Fluvastatin potentiates anticancer activity of vorinostat in renal cancer cells

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Abstract
Drug repositioning is an emerging approach to developing novel cancer treatments. Vorinostat is a histone deacetylase inhibitor approved for cancer treatment, but it could attenuate its anticancer activity by activating the mTOR pathway. The HMG-CoA reductase inhibitor fluvastatin reportedly activates the mTOR inhibitor AMP-activated protein kinase (AMPK), and we thought that it would potentiate vorinostat’s anticancer activity in renal cancer cells. The combination of vorinostat and fluvastatin induced robust apoptosis and inhibited renal cancer growth effectively both in vitro and in vivo. Vorinostat activated the mTOR pathway, as evidenced by the phosphorylation of ribosomal protein S6, and fluvastatin inhibited this phosphorylation by activating AMPK. Fluvastatin also enhanced vorinostat-induced histone acetylation. Furthermore, the combination induced endoplasmic reticulum (ER) stress that was accompanied by aggresome formation. We also found that there was a positive feedback cycle among AMPK activation, histone acetylation, and ER stress induction. This is the first study to report the beneficial combined effect of vorinostat and fluvastatin in cancer cells.

Keywords
AMP-activated protein kinase, endoplasmic reticulum stress, fluvastatin, histone acetylation, vorinostat

1 INTRODUCTION

Histone deacetylase (HDAC) inhibitors have been shown in various cancer cells to promote histone acetylation and thereby induce apoptosis. Vorinostat became, in 2006, the first pan-HDAC inhibitor approved for the treatment of cutaneous T-cell lymphoma. We have previously shown that the HDAC inhibitor panobinostat predisposes bladder cancer cells to apoptosis by inhibiting HDAC, but it also results in mTOR activity with consequent ribosomal protein S6 (S6) phosphorylation and these are antiapoptosis forces within a cell. Several recent studies have shown that combinations of an HDAC inhibitor and an mTOR inhibitor act cooperatively against renal cancer cells and S6 phosphorylation is thought to be especially relevant in renal cancer proliferation because it is associated with unfavorable prognosis in patients with renal cancer. We therefore thought that vorinostat might phosphorylate S6 in renal cancer cells and thereby attenuate its ability to induce apoptosis.

Fluvastatin is one of the HMG-CoA reductase inhibitors widely used for treating dyslipidemia and coronary artery disease. It also has anticancer activity and recently been shown to activate AMP-activated protein kinase (AMPK), which acts against...
cancer by suppressing the mTOR pathway and inducing histone acetylation. 16-21

In the present study, we postulated that the combination of vorinostat and fluvastatin would suppress the vorinostat-activated mTOR pathway and also enhance vorinostat-induced histone acetylation, thereby killing renal cancer cells cooperatively.

2 MATERIALS AND METHODS

2.1 Cell cultures

Human renal cancer cells (ACHN, A498) and murine renal cancer cells (Renca) purchased from the ATCC were cultured in minimum essential
medium (MEM) containing 10% FBS and 1.0% penicillin/streptomycin (Invitrogen) at 37°C under 5% CO2 in a humidified incubator.

2.2 | Reagents

Vorinostat and fluvastatin purchased from Cayman Chemical, panobinostat purchased from LC Laboratories, belinostat purchased from Selleck Chemicals, and tunicamycin purchased from Enzo Life Sciences were dissolved in DMSO. Compound C dihydrochloride purchased from R&D Systems and cycloheximide purchased from Enzo Life Sciences were dissolved in distilled water. These reagents were stored at −80°C or −20°C until use.

2.3 | Cell viability assay

Cell viability was evaluated by MTS assay (CellTiter 96 Aqueous kit; Promega) according to the manufacturer’s protocol. Cells (5 × 10^3) were seeded into each well of a 96-well culture plate 1 day before being treated under indicated conditions for 48 hours. After treatment, 20 μL MTS solution was added to the medium and the plates were incubated for 30–60 minutes. The plates were then read at a wavelength of 490 nm in a microplate autoreader.

2.4 | Clonogenic assay

Cells (0.5-1 × 10^3) were seeded into each well of a 6-well culture plate 1 day before being treated for 48 hours with 5 μmol/L vorinostat and/or 10 μmol/L fluvastatin. The cells were then given fresh medium and cultured for 1-2 weeks. The colonies were stained with Giemsa’s solution after being fixed with 100% methanol and were quantified using the ImageJ plugin ColonyArea developed by Guzmán et al. 22

2.5 | In vitro cell invasion assay

The invasiveness of renal cancer cells was assessed by examining the invasion of 24 wells through Matrigel-coated Transwell inserts with 8 μm pores (BD Biosciences) according to the manufacturer’s protocol. Cells (2.0 × 10^5) in 500 mL serum-free MEM were added to each insert, and 500 mL of MEM containing 10% fetal bovine serum with or without 5 μmol/L vorinostat and/or 10 μmol/L fluvastatin was added to the bottom of each well. Forty-eight hours later, the cells that had remained inside the inserts were removed and cells that had migrated through the inserts’ membranes were fixed in methanol and stained with 1% toluidine blue (Kanto Chemicals) in 1% borax (Sigma). The cells were counted in 3 randomly chosen visual fields at ×200 magnification.

2.6 | In vivo study

The experimental protocol for this in vivo experiment was approved by the institutional Animal Care and Use Committee of National Defense Medical College. Renca cells (1 × 10^7) were implanted s.c. into nude mice purchased from CLEA Japan and treatment was initiated 5 days later (day 1), when all the mice showed measurable tumors. The mice were divided into control and treatment groups (n = 5 per group). The treated mice received i.p. injections of either vorinostat (25 mg/kg) or fluvastatin (10 mg/kg) or both; the control mice received vehicle only. The injections were given once a day for 14 days (5 days on, 2 days off). Tumor volume and body weights were measured every 2 or 3 days. Tumor volumes were estimated using the following formula: volume = 0.5 × length × width^2. After 14 days of treatment, the animals were killed in compliance with the ethical policy for animal experiments worldwide23 and the s.c. tumors were harvested.
2.7 | Flow cytometry

Cells (1.0 \times 10^5) were seeded into each well of a 12-well culture plate 1 day before being cultured for 48 hours in medium with or without 5 \mu M vorinostat and/or 10 \mu M fluvastatin. The cells were then washed with PBS and harvested by trypsinization. For the annexin-V assay, the cells were stained with annexin V and 7-AAD following the protocol of the assay kit’s manufacturer (Beckman Coulter). For cell cycle analysis, the cells were resuspended in citrate buffer, stained with propidium iodide, and then analyzed using a flow cytometer and CellQuest Pro Software (BD Biosciences). This cytometric analysis was carried out 3 times.

2.8 | Western blot analysis

Cells were treated under the indicated conditions for 48 hours and whole cell lysates were obtained using RIPA buffer. Tumor specimens harvested from mice were homogenized using RIPA buffer, and whole cell lysates were obtained. Equal amounts of protein were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. After the membranes were blocked by 5% skimmed milk, they were incubated overnight at 4°C with primary Abs: anti-AMPK from Proteintech; anti-phospho-AMPK, anti-phospho-S6, anti-S6, anti-phospho-eukaryotic translation initiation factor 4E-binding protein 1 (p-4EBP1), anti-4EBP1, and anti-endoplasmic reticulum resident protein (ERp) 44 from Cell Signaling Technology; anti-glucose-regulated protein (GRP) 78, anti-cyclin D1, anti-cyclin-dependent kinase (CDK) 4, anti-HDAC1, anti-HDAC3, and anti-HDAC6 from Santa Cruz Biotechnology; anti-acetylated histone from Abcam; and anti-actin from Millipore. Then the protein was detected by reaction with HRP-tagged goat anti-mouse or goat anti-rabbit Ab (Bio-Rad) and staining with chemiluminescence solution by using the ECL Plus system (GE Healthcare).

2.9 | Immunohistochemical detection of active caspase 3

Tumor tissue samples derived from the mice models were fixed in 10% formalin, embedded in paraffin, and sliced into sections 4 \mu m thick. The sections were deparaffinized in xylene and rehydrated through graded alcohols and distilled water. After antigen retrieval, the sections were incubated in 10% normal goat serum in PBS for 1 hour at room temperature. They were then incubated overnight at 4°C with anti-active caspase 3 Ab (Abcam), and this incubation was followed by incubation with HRP-tagged anti-rabbit Ab (Dako) for 1 hour at room temperature. The sections were developed in diaminobenzidine (Dako) and counterstained with hematoxylin.

2.10 | Detection of aggresome formation

Cells (1.0 \times 10^5) were seeded into each well of 2-well chamber slides 1 day before treatment and incubated for 48 hours in media with or without 5 \mu M vorinostat and/or 10 \mu M fluvastatin. Cells were then fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X before being incubated for 30 minutes with Hoechst 33342 and PROTEOSTAT dye (Enzo Life Sciences). Aggresomes and the nucleus were then detected using a fluorescence microscope (Carl Zeiss).

2.11 | Statistical analysis

CalcuSyn software (Biosoft) was used for calculating the combination indexes according to the method developed by Chou and Talalay.\textsuperscript{24} The statistical significance of observed differences
between samples was evaluated using the Mann-Whitney U test (JMP Pro 14 software; SAS Institute), and differences for which \( P < .05 \) were considered statistically significant.

3 | RESULTS

3.1 | Anticancer activity of vorinostat in renal cancer cells

Vorinostat impaired renal cancer viability in a dose-dependent manner (Figure 1A). Mechanistically, it induced not only histone acetylation but also endoplasmic reticulum (ER) stress evidenced by the increased expression of the ER stress markers GRP78 and ERp44 (Figure 1B). Of note, vorinostat activated the mTOR pathway by increasing the phosphorylation of S6, one of the pathway’s most important downstream proteins associated with renal cancer proliferation.7,8 Interestingly, vorinostat decreased the phosphorylation of 4EBP1, another downstream protein of the mTOR pathway, thus inhibiting another mTOR signaling cascade (Figure 1B). To confirm that HDAC inhibition activates the mTOR pathway, we then treated the cancer cells with other HDAC inhibitors. Both of the HDAC inhibitors panobinostat and belinostat inhibited the growth of renal cancer cells in a dose-dependent manner (Figure 1C). As expected, each induced histone acetylation and ER stress but activated the mTOR pathway by increasing the phosphorylation of S6 (Figure 1D). Both also decreased the phosphorylation of 4EBP1, confirming that HDAC inhibition activated one downstream protein in the mTOR pathway while inhibiting another.

3.2 | Fluvastatin not only activated AMPK but induced histone acetylation

We next evaluated fluvastatin’s anticancer activity and its mechanism of action in renal cancer cells. Fluvastatin reduced the viability of renal cancer cells in a dose-dependent manner (Figure 2A). Mechanistically, it increased the phosphorylation of AMPK and decreased the phosphorylation of both S6 and 4EBP1 (Figure 2B), suggesting that it reduced renal cancer viability by inhibiting parts of the mTOR pathway through AMPK activation. We also found that fluvastatin induced histone acetylation in renal cancer cells (Figure 2B).

3.3 | Vorinostat and fluvastatin in combination inhibited renal cancer growth in vitro and in vivo

The combination of vorinostat and fluvastatin inhibited renal cancer growth effectively (Figure 3A). The combined effect was synergistic in most of the treatment conditions (Figure 3B and Table 1). The clonogenic survival of renal cancer cells was also significantly inhibited by the combination (Figure 3C). Furthermore, the combination significantly impaired the cancer cells’ invasiveness (Figure 3D).

In murine s.c. allograft tumor models using Renca cells, a 14-day treatment with the combination of fluvastatin and vorinostat suppressed tumor growth significantly (Figure 3E). Furthermore, the combination caused no remarkable weight loss (Figure 3F).
Combination of vorinostat and fluvastatin induced apoptosis cooperatively

The combination significantly caused the cancer cells to accumulate in the sub-G₁ fraction (Figure 4A), suggesting that it caused DNA fragmentation and induced apoptosis. Accordingly, the combination decreased the expression of cyclin D1 and CDK4 (Figure 4B). The induction of apoptosis was further confirmed by the annexin-V assay: the combination significantly increased the percentage of the cell population that was annexin V-positive (Figure 4C).

Immunohistochemical analyses of the tumor specimens revealed that the combination increased the expression of active caspase 3 more than each agent itself did (Figure 4D), suggesting that the combination would induce apoptosis cooperatively also in vivo.
3.5 | Combination of vorinostat and fluvastatin inhibited the mTOR pathway

As expected, fluvastatin increased the phosphorylation of AMPK and decreased the vorinostat-increased S6 phosphorylation (Figure 5), showing that fluvastatin indeed suppressed the vorinostat-activated mTOR pathway. Further dephosphorylation of 4EBP1 is also evidence of the suppression of the mTOR pathway by fluvastatin. Thus, the combination was shown to inhibit the mTOR pathway.

3.6 | Pivotal role of AMPK in the combination’s action

The AMPK inhibitor compound C attenuated not only the cytotoxicity of the combination (Figure 6A), but also its ability to induce apoptosis (Figure 6B). The increased phosphorylation of S6 proved that compound C inhibited the function of AMPK, and compound C reactivated the combination-suppressed mTOR pathway evidenced by the increased phosphorylation of S6 and 4EBP1 (Figure 6C). These findings show that AMPK activation plays a pivotal role in the combination’s action.

3.7 | Vorinostat-fluvastatin combination induced histone acetylation and ER stress cooperatively

Vorinostat is an HDAC inhibitor and induces histone acetylation. Fluvastatin was also shown to induce histone acetylation, as described above. Furthermore, AMPK activation itself is also known to induce histone acetylation. We therefore thought that the combination of fluvastatin and vorinostat would induce histone acetylation cooperatively. Western blot analysis showed that vorinostat induced histone acetylation in a dose-dependent manner and fluvastatin enhanced this acetylation (Figure 7A). Interestingly, the expression of HDACs was also decreased by the combination (Figure 7A). Because vorinostat itself induced both histone acetylation and ER stress (Figure 1B), we thought that the combination too would induce ER stress. As expected, the expression of the ER stress markers GRP78 and ERp44 was drastically increased by the combination,
whereas fluvastatin or vorinostat alone increased their expression only moderately (Figure 7A).

Aggresomes are formed when ER stress triggers the aggregation of unfolded proteins,25,26 so we next examined whether the combination facilitated aggresome formation. Notably, extensive aggresome formation was observed only when cells were treated with the combination (Figure 7B).

3.8 | Vorinostat-fluvastatin combination caused AMPK activation, histone acetylation, and ER stress in vivo

We then undertook western blot analysis using the specimens obtained in the in vivo experiment. The combination of vorinostat and fluvastatin increased the phosphorylation of AMPK and the expression of acetylated histone and GRP78 (Figure 8), indicating that the combination has the same mechanism of action in vivo that it does in vitro.

3.9 | Cross-talk among AMPK activation, histone acetylation, and ER stress induction

Because the combination of vorinostat and fluvastatin cooperatively induced histone acetylation and ER stress, we further investigated the contribution of ER stress induction to the combination’s action. The protein synthesis inhibitor cycloheximide suppresses ER stress induction,27 so we examined whether it impaired the combination’s action. Cycloheximide significantly attenuated the combination’s ability to inhibit cell proliferation (Figure 9A) and induce apoptosis (Figure 9B). Western blot analysis indicated that cycloheximide inhibited the combination-increased expression of ER stress markers, showing that it suppressed the ER stress induced by the combination (Figure 9C). Cycloheximide also inhibited the combination-increased histone acetylation (Figure 9C). Interestingly, cycloheximide also suppressed the combination-induced AMPK activation and thereby activated the combination-suppressed mTOR pathway as evidenced by the increased phosphorylation of S6 and 4EBP1 (Figure 9D). In contrast, the AMPK inhibitor compound C attenuated the combination-induced histone acetylation and ER stress (Figure 9E). Furthermore, the massive aggresome formation caused by the combination was inhibited by both compound C and cycloheximide (Figure 9F), which showed that both agents suppressed the combination-induced ER stress.

We inferred from these results that there was cross-talk among ER stress induction, histone acetylation, and AMPK activation.

4 | DISCUSSION

Histone deacetylase inhibitors are considered to be innovative anticancer drugs,28-30 but their efficacy as single agents is limited, especially in solid tumors.31-36 We have found that some HIV protease inhibitors and proteasome inhibitors enhance the activity of HDAC...
inhibitors.\textsuperscript{37-40} We recently found that mTOR activation could be one of the important mechanisms of bladder cancer cells' resistance to the HDAC inhibitor panobinostat.\textsuperscript{4} In the present study, we postulated that vorinostat might induce mTOR activation, attenuating its anticancer activity in renal cancer cells, and that mTOR inhibition would overcome this attenuation.

Vorinostat indeed activated the mTOR pathway by increasing the phosphorylation of S6. The experiments with panobinostat and belinostat, hydroxamic acid-based HDAC inhibitors like vorinostat,\textsuperscript{41,42} provided further evidence that HDAC inhibition itself causes this phosphorylation. S6 phosphorylation reportedly regulates protein synthesis, glucose homeostasis, and cell size,\textsuperscript{43} but its molecular mechanism of action has not been clarified.\textsuperscript{44} Activation of mTOR is also an important mechanism of drug resistance,\textsuperscript{45-48} so inhibiting the mTOR pathway is a reasonable approach to enhancing vorinostat's anticancer activity. Accordingly, we first treated renal cancer cells with vorinostat and the mTOR inhibitor temsirolimus and found that temsirolimus indeed enhanced the cytotoxicity of vorinostat (Figure S1A). However, the combined effect was only slightly synergistic in the limited treatment conditions (Figure S1B and Table S1). We therefore thought that only directly inhibiting mTOR would be insufficient to enhance the anticancer activity of vorinostat.

In the present study, we used the HMG-CoA reductase inhibitor fluvastatin. HMG-CoA reductase inhibitors reportedly have antiproliferative effects in various cancer cells,\textsuperscript{12-14,49-52} but they do not have demonstrable clinical anticancer activity (Table 2).\textsuperscript{53-59} One of fluvastatin's important mechanisms of action is AMPK activation.\textsuperscript{15} Statins are reported to activate AMPK by causing its phosphorylation through liver kinase B1 activation.\textsuperscript{60,61} AMPK plays a key role in the regulation of energy balance.\textsuperscript{16} Because AMPK also controls cellular metabolism essential for cancer progression,\textsuperscript{62,63} AMPK activation is crucial in the regulation of cancer cell growth and proliferation.\textsuperscript{16} We searched The Cancer Genome Atlas database by using the UCSC Cancer Browser UCSC Xena (https://xena.ucsc.edu/welcome-to-ucsc-xena/) and found that the expression of AMPK genes PRKAA1 and PRKAA2 was higher in normal tissue than cancer tissue (Figure S2A) and that renal cancer patients with higher expression of these genes had significantly longer overall survival (Figure S2B). These results also support the idea that activating AMPK is a promising way to treat renal cancer.

To further develop this AMPK-targeting strategy, the combined effect of vorinostat and other clinically available AMPK activators should be investigated. Our preliminary results showed that the antipsychotic olanzapine\textsuperscript{64} enhanced vorinostat's cytotoxicity only slightly (Figure S3 and Table S2), whereas the antidiabetic metformin\textsuperscript{65} synergized with vorinostat by a mechanism similar to that of fluvastatin (Figures S4-6 and Table S3).

Activation of AMPK not only suppresses the mTOR pathway\textsuperscript{16-19} but also induces histone acetylation.\textsuperscript{20,21} We found that the AMPK activation played a pivotal role in the combination's action by showing that the AMPK inhibitor compound C impaired the combination's anticancer effects. Interestingly, compound C also inhibited the combination-induced histone acetylation, confirming that AMPK activation played a role in regulating histone acetylation.

The combination of vorinostat and fluvastatin also induced ER stress. ER stress is caused by the accumulation of unfolded proteins, and profound ER stress inhibits the growth of malignant cells and causes their apoptosis.\textsuperscript{66,67} The ER stressor tunicamycin reduced renal cancer cell viability in a dose-dependent manner (Figure S7A). Furthermore, we have previously reported that ER stress-inducing drug combinations killed urological cancers effectively.\textsuperscript{68-71} The ER stress induction was also found to be crucial in the combination's action because the ER stress inhibitor cycloheximide significantly reduced combination-caused apoptosis and the combination's cytotoxicity.

Our study showed that AMPK activation enhanced vorinostat-induced histone acetylation and ER stress and that the AMPK

| Statin       | Cancer type       | Disease stage | Number of patients, statin/control | Study design | Median OS, statin/control (mo) | P value | Reference         |
|--------------|-------------------|---------------|-----------------------------------|--------------|--------------------------------|---------|-------------------|
| Pravastatin  | Small-cell lung cancer | Limited or extensive disease | 422/424 | Phase III | 10.7/10.6 | .76 | Seckl et al\textsuperscript{53} |
| Simvastatin  | Non-ADC NSCLC     | Advanced      | 36/32                | Phase II     | 10.0/7.0       | .93 | Lee et al\textsuperscript{54} |
| Simvastatin  | Any               | Brain metastases | 25/25              | Phase III   | 3.4/3.0         | .88 | El-Hamamsy et al\textsuperscript{55} |
| Simvastatin  | Colorectal cancer | Metastatic    | 134/135              | Phase III   | 15.3/19.2       | .83 | Lim et al\textsuperscript{56} |
| Simvastatin  | Gastric cancer    | Metastatic    | 120/124              | Phase III   | 11.6/11.5       | .82 | Kim et al\textsuperscript{57} |
| Simvastatin  | Pancreatic cancer | Locally advanced or metastatic | 58/56          | Phase II     | 6.6/8.9          | .74 | Hong et al\textsuperscript{58} |
| Simvastatin  | NSCLC             | Locally advanced or metastatic | 52/54          | Phase II     | 13.6/12.0       | .49 | Han et al\textsuperscript{59} |

ADC, adenocarcinomatous; NSCLC, non-small-cell lung cancer; OS, overall survival.
inhibitor compound C attenuated the combination-induced histone acetylation and ER stress. Similarly, the ER stressor tunicamycin caused AMPK activation and histone acetylation (Figure S7B), whereas the ER stress inhibitor cycloheximide attenuated the combination-induced AMPK activation and histone acetylation. Both compound C and cycloheximide inhibited massive aggresome formation by the combination, which confirmed that both agents suppressed the combination-induced ER stress. Furthermore, the HDAC inhibitors vorinostat, panobinostat, and belinostat all caused histone acetylation and ER stress (Figures 1B and D). These findings are compatible with those of previous studies, which showed that AMPK activation induces histone acetylation,\cite{4,20,21} ER stress induction is associated with calcium/calmodulin-dependent kinase (CaMKK)-beta, which is an activator of AMPK,\cite{72-74} ER stress induction causes histone acetylation in urological cancer cells,\cite{68-70} and decreased HDAC function causes ER stress by acetylating molecular chaperones and suppressing their function, thereby leading to an increased amount of unfolded proteins.\cite{75-77}

This cross-talk causes a positive feedback cycle, suppressing cancer growth (Figure 10).

Finally, safety is an important issue when clinical application of the combination is considered. The combination caused no remarkable weight loss in vivo. Furthermore, the combined effect was weaker in normal cells, even though its mechanisms of action were similar to those in cancer cells (Figures S8-10 and Table S4). Thus, the vorinostat-fluvastatin combination would be a safe combination therapy. However, careful clinical trials are needed before its clinical application.

DISCLOSURE

None.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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