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ORIGINAL ARTICLE

Antitumor effects of (*S*)-HDAC42, a phenylbutyrate-derived histone deacetylase inhibitor, in multiple myeloma cells

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Abstract
Purpose Epigenetic agents are among the newly targeted therapeutic strategies being studied with intense interest for patients with multiple myeloma. Here, we demonstrate the antitumor activity of a phenylbutyrate-based histone deacetylase (HDAC) inhibitor, (*S*)-HDAC42, and identify its possible targets in myeloma cells.
Methods The antiproliferative effect of (*S*)-HDAC42 was compared with suberoylanilide hydroxamic acid (SAHA) in three myeloma cell lines, IM-9, RPMI-8226, and U266. Flow cytometry and terminal transferase dUTP nick-end labeling (TUNEL) assay were used to demonstrate the induction of apoptosis by (*S*)-HDAC42. Moreover, the proposed mechanisms of action, such as modulation of Akt, NF-κB pathway, and cell cycle–related proteins, were investigated by western blotting.
Results (*S*)-HDAC42 exhibited four- to sevenfold higher potency relative to SAHA in suppressing myeloma cell viabilities. The apoptotic effect induced by (*S*)-HDAC42 was through both intrinsic and extrinsic pathways, as evidenced by increased cleavage of caspase-3, caspase-8, and caspase-9 and release of cytochrome *c* from mitochondria. In addition to HDAC inhibition, (*S*)-HDAC42 also disturbed signaling pathways governing cell survival, including downregulating Akt phosphorylation and NF-κB signaling. The modulation of cell cycle–related proteins by (*S*)-HDAC42 suggested its inhibitory effect on cell cycle propagation.
Conclusion These data suggest the translational value of (*S*)-HDAC42 in developing new therapeutic strategies for myeloma, which warrants further investigations.

Keywords Histone deacetylase · (*S*)-HDAC42 · Myeloma · Akt · NF-κB · Cell cycle

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Introduction

Multiple myeloma is a malignant disorder of plasma cells characterized by high levels of monoclonal protein in serum or urine. The tumor cells are derived from post-germinal center plasmablasts or plasma cells. Despite the advances of high-dose chemotherapy and new active agents, including thalidomide, lenalidomide, and bortezomib, this disease remains incurable. The clinical demise of some patients with multiple myeloma is the inevitable development of drug resistance, which highlights the necessity of new agents and treatment strategies for multiple myeloma [1, 2].

Epigenetic agents are among the newly targeted therapeutic strategies being studied with intense interest for

patients with multiple myeloma [3]. Of this class of compounds, histone deacetylase (HDAC) inhibitors showed promising activities against many solid and hematological malignancies, as well as to multiple myeloma [4–7], by apoptosis induction, cell cycle arrest, cell differentiation, antiangiogenesis, and immunomodulation [6, 8]. Suberoylanilide hydroxamic acid (SAHA; vorinostat) is a well-known HDAC inhibitor with demonstrated antimyeloma effects both in vivo and in vitro [9, 10]. Preliminary data from clinical trials suggest that SAHA alone is well tolerated but with only modest efficacy [11]. The efficacy is more promising when SAHA is used in combination with other antimyeloma agents [12]. Furthermore, studies of other histone deacetylase inhibitors report variable activity against myeloma [13–17].

(*S*)-HDAC42 [(*S*)-(+)-*N*-hydroxy-4-(3-methyl-2-phenylbutyrylamino) benzamide] is a phenylbutyrate-based HDAC inhibitor with potent antitumor effects in prostate cancer, ovarian cancer, and hepatocellular carcinoma both in vitro and in vivo [18–21]. Substantial evidence indicates that (*S*)-HDAC42 mediates antitumor effects through both histone acetylation–dependent and histone acetylation–independent mechanisms [18, 22]. This broad spectrum of antitumor actions renders (*S*)-HDAC42 more potent relative to SAHA in suppressing the growth of various types of cancer cells, including those of prostate, ovary, and liver [18–21].

Here, we demonstrate the antitumor activity of (*S*)-HDAC42 in several multiple myeloma cell lines. The results suggest that (*S*)-HDAC42 has potent antimyeloma activity that was mediated by both histone acetylation–dependent and histone acetylation–independent mechanisms. Based on this study, (*S*)-HDAC42 warrants further evaluations as a monotherapy or in combination with other agents in the treatment of multiple myeloma.

Materials and methods

Cells and culture conditions

Three multiple myeloma cell lines, IM-9, RPMI-8226, and U266, were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cells were incubated in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (Invitrogen) at 37°C in the presence of 5% CO_2.

Reagents

(*S*)-HDAC42 and SAHA (chemical structure shown in Fig. 1a) were synthesized in our laboratory with purity greater than 99% according to published procedures [23]. The identity and purity of these agents were confirmed by nuclear magnetic resonance and mass spectrometry.

Cell viability assay

Cell viability was assessed by using CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay kit (Promega; Madison, WI). Cells (2×10^5/ml) were placed in 100-μl volume in 96-well microtiter plates with indicated reagents and incubated in 37°C. MTS solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and PMS (phenazine methoxulfate) solution were mixed in 20:1 in volume. The colorimetric measurements were taken 3 h later at 490-nm wavelength by a multimode microplate reader Synergy HT (Bio-Tek). Cell viability was expressed as a percentage of absorbance value in treated samples compared to that observed in control vehicle–treated samples.

Apoptosis assay

Dual staining with annexin V conjugated to fluorescein isothiocyanate (FITC) and propidium iodide (PI)

Cells (1×10^6) were stained by annexin V-FITC (BD Pharmingen, San Diego, CA) and PI (BD Pharmingen). Cells were analyzed by a Beckman-Coulter EPICS XL cytometer (Beckman-Coulter, Miami, FL). Annexin V-FITC- and/or PI-positive cells were identified as apoptotic cells. Viable cells were those with both annexin V-FITC-negative and PI-negative staining. The viable cells in each sample were expressed as a percentage by normalizing annexin V^-/PI^- cells to untreated controls.

Terminal transferase dUTP nick-end labeling (TUNEL) assay

IM-9 and U-266 cells (2×10^5/ml) were cultured in six wells in the absence or presence of (*S*)-HDAC42. After fixation in 4% formaldehyde for 1 h, the apoptotic cells were treated with Triton-X 100 for 10 min. Then, the cells were stained by the TUNEL method using In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Taipei, Taiwan) for 2 min and were observed by a fluorescence microscope. Staining with 4,6-diamidino-2-phenylindole (DAPI) was used for nuclear localization.

Western blotting

Cell lysates were prepared using RIPA buffer (150 mM NaCl, 50 mM Tris PH 8.0, 1% NP40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Protease inhibitor (Sigma–Aldrich; Saint Louis, MO) and

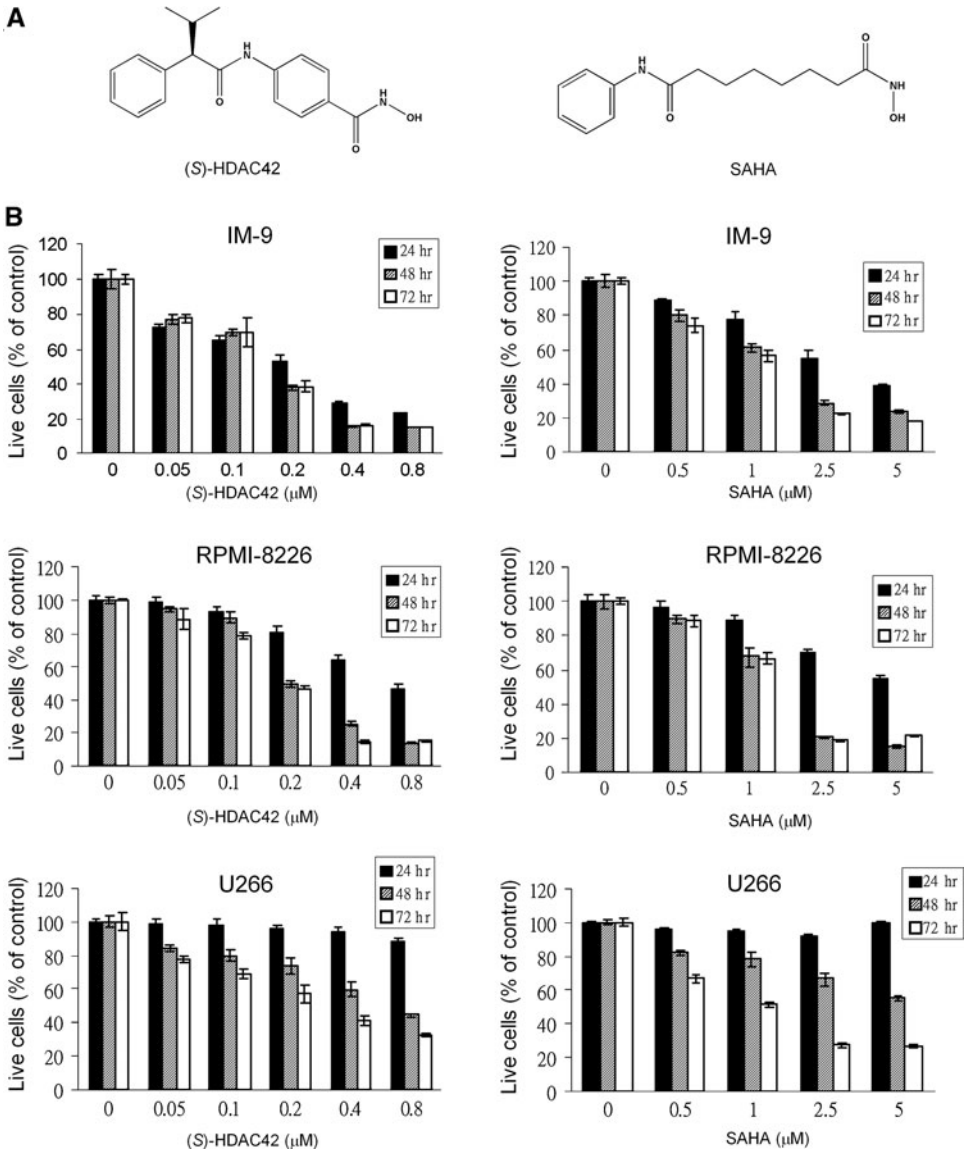

Fig. 1 The structure and anti-proliferative effect of (*S*)-HDAC42 and suberoylanilide hydroxamic acid (SAHA) in three multiple myeloma cell lines. **a** Chemical structure. **b** MTS assay. Cells (2×10^5/ml) were exposed to (*S*)-HDAC42 or SAHA for 24, 48, and 72 h. ($n = 6$ each in three independent experiments)

phosphatase inhibitor cocktail (Calbiochem, Gibbstown, NJ) were added to RIPA buffer before lysing the cells. Antibodies against various proteins were obtained from the following sources: p-Akt (Ser473), p-Akt (Thr308) (Santa Cruz Biotechnology, Santa Cruz, CA); α-tubulin, HDAC1, HDAC4, Akt, NF-κB p65, IKKα, IKKβ, IκBα, p-IκBα (Ser32/36), cytochrome *c*, COX IV, caspase-3, caspase-8, caspase-9, poly-ADP-ribose polymerase (PARP), X-chromosome-linked inhibitor of apoptosis (XIAP), cyclin B1, cyclin D1, CDK6, BIRC5 (Cell Signaling, Danvers, MA); nucleolin, p19 (Abcam Inc, Cambridge, MA); acetylated histone H3 (Upstate, Temecula, CA); β-actin (Sigma–Aldrich, St. Louis, MO). The goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugates and goat anti-mouse IgG-HRP conjugates were purchased from PerkinElmer life Sciences, Inc. (Boston, MA).

Cytochrome *c* release from mitochondria

Cells (2×10^5/ml) were treated with (*S*)-HDAC42 at the indicated concentrations for 48 h. The mitochondria isolation kit (Pierce, Rockford, IL) was used according to the manufacturer's instructions to obtain the mitochondrial fraction. Cytochrome *c* was detected by western blot analysis using COX IV as the mitochondrial loading control.

Nuclear protein extraction

Nuclear extracts were prepared from IM-9 and U266 cells (2×10^5/ml) after treatment with (*S*)-HDAC42 at the indicated concentrations for 48 h using the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit according to the manufacturer's protocol (Thermo Fisher Scientific, Rockford, IL).

Statistical analysis

All experiments were performed at least in triplicates, and results were expressed as means ± standard deviation (SD) unless otherwise indicated.

Results

(*S*)-HDAC42 mediates greater cytotoxicity than SAHA in multiple myeloma cell lines

The cytotoxicity of (*S*)-HDAC42 vis-à-vis SAHA was assessed in three myeloma cell lines, IM-9, RPMI-8226, and U266, in a dose- and time-dependent manner by MTS assays (Fig. 1b). (*S*)-HDAC42 and SAHA exhibited a differential, suppressive effect on the cell viability of these three cell lines, with the susceptibility in the order of IM-9 > RPMI-8226 > U266. The respective IC_{50} values at different time points were summarized in Table 1. As shown, the antiproliferative activities of (*S*)-HDAC42 against these three cell lines were four- to sevenfold higher than those of SAHA (IC_{50} at 72 h, 0.17 ~ 0.27 μM vs. 1.2 ~ 1.4 μM). The potency of (*S*)-HDAC42 is consistent with its dose response in inducing histone H3 hyperacetylation (Fig. 2). The stronger inhibition of histone H3 deacetylation in IM-9 than that in U266 is compatible with the more susceptibility of IM-9 to (*S*)-HDAC42. It is also interesting to note that (*S*)-HDAC42, at high doses, suppressed the expression of HDAC1 and HDAC4, resulting in substantially higher degree of acetylated histone H3 accumulation.

(*S*)-HDAC42 induces apoptotic cell death

Annexin V/PI staining indicates that the treatment of IM-9 and U266 cells with (*S*)-HDAC42 led to a dose-dependent increase in the proportion of apoptotic cells (Fig. 3a), suggesting that (*S*)-HDAC42-induced cell death was, at least in part, attributable to apoptosis. The effect of (*S*)-HDAC42 on apoptosis induction was further confirmed by the TUNEL assay in both IM-9 (Fig. 3b) and U266 (Fig. 3c). These results indicated a dose-dependent DNA damage in response to (*S*)-HDAC42.

(*S*)-HDAC42 activates both intrinsic and extrinsic pathways

To investigate the involvement of the intrinsic and extrinsic apoptosis pathways in (*S*)-HDAC42-mediated cytotoxicity, protein extracts of IM-9 and U266 cells were examined by western blotting (Fig. 4a). (*S*)-HDAC42 induced dose-dependent increases in the proteolytic cleavage of PARP, caspase-3, caspase-8, and caspase-9, indicative of the involvement of both intrinsic and extrinsic apoptosis pathways. To confirm the involvement of intrinsic pathway, mitochondrial extracts were collected from (*S*)-HDAC42-treated IM-9 and U266 cells. As shown in Fig. 4b, (*S*)-HDAC42 caused a dose-dependent decrease in the level of mitochondrial cytochrome *c*.

(*S*)-HDAC42 downregulates Akt phosphorylation and NF-κB signaling

As Akt and NF-κB pathways play a crucial role in regulating cellular survival and proliferation, we assessed the

Table 1 IC_{50} values (means ± SD; $N = 6$ in three independent experiments)

	(*S*)-HDAC42 (μM)			SAHA (μM)		
	24 h	48 h	72 h	24 h	48 h	72 h
IM-9	0.21 ± 0.05	0.17 ± 0.08	0.17 ± 0.07	2.9 ± 0.10	1.4 ± 0.56	1.2 ± 0.67
RPMI-8226	0.68 ± 0.03	0.23 ± 0.03	0.19 ± 0.03	>5	1.4 ± 0.89	1.4 ± 0.08
U266	>0.8	0.55 ± 0.12	0.27 ± 0.11	>5	>5	1.2 ± 0.37

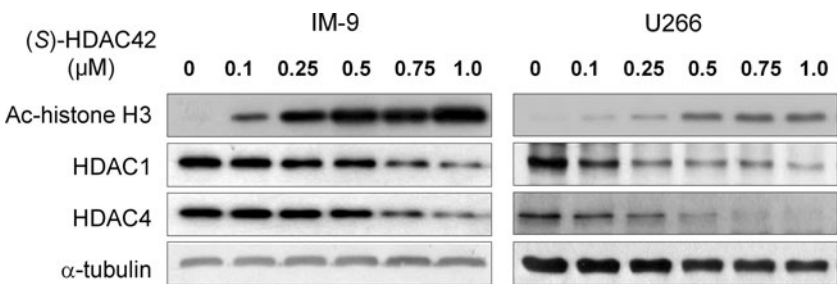

Fig. 2 Effects of (*S*)-HDAC42 on HDAC inhibition. Western blotting analysis of effect of (*S*)-HDAC42 on the expression level of acetylated histone H3 (Ac-histone H3), HDAC1, and HDAC4 in IM-9 and U266 cells (2×10^5/ml) treated with (*S*)-HDAC42 or DMSO for 48 h

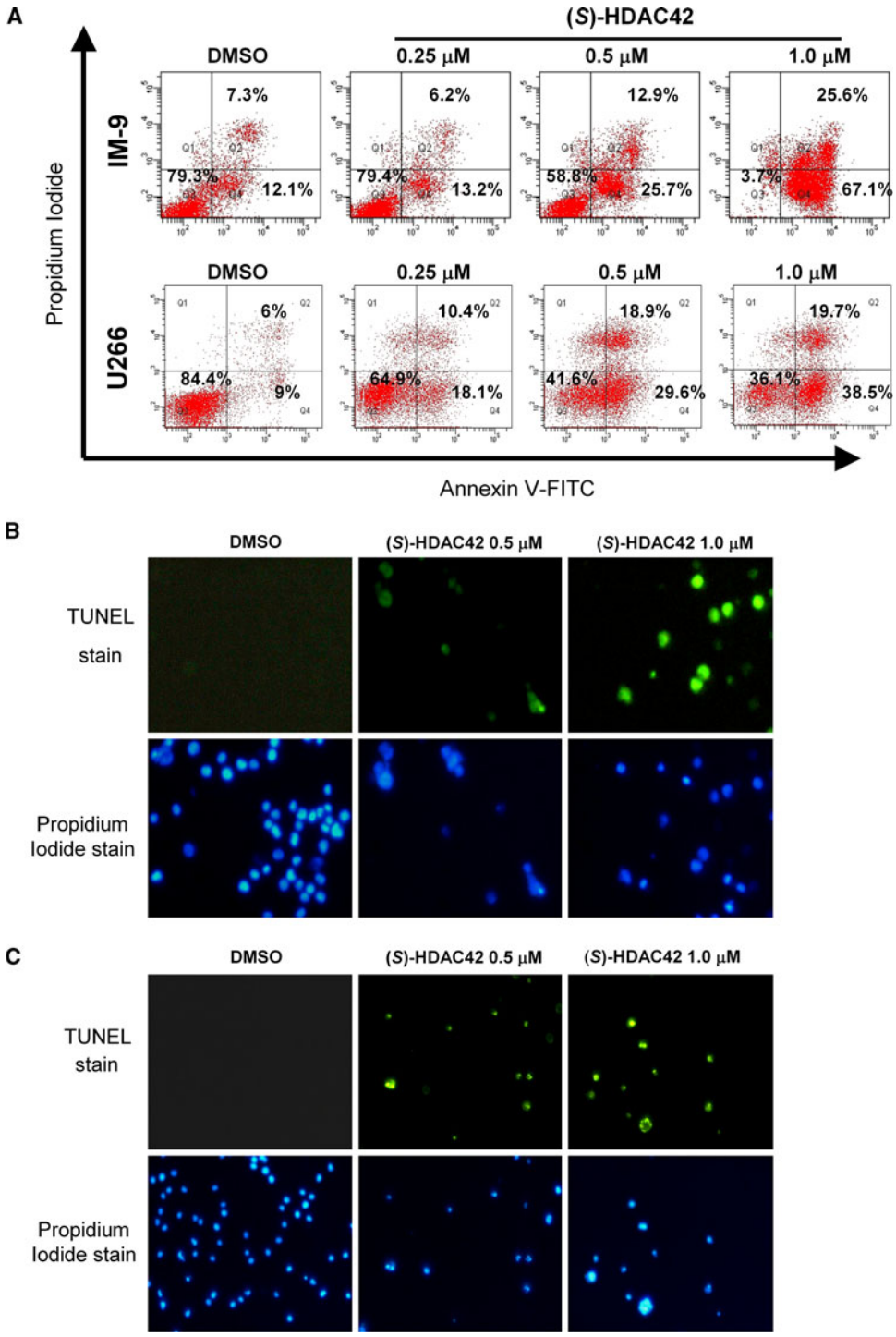

Fig. 3 Apoptotic assay of IM-9 and U266 cells treated with (*S*)-HDAC42 for 48 h. The *data* shown is a representative of three independent experiments with similar results. **a** Annexin V-FITC / propidium iodide staining. **b** TUNEL assay in IM-9. **c** TUNEL assay in U266. The *green color* in TUNEL stain denotes DNA fragmentation. DAPI (4,6-diamidino-2-phenylindole) stain was used as for nuclear localization

effects of (*S*)-HDAC42 on the phosphorylation and/or expression levels of Akt and proteins of NF-κB pathway in IM-9 and U266 cells (Fig. 5a). As shown, (*S*)-HDAC42 mediated robust Akt dephosphorylation at Thr308 and, to a lesser extent, Ser473 even at low doses. In addition, (*S*)-HDAC42 suppressed the expression level of NF-κB p65 with parallel decreases in the expression of its two target gene products, BIRC5 and XIAP. To further confirm the implication of NF-κB pathway, the nuclear portions of IM-9 and U266 cells treated with (*S*)-HDAC42 were isolated and were blotted with NF-κB p65 antibody (Fig. 5b). There was a downregulation of NF-κB p65 expression in nuclear extract. Together, these data suggest the involvement of Akt and NF-κB pathways in (*S*)-HDAC42-induced apoptosis.

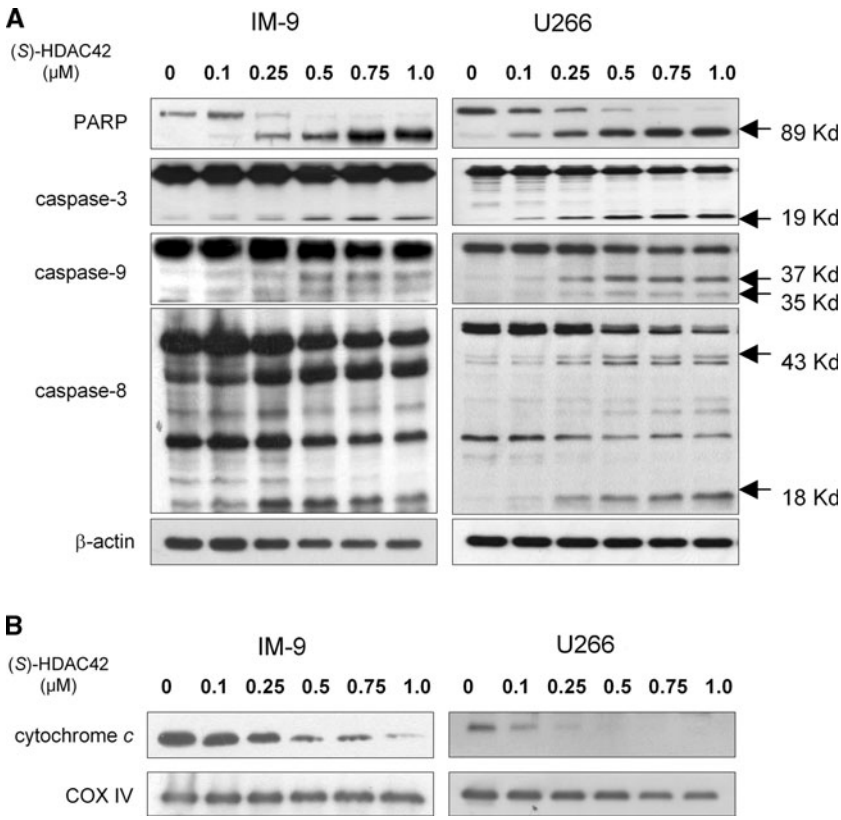

Fig. 4 Western blot analysis of apoptotic pathway in IM-9 and U266 cells treated with (*S*)-HDAC42 for 48 h. **a** Total cell lysates were immunoblotted using antibodies against poly-ADP-ribose polymerase (PARP) and caspase-3, -9 and -8. **b** Mitochondrial extract were analyzed using antibodies against cytochrome *c* and COX IV

Cell cycle–related proteins are regulated by (*S*)-HDAC42

To investigate the influence of (*S*)-HDAC42 on cell cycle, the cell lysates of IM-9 and U266 cells treated with (*S*)-HDAC42 were blotted with antibodies of cell cycle–related proteins (Fig. 5c). Cyclin B1, cyclin D1, and CDK6 were all downregulated in a dose-dependent manner. In contrast, p19, a cyclin-dependent kinase inhibitor was upregulated in both cell lines.

Discussion

Here, we describe the antitumor effects of (*S*)-HDAC42 in myeloma cells through both intrinsic and extrinsic apoptotic pathways. Compatible with its pleiotropic mode of action reported in solid tumor cells [18–22], (*S*)-HDAC42 mediates apoptosis in myeloma cells through the concerted effects on HDAC inhibition, downregulation of Akt and NF-κB signaling, and modulation of cell cycle–related proteins.

Increasing knowledge of the pathogenesis of multiple myeloma has facilitated the identification of target pathways for designing effective therapeutic strategies [3]. Among them, NF-κB, Akt, and epigenetic modulation represent three important targets, all of which are downregulated or affected by (*S*)-HDAC42. Previous studies suggest the importance of NF-κB signaling in multiple myeloma, specifically by the nuclear localization of NF-κB in myeloma cells and the effect of NF-κB inhibitors in myeloma [24–26]. Bortezomib is one compound that has successfully changed the clinical course of patients with multiple myeloma through mechanisms involving the NF-κB pathway [1]. On the other hand, perifosine, by inhibiting PI3K/Akt pathway, has demonstrated therapeutic value in relapsed and refractory myeloma [27, 28]. Besides, the downregulation of CDK6, cyclin B1, and cyclin D1, as well as upregulated p19, suggest the inhibition of cell cycle propagation by (*S*)-HDAC42. Through the combined effect on HDAC inhibition and a broad spectrum of cellular targets, (*S*)-HDAC42 may foster new therapeutic strategies to improve myeloma patient survival.

Data from previous studies suggest that (*S*)-HDAC42 has the following advantages in addition to the aforementioned pleiotropic effect. First, it has lower IC_{50} than SAHA in suppressing the growth of solid tumor cells [18]. This higher potency is also noted in the present myeloma study. Second, mice have been shown to tolerate the therapeutic range of (*S*)-HDAC42 well without overt signs of toxicity [18–22]. Repeated oral administration of (*S*)-HDAC42 to mice was associated with reversible testicular and thymic atrophy and leukopenia but without mortality or significant effects on body weight [18, 19]. Third, (*S*)-HDAC42 is orally bioavailable, which is advantageous for clinical

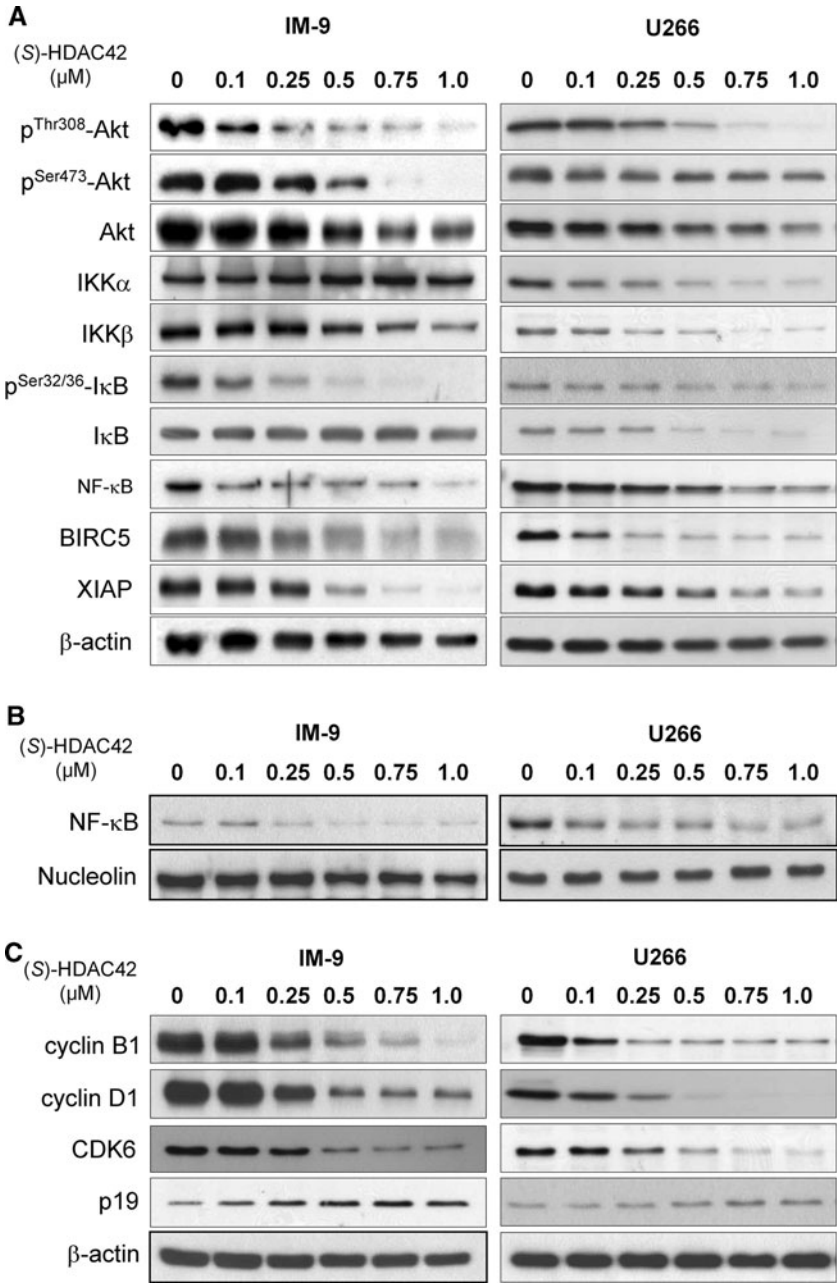

Fig. 5 Western blot analysis of Akt and NF-kB pathways and cell cycle related proteins in cells treated with (*S*)-HDAC42 for 48 h. **a** Total cell lysates were immunoblotted using antibodies against proteins of Akt and NF-kB pathway. **b** Nuclear extract proteins were immunoblotted with antibodies against NF-kB p65. Nucleolin was used as an internal loading control. **c** Total cell lysates were immunoblotted using antibodies against cell cycle related proteins

administration. Bioanalytical data are currently being evaluated by Arno Therapeutics (Parsippany, NJ), the biopharmaceutical company to which the compound is licensed.

In conclusion, (*S*)-HDAC42 has promising antitumor activities against myeloma cells. It induces apoptosis in myeloma cells through HDAC inhibition, the downregulation of Akt and NF-κB signaling, and modulation of cell cycle–related proteins. Further studies are warranted to validate the antimyeloma effect of (*S*)-HDAC42 clinically.

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Conflict of interest The authors declare no competing financial interest.

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