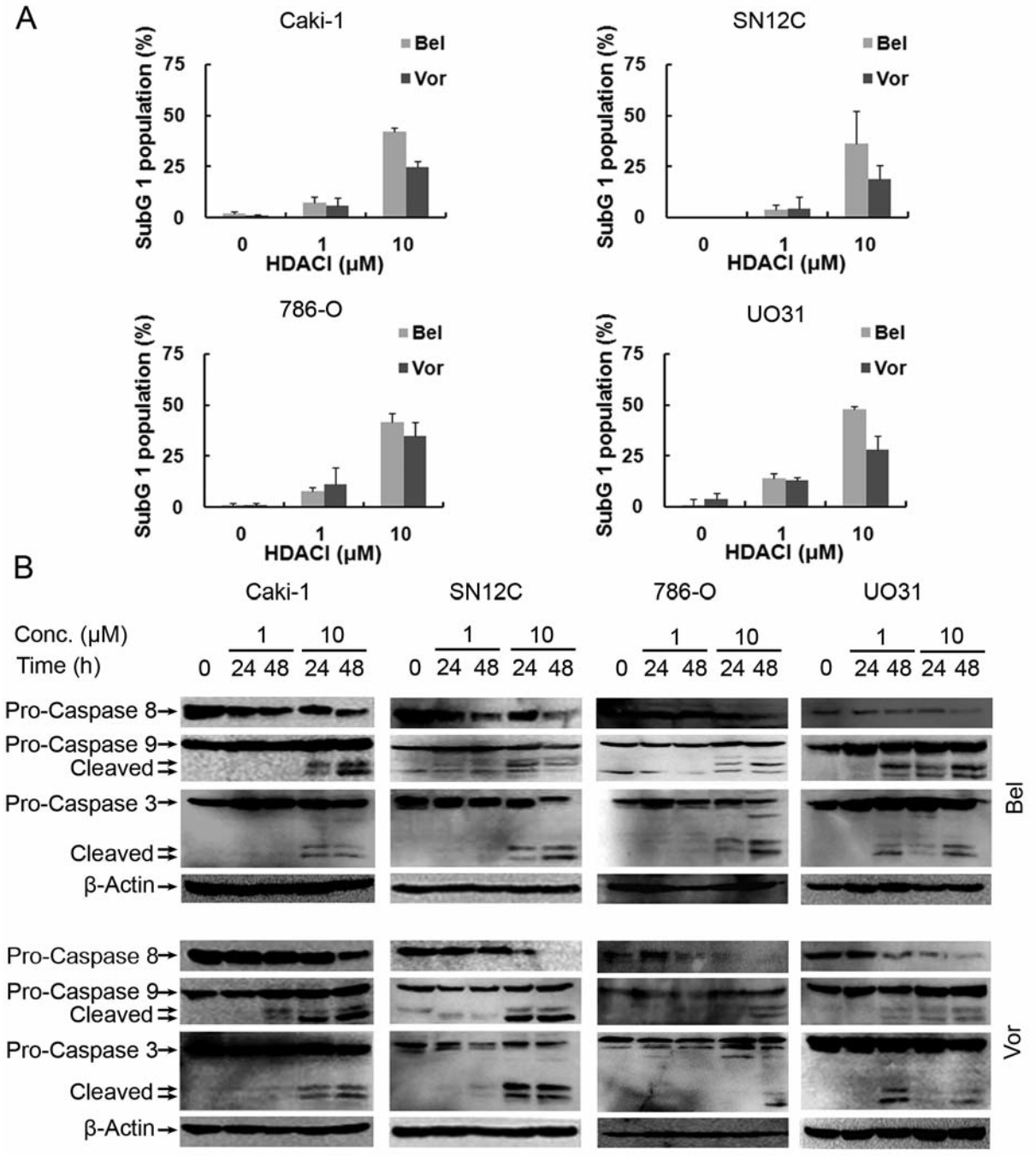

Figure 3. *The apoptosis-inducing effects of histone deacetylase inhibitors (HDACI) in clear cell renal cell carcinoma (CCRCC) cell lines. A: Bars (means±SD; n=2) denote the sub-G_1 population in Caki-1, SN12C, 786-O, and UO31 cells treated with 1 or 10 μM belinostat or vorinostat for 48 h. The sub-G_1 population is presented as a percentage of the total number of cells analyzed. B: Caspases-3, -8, and -9 were analyzed by western blotting in four cell lines following exposure to 1 or 10 μM belinostat or vorinostat for the indicated times. β-Actin was used as a loading control.*

Acknowledgements

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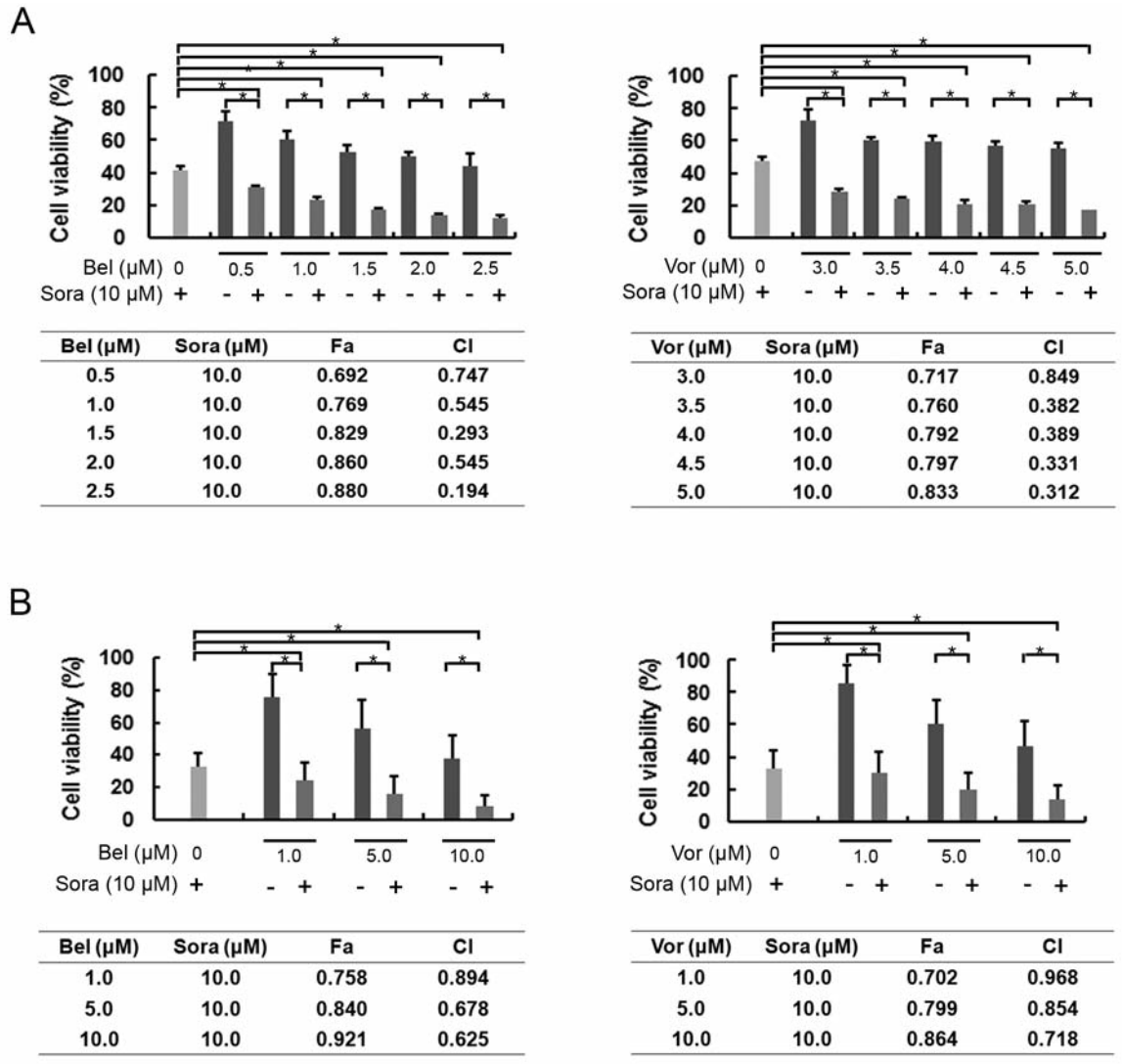

Bel (μM)	Sora (μM)	Fa	CI
0.5	10.0	0.692	0.747
1.0	10.0	0.769	0.545
1.5	10.0	0.829	0.293
2.0	10.0	0.860	0.545
2.5	10.0	0.880	0.194

Vor (μM)	Sora (μM)	Fa	CI
3.0	10.0	0.717	0.849
3.5	10.0	0.760	0.382
4.0	10.0	0.792	0.389
4.5	10.0	0.797	0.331
5.0	10.0	0.833	0.312

Bel (μM)	Sora (μM)	Fa	CI
1.0	10.0	0.758	0.894
5.0	10.0	0.840	0.678
10.0	10.0	0.921	0.625

Vor (μM)	Sora (μM)	Fa	CI
1.0	10.0	0.702	0.968
5.0	10.0	0.799	0.854
10.0	10.0	0.864	0.718

Figure 4. *Growth-inhibitory effects of histone deacetylase inhibitors (HDACI) in combination with sorafenib. A: Caki-1 cells were pre-treated with the indicated concentrations of HDACI belinostat (Bel) or vorinostat (Vor) for 24 h, and then treated with 10 μM sorafenib (Sora) for an additional 48 h. Cell viability, determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays and expressed as the mean ± SD (n=3), was calculated as a percentage of the amount found in untreated cells (*p<0.05; one-way ANOVA). The interaction between HDACIs and sorafenib was determined by combination index (CI) and by the fractional effect analysis (Fa). B : 786-O cells were pre-treated with 1 to 10 μM HDACIs for 24 h followed by incubation with 10 μM sorafenib for an additional 48 h. Cell viability and CI values, expressed as the mean ± SD (n=6), were calculated as described above (*p<0.05; one-way ANOVA).*

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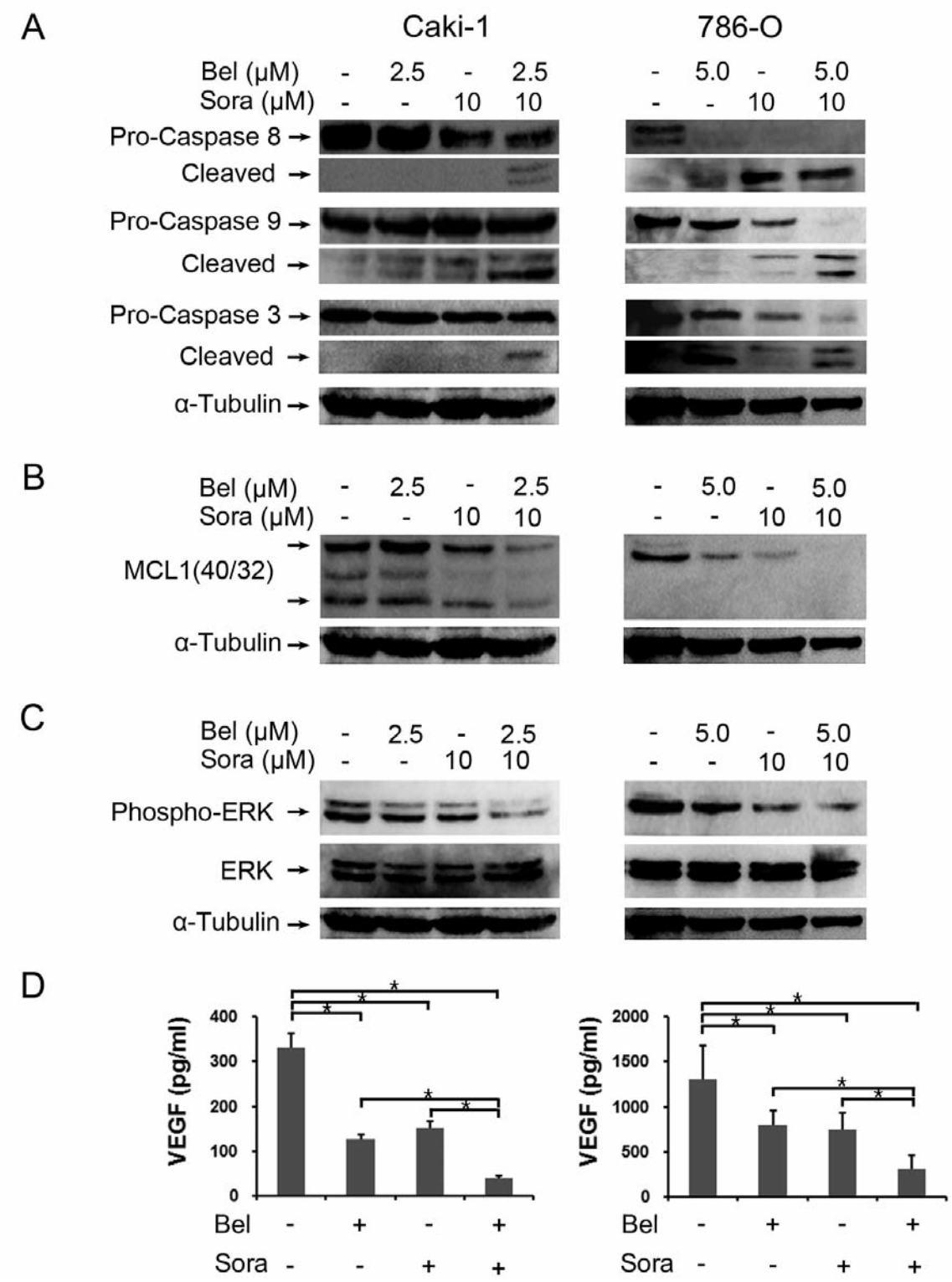

Figure 5. *The cell death mechanism of combined treatment with belinostat (Bel) and sorafenib (Sora) in Caki-1 and 786-O cells. Caki-1 and 786-O cells were incubated with 2.5 μM belinostat for 24 h and exposed to 10 μM sorafenib for an additional 48 h. The levels of activated caspases (A), myeloid leukemia cell differentiation protein 1 (MCL1) (B), and phospho-extracellular signal-regulated kinases (ERK) (C) were analyzed by western blotting. α-Tubulin was used as a loading control. D : The amount of secreted vascular endothelial growth factor (VEGF) in the medium, expressed as the mean ± SD (n=6), was determined by ELISA (*p<0.05; one-way ANOVA).*

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