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A novel dual-epigenetic inhibitor enhances recombinant monoclonal antibody expression in CHO cells

Ming-Ming Han[1,2,3] · Hai-Tong Wang[1,2,3] · Hui-Jie Zhang[1,2,3] · Jiang-Tao Lu[1,2,3] · Jia-Liang Guo[4] · Le-Le Qiu[5] · Xi Zhang[1,2,3] · Xiao-Yin Wang[2,3,5] · Tian-Yun Wang[2,3,5] · Yan-Long Jia[1,2,3]

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Abstract

Epigenetic regulation plays a central role in the regulation of a number of cellular processes such as proliferation, differentiation, cell cycle, and apoptosis. In particular, small molecule epigenetic modulators are key elements that can effectively influence gene expression by precisely regulating the epigenetic state of cells. To identify useful small-molecule regulators that enhance the expression of recombinant proteins in Chinese hamster ovary (CHO) cells, we examined a novel dual-HDAC/LSD1 inhibitor I-4 as a supplement for recombinant CHO cells. Treatment with 2 μM I-4 was most effective in increasing monoclonal antibody production. Despite cell cycle arrest at the G1/G0 phase, which inhibits cell growth, the addition of the inhibitor at 2 μM to monoclonal antibody-expressing CHO cell cultures resulted in a 1.94-fold increase in the maximal monoclonal antibody titer and a 2.43-fold increase in specific monoclonal antibody production. In addition, I-4 significantly increased the messenger RNA levels of the monoclonal antibody and histone H3 acetylation and methylation levels. We also investigated the effect on HDAC-related isoforms and found that interference with the *HDAC5* gene increased the monoclonal antibody titer by 1.64-fold. The results of this work provide an effective method of using epigenetic regulatory strategies to enhance the expression of recombinant proteins in CHO cells.

Key points

- *HDAC/LSD1 dual-target small molecule inhibitor can increase the expression level of recombinant monoclonal antibodies in CHO cells.*
- *By affecting the acetylation and methylation levels of histones in CHO cells and downregulating HDAC5, the production of recombinant monoclonal antibodies increased.*
- *It provides an effective pathway for applying epigenetic regulation strategies to enhance the expression of recombinant proteins.*

Keywords Epigenetic regulation · Recombinant protein expression · CHO cells · Acetylation modification · Small molecule additives

✉ Tian-Yun Wang
wtianyuncn@126.com

✉ Yan-Long Jia
yanlongjia@126.com

[1] School of Pharmacy, Xinxiang Medical University, Xinxiang 453003, Henan, China

[2] International Joint Research Laboratory for Recombinant Pharmaceutical Protein Expression System of Henan, Xinxiang Medical University, Xinxiang 453003, Henan, China

[3] Henan Engineering Research Center for Biopharmaceutical Innovation, Xinxiang Medical University, Xinxiang 453003, Henan, China

[4] Junji College, Xinxiang Medical University, Xinxiang 453003, Henan, China

[5] School of Basic Medicine, Xinxiang Medical University, Xinxiang 453003, Henan, China

Introduction

In recent years, in the field of biopharmaceuticals, Chinese hamster ovary (CHO) cells have emerged as the mammalian host cells of choice for the large-scale production of recombinant therapeutic proteins due to their excellent properties (Walsh and Walsh 2022). It has significant advantages over other mammalian expression systems (Fischer et al. 2015a, b), including high safety, high yield, precise post-translational modification, ease of cultivation in large reactors and serum-free media, and flexible genetic modification (Bryan et al. 2021). However, increasing the yield of recombinant proteins in cell culture remains a challenge. The use of a variety of small molecule epigenetic modulators can effectively improve antibody expression without compromising cell growth and stability, such as sodium butyrate (NaBu) (Rodrigues Goulart et al. 2010) and valproic acid (VPA) (Muthaffar et al. 2021). Epigenetic silencing is a potential limiting factor in the efficient and stable production of recombinant proteins in rCHO cells (Jia et al. 2018).

The addition of histone deacetylase (HDAC) inhibitors (HDACis) may boost the production of recombinant proteins in mammalian cell-based manufacturing systems (Su et al. 2021) and play important roles in various cancers and therapeutics (Li and Seto 2016). HDACs regulate the acetylation of histones in nucleosomes, which may influence chromatin conformation and lead to altered gene expression (Kim et al. 2022). They similarly regulate the acetylation status of other non-histone substrates, such as important tumor suppressor proteins and oncogenes (Mrakovcic et al. 2019). HDACis are potent antiproliferative agents that regulate acetylation by targeting HDACs (Cao et al. 2021). HDACs regulate a variety of cellular processes in eukaryotes, including apoptosis (Fulda 2008, 2012), cell cycle arrest (Gu et al. 2023; Li et al. 2012), cellular differentiation (Nalawansha et al. 2017), anti-angiogenesis (Deng et al. 2020), and autophagy (Bondarev et al. 2021). Lysine-specific demethylase 1 (LSD1) functions as a flavin adenine dinucleotide (FAD)-reliant enzyme, tasked with eliminating both single and double methylation occurrences on histone H3 lysine 4 (H3K4) and histone H3 lysine 9 (H3K9) (Egolf and Capell 2020), with a tight regulatory relationship with HDAC, leading to their synergistic action (Anastas et al. 2019). The combination of HDAC and LSD1 small-molecule inhibitors has shown significant synergistic antitumor effects in a variety of malignancies, including rhabdomyosarcoma (Haydn et al. 2017), glioblastoma, and acute myeloid leukemia (Bulut et al. 2022). CHO cell lines display epigenetic features or chromatin marks associated with recombinant mRNA expression during long-term culture, and the cell lines with the highest recombinant protein mRNA expression are significantly characterized by the enrichment of histone-modified H3K9ac (Veith et al. 2016). The degree of histone acetylation can be regulated by HDACis, and transgene expression in recombinant CHO monoclonal antibodies can be enhanced after treatment with NaBu HDACi (Nmagu et al. 2021).

A small-molecule dual-target inhibitor with anti-tumor activity has been designed and synthesized to specifically inhibit both HDAC and LSD1 (Duan et al. 2021), but it is not clear whether it has any effect on CHO cells. In this study, we identified unique small molecule epigenetic modulators that promote the expression of recombinant proteins. As an additive, a novel dual-HDAC/LSD1 inhibitor I-4 (kindly provided by Prof. Duan) was used to enhance the performance of recombinant CHO cells. I-4 increases the production of monoclonal antibodies in rCHO cell culture by affecting histone acetylation and methylation levels and downregulating the expression of *HDAC5*. The results of this work provide an effective method of using epigenetic regulatory strategies to increase recombinant protein expression in CHO cells.

Materials and methods

Cell lines and culture media

Recombinant CHO cells expressing adalimumab (ADM) monoclonal antibody (CHO-ADM), obtained by screening in our laboratory, were used in this study. The sequences of the components, including CMV promoter, SV40 polyA, IRES, EF-1α promoter, HC chain (GenBank No: LQ506328.1) and LC chain (GenBank No: LQ506329.1) of adalimumab, Blasticidin, BSD resistance gene, puromycin, and Puro resistance gene sequences, were synthesized to construct a dual promoter vector pLC-pHC expressing recombinant ADM (Yang et al. 2022). Brifely, pLC-pHC plasmid was transfected into CHO-S cells (Life Technologies, A11557-01). On the day before transfection, the cells were seeded at a concentration of 300,000 per milliliter, and the liposome transfection process was started when the cells had grown to 70% coverage on the second day: 50 µL basal DMEM/F12 medium and 0.8 µg ADM plasmid were gently mixed. Liposome transfection was started when the cells reached 70% on the second day: 50 µL basal DMEM/F12 medium and 0.8 µg ADM plasmid were gently mixed, followed by 50 µL basal DMEM/F12 medium and 4 µL Lipofectamine® 2000. Both components were left untouched for 5 min at room temperature, subsequently combined, and further incubated for 20 min at room temperature to facilitate thorough binding and the development of stable transfection reagent complexes. Finally, the complex was added to the 12-well plate after liquid exchange, waited for about 5 h, replaced

with 1 mL of DMEM/F12 complete medium to continue the culture, cultured for 24–48 h, digested and then transferred to the 6-well plate for culture, and screened with 15 μg/mL of Blasticidin S (ST018, Beyotime) at progressively decreasing concentrations, successfully screening out the desired pooled polyclonal cells, named CHO-ADM. The cell line was accustomed to proliferating in a suspension culture environment. The suspension medium was Power CHO 2CD medium; the CHO cell culture medium was DMEM/F12 (319–075-CL; Wisent).

Addition of small-molecule inhibitor to cell cultures

Cells in the exponential growth phase were inoculated at a concentration of 300,000 cells per milliliter into 125-mL Erlenmeyer flasks, each containing 30 mL of culture medium. These flasks were then placed in a humidified Eppendorf CellXpert incubator, set at 37 ℃, 120 rpm, and 5% CO_2 for cultivation. Following a 3-day incubation period, the small-molecule epigenetic inhibitor I-4, provided by Professor Duan (Ma et al. 2023), dissolved in dimethyl sulfoxide (DMSO), was introduced into the cultures at varying concentrations. Daily, samples from the cultures were gathered to quantify viable cell counts and monoclonal antibody concentrations. The collected samples were stored at − 80 ℃ for subsequent analysis.

Transfection

Nonviral siRNA transfection was performed using Lipo2000 transfection reagent (BL623B; White Shark) as previously described (Ozbalci et al. 2019). siRNA transfection was performed in 6-well suspension culture plates at a final siRNA concentration of 100 pmol, unless otherwise stated. Functional transfection controls included a Chinese hamster targeting siRNA mixture (cgr)-*HDAC5* (siHDAC5) and a nontargeting control NC.

Flow cytometric analysis

Cells were collected in suspension on day 7 and assayed using Apoptosis Kits (KGA111; KGI Bio and C1052; Beyotime). The apoptosis and cell cycle distributions were analyzed using an Invitrogen flow cytometry analyzer (ThermoFisher) and the FlowJo software (Tree™).

Quantitative reverse transcriptase- polymerase chain reaction (qRT-PCR) analysis

Previously outlined procedures were followed to collect cultured cells for the extraction of messenger RNA (mRNA) and subsequent qRT-PCR analysis (Chervoneva et al. 2018). Briefly, during the logarithmic growth phase, CHO-ADM cells were gathered, and a suitable quantity of TRIzol was administered to each set of cells, which were completely lysed and collected at low temperature. RNA was extracted with chloroform, isopropanol, and 75% anhydrous ethanol, and then, total mRNA was reverse transcribed to cDNA using oligo-dT. The qRT-PCR amplification assay was executed utilizing a reaction system comprising 10 μL of stock solution, 4 μL of 5 × Evo M-MLV RT reaction mix, and 6 μL of RNase-free water, resulting in a final volume of 20 μL. The ΔΔCt method, calculated from the number of cycles (Ct value) at which the fluorescence signal reached a threshold during PCR amplification, was used to determine relative gene expression. The primers utilized for conducting the qRT-PCR analysis are detailed in Table 1.

Table 1 The siRNA sequences for the targeted genes

Genes	Orientations	Sequences (5′-3′)
HC	Forward	5′-CCCAGAACCCGTTACCGTTAG TTG-3′
	Reverse	5′-CCAAGGCTTGAGGAAGGTACA GTG-3′
LC	Forward	5′-TTTCCCTCCAAGTGACGAACAGC-3′
	Reverse	5′-TTGTGAGTTCCCAGACTGAAG TGC-3′
HDAC1	Forward	5′-ACAGCAAGCAGATGCAGAGA-3′
	Reverse	5′-TTCACAGCACTTGCCACAGA-3′
HDAC2	Forward	5′-AAGCGGATTTCGATTCGTGC-3′
	Reverse	5′-GACCTCCTTCGCCTTCATCC-3′
HDAC3	Forward	5′-ATCATGGCGATGGTGTGGAA-3′
	Reverse	5′-CAGCCCCAATATCCCGAAGG-3′
HDAC4	Forward	5′-CACCCCTTGTCACAGACTGG-3′
	Reverse	5′-GAATGGATGGGGACACCCTG-3′
HDAC5	Forward	5′-GTCACCGCCAGATGTTTTGG-3′
	Reverse	5′-TGATGCTGGGTTTTTGCTGC-3′
HDAC6	Forward	5′-ACACTACGAACTGGACCTCT-3′
	Reverse	5′-GTCACAGCATAAAATACATCC TGG-3′
HDAC7	Forward	5′-GCAGGAGGTGCAGTGGTATT-3′
	Reverse	5′-AGCCCCAGTATTTCCTGTGC-3′
HDAC8	Forward	5′-GGGATCGGCAAGTGTCTGAA-3′
	Reverse	5′-GCAAGGTTATAGCCTCCTCCTC-3′
HDAC9	Forward	5′-CAGAGGACGAGAAAGGGCAG-3′
	Reverse	5′-GTGGTGAGCAGCCCAGAG-3′
HDAC10	Forward	5′-GGAAGTTGCTGCAGGTCCTC-3′
	Reverse	5′-TGTGTGCGCTCTTCTCCAAA-3′
HDAC11	Forward	5′-GAGGCTGGGCCATCAATGTT-3′
	Reverse	5′-CCGCCTGATGGCCTTGATAG-3′
si HDAC5	Sense	5′-GCAACAGAGCACGCUUAUATT-3′
	Antisense	5′-UAUAAGCGUGCUCUGUUGCTT-3′
NC	Sense	5′-UUCUCCGAACGUGUCACGUTT-3′
	Antisense	5′-ACGUGACACGUUCGGAGAATT-3′

Determination of viable cells and monoclonal antibody concentration

The viable cell density and viability of the suspension cells were measured daily with the Count star automatic cell counter, utilizing the Taipan blue dye exclusion technique. Culture supernatants were collected to determine the monoclonal antibody concentration using protein immunoblotting and enzyme-linked immunosorbent assay. The specific monoclonal antibody productivity (qmAb) was assessed via the time-integrated value of monoclonal antibody concentration versus live cell concentration. For six consecutive days, Cell Counting Kit-8 (NCM Biotech, C6005) was employed to monitor cell proliferation and growth.

Protein blotting analysis

CHO-ADM cells in the logarithmic growth stage were harvested. Based on the cell count, RIPA lysate was administered, and the samples were promptly relocated to a consistently chilled freezer for storage. Next, the cells were centrifuged at low temperature, and the supernatant was collected for subsequent experiments. SDS-PAGE was utilized to separate the protein samples. The gels were thoroughly wetted in the transmembrane solution and transferred to PVDF membranes to remove the effect of nonspecific adsorption. PVDF membranes were incubated overnight at 4 °C with the appropriate primary antibody, followed by incubation with the appropriate secondary antibody for 2 h on a shaker. The blots were developed using a Biotronik chemiluminescent HRP substrate. For protein analysis, the following primary antibodies were employed: anti-HDAC5 mouse polyclonal antibody (1:2000), anti-total histone H3 rabbit polyclonal antibody (1:2000), anti-acetylated histone H3K27 mouse monoclonal antibody (1:2000), anti-acetylated histone H3K9 mouse monoclonal antibody (1:2000), anti-histone H3K4me2 mouse monoclonal antibody (1:2000), and anti-β-actin (1:10,000) and anti-LSD1 mouse monoclonal antibody (1:2000), all purchased from Abways, China.

Statistical analysis

ANOVA was performed to compare differences between multiple groups, and independent samples were analyzed using *t*-tests. $*P < 0.01$ was considered to indicate high significance, $***P < 0.001$ was considered to indicate high statistical significance, $****P < 0.0001$ was considered extremely statistically significant, $**P < 0.05$ was considered to indicate significance, and $P > 0.05$ (ns) was considered to indicate nonsignificant results. Images were analyzed for gray level using the ImageJ software.

Results

Small-molecule inhibitor I-4 significantly increases ADM monoclonal antibody production

To determine whether treatment with the small-molecule inhibitor I-4 could increase monoclonal antibody production and the most effective concentration, different concentrations of I-4 (0.5, 1, 2, or 4 μM) were added to CHO-ADM cells on the third day of suspension culture. Figure 1a shows the molecular structural formula of I-4. Compared with the control, the addition of I-4 increased monoclonal antibody expression in a manner that is contingent upon the dosage administered (Fig. 1b). Specifically, a marked decrease was observed at 4 μM, and as the experiment progressed, cell growth inhibition, and a decrease in cell viability became evident in later stages (Fig. 1c, d). Despite the slight inhibition in cell growth, the addition of I-4 resulted in a significant increase in ADM monoclonal antibody concentration, which increased approximately 1.94-fold at 2 μM. The observed increase in monoclonal antibody titer was due to an increase in qmAb of approximately 2.43-fold (Fig. 1e). In conclusion, the small-molecule inhibitor I-4 significantly increased ADM monoclonal antibody production, and treatment was most effective at 2 μM.

Currently, CHO cell cultures employ a diverse range of HDAC-associated small-molecule inhibitors to enhance the yield of recombinant antibodies. Sodium butyrate (NaBu) and valproic acid (VPA) have been utilized in the culture of recombinant CHO (rCHO) cells. Vorinostat (SAHA) is a potent non-selective HDAC inhibitor, with non-documented use in CHO cells to enhance recombinant protein production. We compared the effects of three inhibitors—SAHA (1.42-fold), sodium butyrate (1.47-fold), and valproic acid (1.82-fold)—with that of I-4 (2.36-fold) and found that I-4 had a great advantage in enhancing the production of ADM recombinant proteins (Fig. 2a, b).

The small-molecule inhibitor I-4 blocks the rCHO cell cycle

We examined the effect of the small-molecule inhibitor I-4 on cell growth characteristics and found that exposure to 2 μM and 4 μM of I-4 resulted in a moderate growth suppression of CHO-ADM cells using the CCK8 assay (Fig. 3a). This finding was consistent with the trend in the pre-suspension assay; thus, in combination with Fig. 1, the most potent 2-μM concentration was selected for subsequent studies. The analysis of the cell cycle outcomes revealed that treatment with I-4 led to an augmentation in

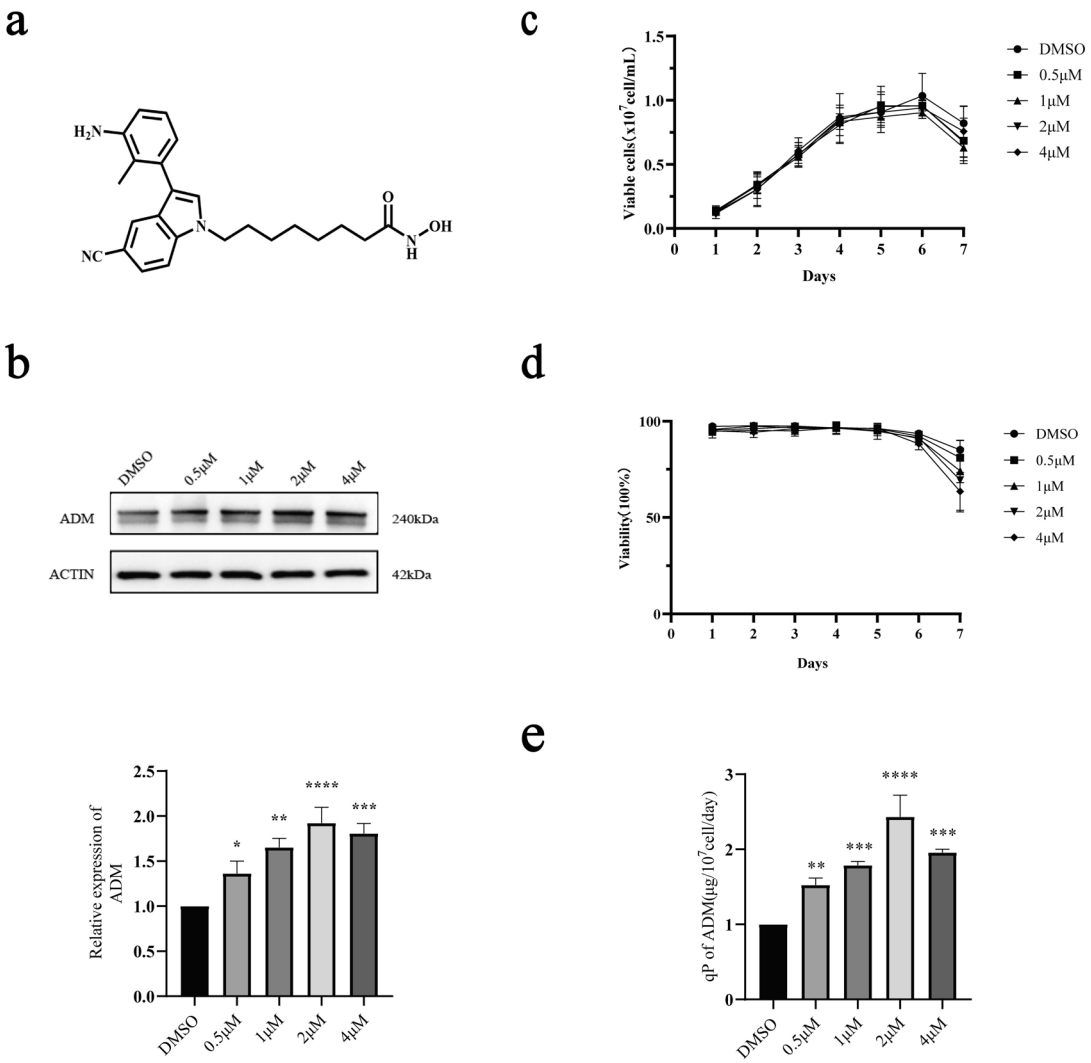

Fig. 1 CHO-ADM cells treated with DMSO (closed circle), 0.5 μM (closed square), 1 μM (rising triangle), 2 μM (falling triangle), and 4 μM (open diamond) I-4 on day 3 of culture. **a** I-4 molecular structure formula. ADM protein levels were detected using immunoblotting and grayscale normalization analysis (**b**). **c** Number of viable cells, **d** cell viability, and **e** specific monoclonal antibody productivity (qmAb). DMSO, dimethyl sulfoxide; adrenomedullin monoclonal antibody, ADM monoclonal antibody. All conditions were performed in three independent replicates and the data are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for statistical analysis. $^{****}P < 0.0001$ vs. DMSO, $^{***}P < 0.001$ vs. DMSO, $^{**}P < 0.01$ vs. DMSO, and $^{*}P < 0.05$ vs. DMSO

the G1/0 phase cell count, contrasting with untreated cells, whereas a reduction in the proportion of cells occupying the S and G2 phases of the cell cycle was noted (Fig. 3c). Consequently, there is a block in the cell cycle at G1/0 phase, thus affecting cell growth (Avello et al. 2022). We also found that treatment with I-4 had almost no negative effect on CHO cell apoptosis (Fig. 3b).

Effect of the small-molecule inhibitor I-4 on monoclonal antibody mRNA levels and epigenetic modifications in CHO-ADM cells

To investigate how I-4 affects the mRNA expression of recombinant ADM monoclonal antibody, we collected sample cells treated with 2 μM I-4 on day 7 of cell culture,

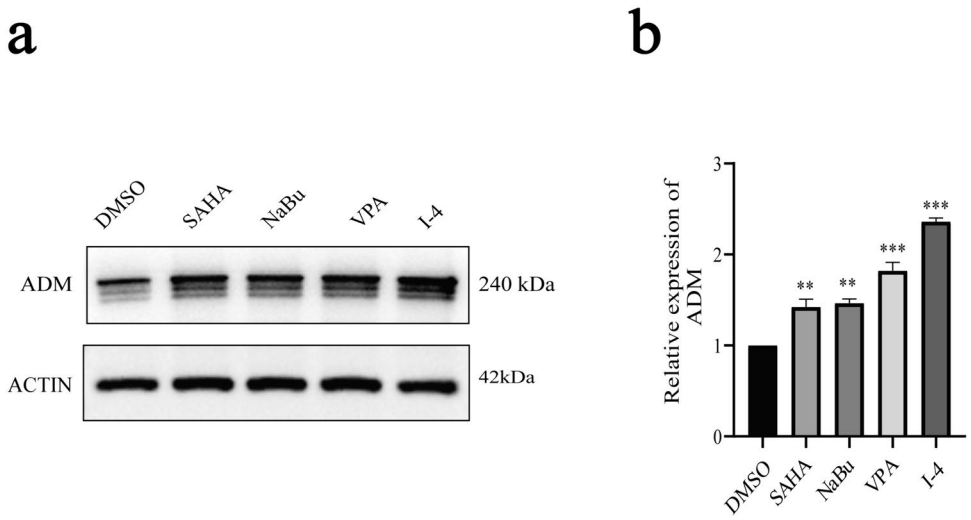

Fig. 2 Effect of HDAC inhibitors [SAHA(2 μM), NaBu(2 mM), VPA(2 mM), and I-4(2 μM)] on recombinant ADM protein production detected using immunoblotting. **a** Expression of recombinant protein and **b** analysis normalized to gray value. All conditions were performed in three independent replicates, and the data are expressed as the mean ± standard deviation (SD). For the statistical analysis, the samples were analyzed using one-way analysis of variance (ANOVA). $^{**}P < 0.01$ vs. DMSO, and $^{***}P < 0.001$ vs. DMSO

as shown in Fig. 3. Subsequently, we applied qRT-PCR to determine the relative levels of antibody light chain (LC) and heavy chain (HC) mRNA. According to the experimental results, the use of I-4 significantly elevated the expression of HC and LC mRNA by approximately 1.60-fold and 1.44-fold, respectively, compared with the control group without I-4 treatment (Fig. 4a, b).

Histone acetylation prevents the tight condensation of chromatin structures, which in turn promotes transcriptional activity and increases gene expression levels (Marmorstein and Zhou 2014). One of the traditional ways of assessing HDAC activity is to analyze the acetylation status of histones H3 and H4, a method that effectively reflects the level of HDAC activity (Bjerling et al. 2002); specifically, LSD1 acts as a regulator of gene transcription through its ability to remove mono- and dimethylation marks at lysine 4 (H3K4) and lysine 9 (H3K9) on histone H3 (including activation or repression) (Fang et al. 2019). We hypothesized that the acetylation status of histones H3 and H4 could be used as a marker for HDAC activity and analyzed the methylation and acetylation status of histone H3 in I-4-treated CHO-ADM cells to test whether I-4 can inhibit HDAC and LSD1 expression at the cellular level. I-4 at a concentration of 2 μM was added to suspension-cultured CHO-ADM cells. After 48 h of exposure, changes in the protein expression levels of the LSD1 protein and LSD1 substrate H3K4me2, and the HDAC substrates Ac-H3K9 Ac-H3K27, and total histone H3 were detected using Western blotting. We found that I-4 had a significant inhibitory effect on the protein expression of LSD1 and that it up-regulated the protein expression levels of H3K4me2, Ac-H3K9, and Ac-H3K27 in cells (Fig. 5a, b). This indicates that I-4 can effectively inhibit the expression of HDAC and LSD1 in cells, thereby increasing the methylation and acetylation levels of histone H3.

Small-molecule inhibitor I-4 downregulates HDAC5 gene expression

Histone deacetylates tightens the DNA structure, thereby stopping gene transcription (Li and Seto 2016). HDAC has different isoforms (Han et al. 2024) (HDAC1–11) that regulate different cellular physiological functions, such as cell proliferation, differentiation, migration, and survival (Parveen et al. 2023). To detect the active HDAC isoforms, 2 μM I-4 was applied to CHO-ADM monoclonal antibody cells. Samples were collected on day 7, and the mRNA expression levels of HDAC1–11 were detected using qPCR. The *HDAC5* mRNA expression was significantly reduced, and *HDAC5* protein expression was also detected using Western blotting (Fig. 6a, b). The selective inhibitory activity of I-4 against class I HDACs, particularly *HDAC5*, was confirmed.

To verify the efficacy of this gene, *HDAC5* siRNA was constructed in this study (Fig. 6c, d) and transfected into CHO-ADM suspension cells on day 3. The supernatants and cells were collected, and the assay revealed that treatment with siHDAC5 increased the expression of recombinant proteins without affecting cell viability or the density of viable cells (Fig. 7b, c), which was approximately 1.64-fold higher than that of the control (Fig. 7a).

Discussion

Small-molecule epigenetic modulators are used to treat a wide range of human pathologies by targeting specific proteins and pathways that affect cellular function (Zhang et al. 2021) and are therefore highly relevant for drug discovery and disease treatment (Singh et al. 2018). Epigenetic modifications are indispensable factors in the process of regulating

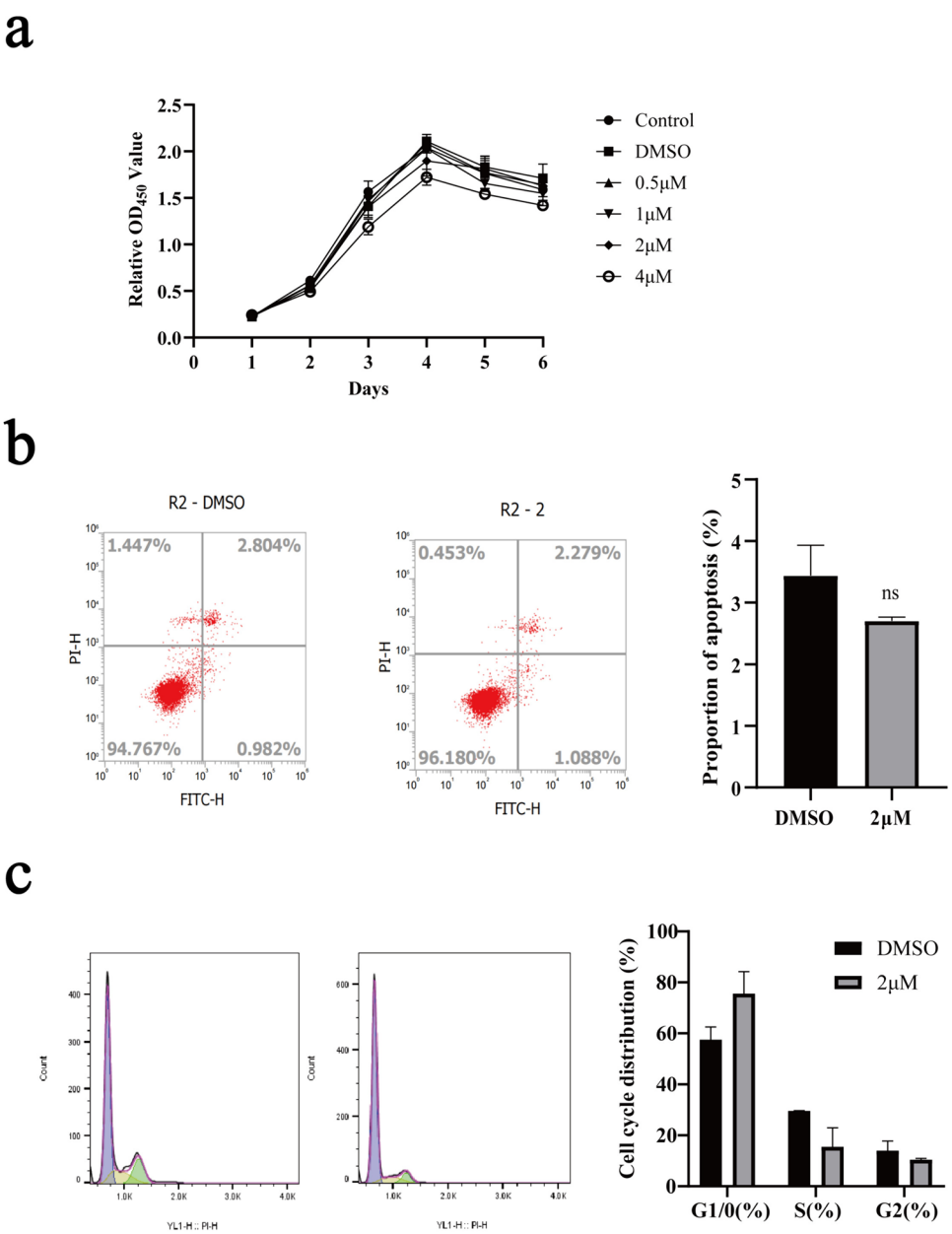

Fig. 3 Comprehensive analysis of cellular properties in ADM monoclonal antibody-generating CHO cells. **a** CCK8 assay to detect the proliferative growth status of CHO-ADM monoclonal antibody cells treated with different concentrations of I-4 in culture for six days. **b** Distribution of apoptosis two days after the addition of I-4 and **c** cell cycle distribution. All conditions were performed in three independent replicates, and the data are expressed as the mean ± standard deviation (SD). A *t*-test and one-way analysis of variance (ANOVA) were used for statistical analysis. ns, not significant vs. DMSO

chromatin structure and gene expression and affect transcriptional and post-transcriptional programs (Chen et al. 2017). Small-molecule epigenetic regulators that reshape the epigenetic signature of cells serve as invaluable assets in gene expression levels. In this study, we used a unique dual-HDAC/LSD1 inhibitor, I-4, which is associated with epigenetic changes, as a means to increase recombinant protein production in rCHO cell culture. We investigated the interactions between I-4 and epigenetic modifications in an attempt to elucidate potential mechanisms underlying the regulation of cellular function and gene expression. We found that a concentration of 2 μM I-4 was the most beneficial in the monoclonal antibody cell line; in addition, administration of I-4 treatment on the third day of incubation resulted in a significant 1.94-fold increase in monoclonal antibody titer. The volumetric yield enhancement observed can be ascribed to the approximately 2.43-fold increase in qmAb. This is a significant advantage over inhibitors such as VPA and NaBu (Hua et al. 2022; Yang et al. 2014).

To investigate the effects of I-4 on monoclonal antibody-producing CHO cells in detail, we assessed changes in cell cycle phase distribution, cell viability, apoptosis rate, and cell proliferation following the action of I-4. Late growth and viability of the cells treated with 2 μM I-4 were found to be impaired compared to those in untreated cells; thus, we cannot exclude that the positive effect of I-4 on monoclonal antibody production is impaired by its negative effect on cell growth. The effect could perhaps be counteracted in a

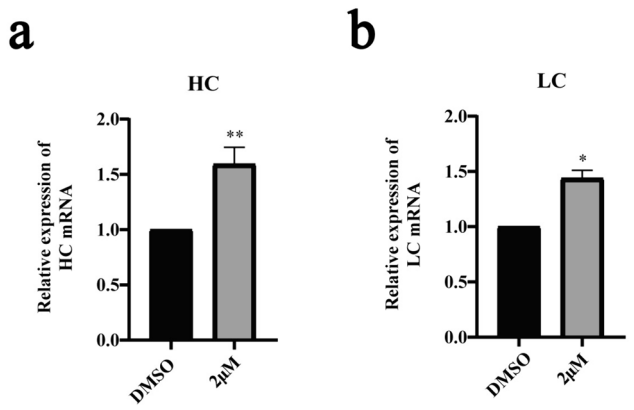

Fig. 4 The relative mRNA expression levels of antibody HC (**a**) and LC (**b**) were detected in CHO-ADM cells treated with DMSO or 2 μM I-4. Cells harvested on day 7 were used in the analysis. All conditions were performed in three independent replicates, and the data are expressed as the mean ± standard deviation (SD). HC, heavy chain; LC, light chain;$^{**}P < 0.01$ vs. DMSO and $^{*}P < 0.05$ vs. DMSO

complementary manner by the addition of chemical additives (Chevallier et al. 2020). The impairment of cell growth compared to the corresponding figures for cells not treated with I-4 appears to be due to an increase in the number of I-4-treated cells entering the G1/0 phase, coupled with a decrease in the percentage of cells progressing to the S and G2 phases. Monoclonal antibody secretion is enhanced by I-4, apparently through its inhibition of cell cycle progression, resulting in cells remaining in the highly productive G0/G1 phase. A number of widely recognized small-molecule inhibitors are known to trigger cell cycle halt and apoptosis in cancerous cells (Ajayi-Smith et al. 2021; Hird et al. 2015), whereas, in this study, I-4 had almost no negative effect on CHO cell apoptosis. SAHA is the first HDAC inhibitor that specifically binds to and blocks the activity of HDACs, which in turn prevents gene expression and induces apoptosis in tumor cells (Wawruszak et al. 2021). In contrast, VPA treatment increases the transient antibody production of CHO-DG44 by at least 1.5-fold. Different

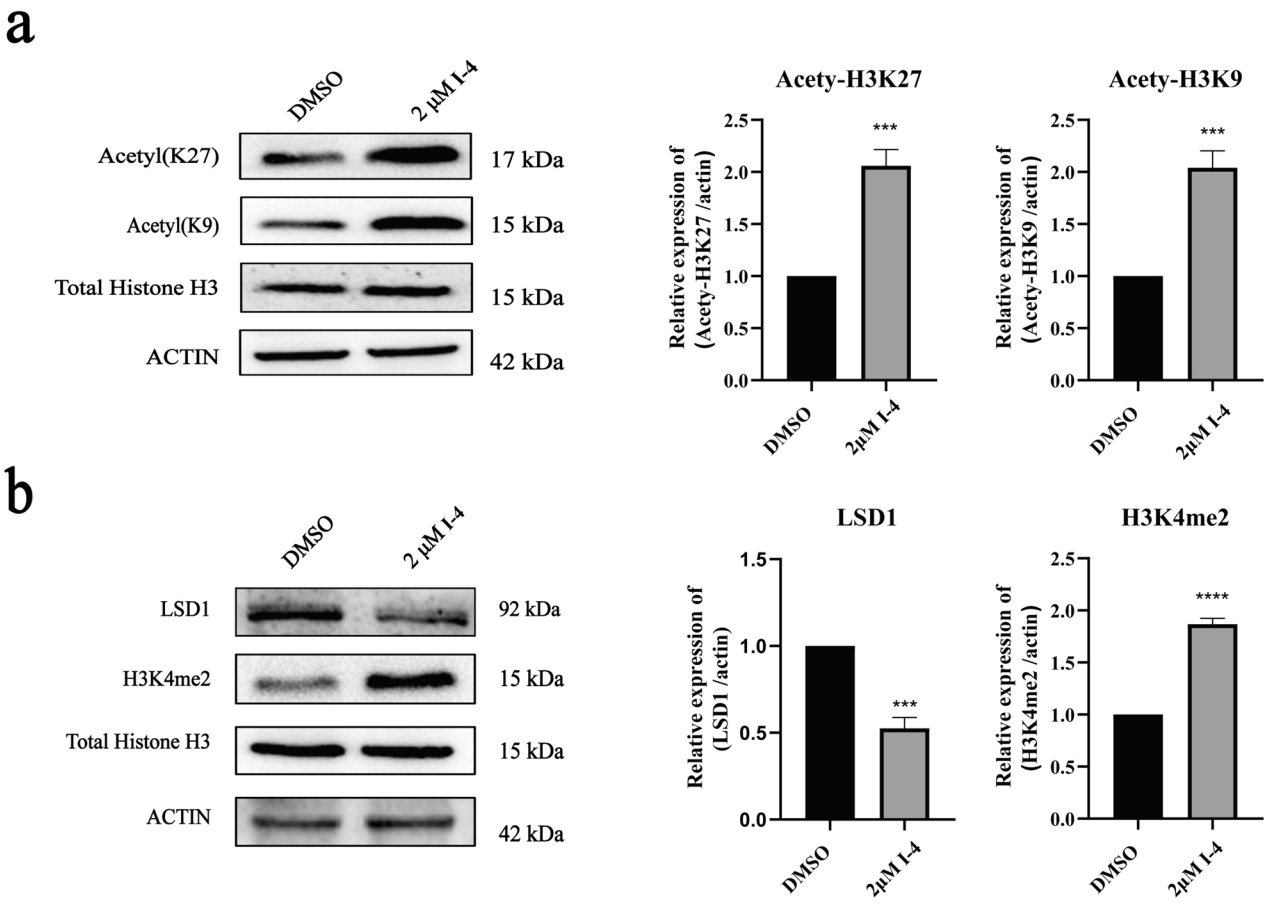

Fig. 5 CHO-ADM cells treated with DMSO or 2 μM I-4 and Western blotting to detect changes in the protein expression levels of HDAC substrates **a** Ac-H3K9 and Ac-H3K27 and LSD1 substrate **b** H3K4me2 and total histone H3, LSD1 proteins. Cells harvested on day 7 were included in the analysis. All conditions were performed in three independent replicates, and the data are expressed as the mean ± standard deviation (SD). DMSO, dimethylsulfoxide; HDACs, histone deacetylases. $^{****}P < 0.0001$ vs. DMSO, and $^{***}P < 0.001$ vs. DMSO

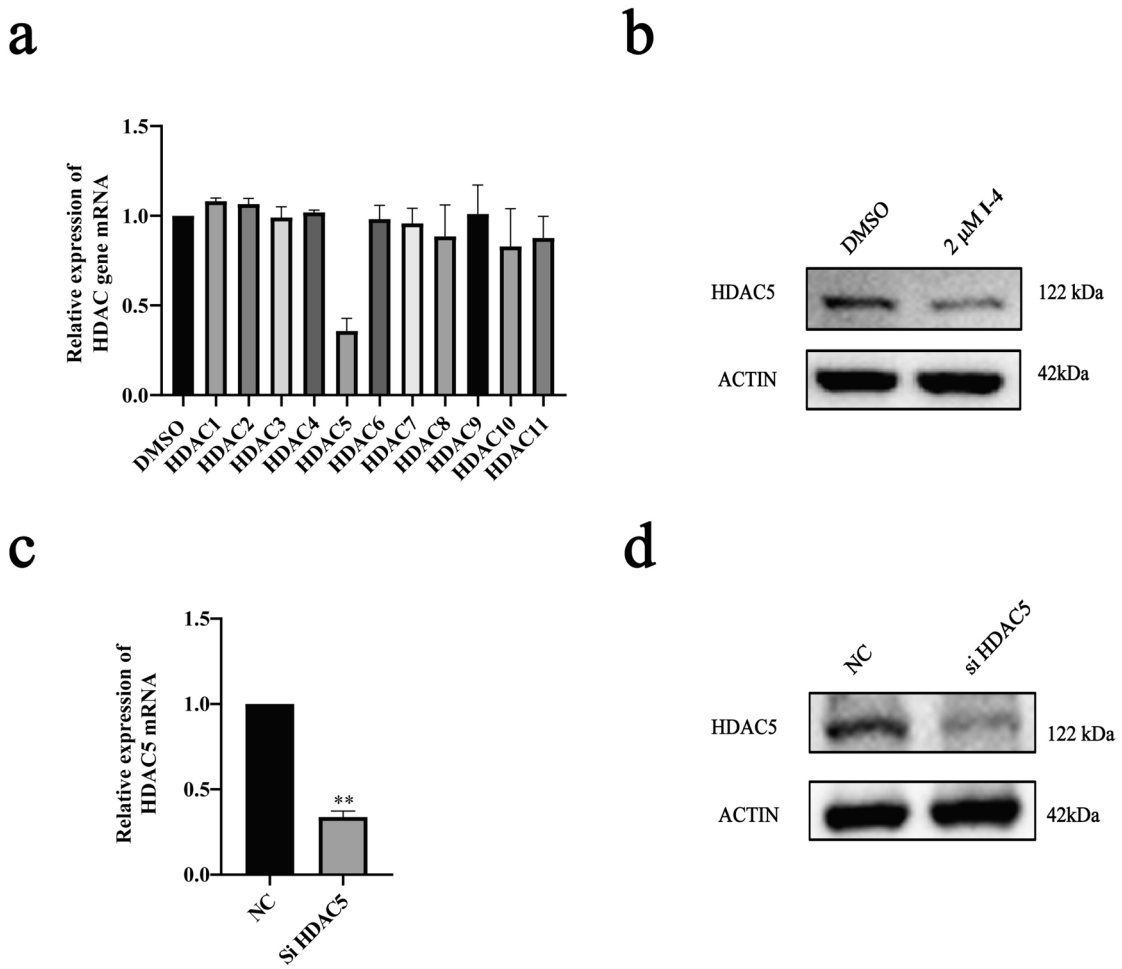

Fig. 6 CHO-ADM cells treated with DMSO or 2 μM I-4 were used to detect the relative mRNA expression level of HDAC1-11 gene (**a**) and the protein expression level of *HDAC5* gene (**b**). Measure the relative mRNA expression and protein levels (**c**, **d**) of *HDAC5* gene in CHO-ADM cells treated with NC (vector control) or siHDAC5. Cells harvested on day 7 were included in the analysis. All conditions were performed in three independent replicates, and the data are expressed as the mean ± standard deviation (SD). HDAC, histone deacetylase, mRNA; siHDAC5, small interfering RNA of *HDAC5*. ns, the difference was not significant. $^{**}P < 0.01$ vs. NC

key nutrients significantly affect the expression of cellular proteins in serum-free medium (Kuang et al. 2021; Ritacco et al. 2018; Tian et al. 2018). In this study, the use of I-4 was significantly more suitable for recombinant protein production than other common small-molecule inhibitors.

The level of gene transcription is correlated with the histone acetylation status of the promoter (Shvedunova and Akhtar 2022), which induces gene expression by remodeling the chromatin structure (Salminen et al. 2016). LSD1 has been shown to regulate gene transcription (including activation or repression) (Li et al. 2023). We explored the impact of I-4 on mRNA abundance and epigenetic alterations by analyzing the transcriptional levels of monoclonal antibody light chain (LC) and heavy chain (HC) mRNAs. We found that both HC and LC mRNA levels increased significantly, which may be the main reason for the increase in specific productivity observed in the preliminary stage.

The dual inhibitors targeting LSD1/HDAC designed and synthesized by Duan et al. (2021) could dose-dependently upregulate the expression of H3K4Me1/2, H3K9Me2, and H3-Ac in cancer cells, suggesting the simultaneous inhibition of intracellular LSD1 and HDAC. In this study, we also found that I-4 as a novel dual inhibitor had similar inhibitory effect targeting LSD1/HDAC in CHO cells. Our research indicates that I-4 has the ability to directly inhibit the expression of LSD1 protein. In addition, it significantly increases the intracellular expression levels of H3K4me2, Ac-H3K9, and Ac-H3K27 proteins. This suggests that I-4 can effectively inhibit the activity of intracellular HDAC and LSD1, thereby increasing the acetylation and methylation level of histone H3. However, this effect is not unique, as HDAC inhibitors such as moxistat (Weber et al. 2022) also increase qmAb by increasing histone acetylation and subsequent mRNA levels; the potential mechanism of action of I-4

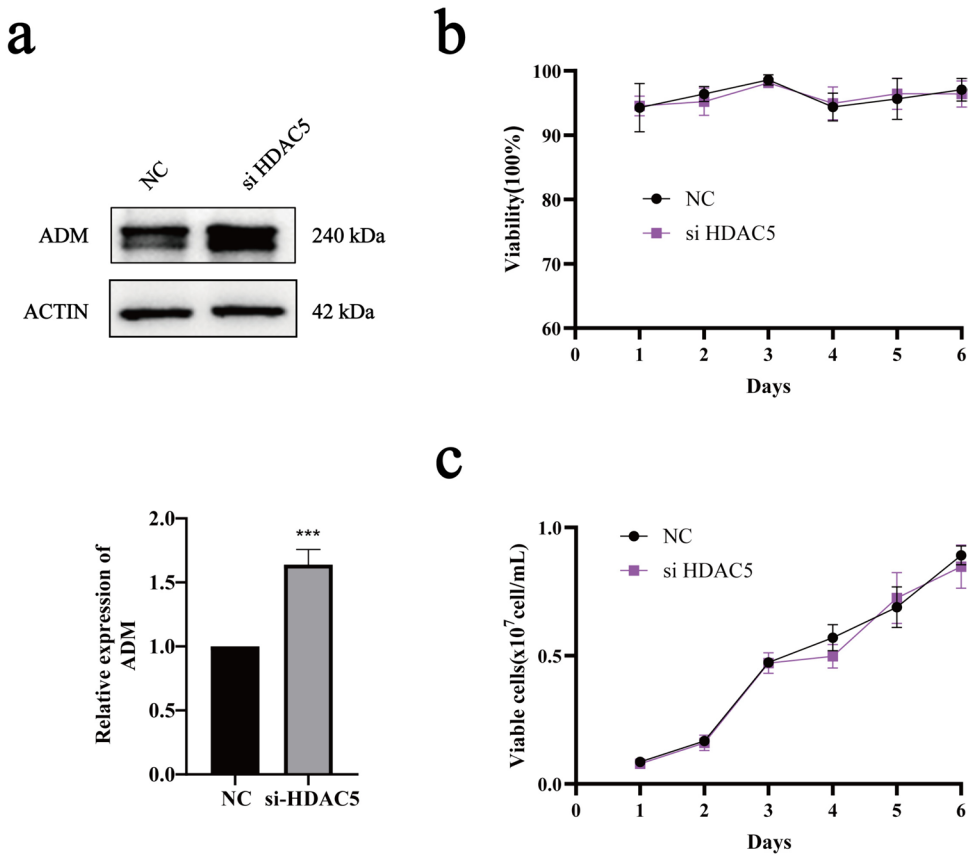

Fig. 7 CHO-ADM cells treated with NC (vector control) or siHDAC5. **a** Detection of monoclonal antibody expression levels and gray value analysis of the supernatant phase on day 6. **b** Cell viability. **c** Live cell density. Supernatants and cells harvested on day 7 were included in the analysis. All conditions were performed in three independent replicates, and the data are expressed as the mean ± standard deviation (SD). siHDAC5, small interfering RNA for *HDAC5*; *HDAC5*, histone deacetylase 5. ***$P < 0.01$ vs. NC

on monoclonal mass remains to be elucidated. We demonstrated that I-4 has selective inhibitory activity against class I HDACs, particularly *HDAC5*, mainly because the mRNA and protein expression of *HDAC5* decreased significantly in a large-scale screening test. To verify the validity of this gene, siHDAC5 was generated and used to examine interference at both the mRNA and protein levels. We found that treatment with siHDAC5 increased the expression of recombinant proteins by approximately 1.64-fold compared to the control without affecting cell viability or viable cell density, which is in agreement with the findings of Simon and colleagues (Fischer et al. 2015a, b). This demonstrates that downregulation of the expression of *HDAC5* can improve recombinant protein production.

In this study, a dual promoter vector expressing the LC/HC genes of adalimumab driven by the CMV and EF-1α promoters, respectively, was used in this study (Yang et al. 2022). Due to epigenetic regulation such as DNA methylation or histone acetylation, the expression and especially the stability of expression of recombinant proteins driven by different promoters in CHO cells yield different results (Jia et al. 2018). The acetylation of histone H3 is generally a marker of the active promoter, and histone acetylation can enhance the expression of CMV promoter (Moritz et al. 2016; Kim et al. 2022). Therefore, we speculate that I-4 inhibition of *HDAC5* may enhance the acetylation level of histones, while H3Kac acts as an enhancer and further binds to the promoter, activating its transcription, which may be the reason for promoting the expression of recombinant proteins. Further studies should be carried out to investigate the underlying mechanism and the use of I-4 for other recombinant proteins driven by different promoters.

Using HDACis to increase productivity has shown variable success, and their effect on productivity may depend on the productivity of the clone itself (Yang et al. 2014; Backliwal et al. 2008). The CHO cells used to study the effect of I-4 on recombinant protein expression are the selected stable cell pool expressing rADM. During the selection process, clones with certain growth profile/host cell gene expression levels may be outgrown. CHO cell line instability can be affected by chromosomal rear-rangement, DNA methylation, and transcriptional/translational silencing, leading to the production instability (cell clonal heterogeneity) (Cordova et al. 2024). Therefore, we plan to establish a stable recombinant antibody CHO cell line through stability testing and identification to ensure that the enhancing effect of I-4 on recombinant protein expression is reproducible during the long-term culture. In addition, the use of a more diverse cell population is necessary to

clarify whether the effect of our strategy in this study is cell specific or universal.

Taken together, the results of the this study suggest that the small-molecule dual-targeting inhibitor I-4 can be used as an effective chemical additive to improve the yield of monoclonal antibodies in recombinant CHO cell cultures.

Author contribution Literature review: HMM; methodology: HMM and WHT; investigation: HMM, WHT, and ZHJ; analysis: HMM and WHT; data collection: WHT and GJL; writing original draft and editing: HMM, LJT, WXY, and ZX; writing review: HMM, QLL, WHT, and WXY; supervision: WTY and JYL.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval This article does not contain any studies with animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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