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A novel HDAC6/HIF-1 α dual inhibitor halts malignant progression and reprograms immune microenvironment in triple-negative breast cancer

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7

8 **Abstract**

9 Triple-negative breast cancer (TNBC) remains the most difficult breast cancer
10 subtype to treat, being highly invasive and prone to metastasis and recurrence.
11 Conventional chemotherapy is limited by acquired resistance, so new
12 strategies are needed. Based on the concept of synergistic combination
13 therapy, we paired the epigenetic regulator histone deacetylase 6 (HDAC6),
14 which controls the acetylation of multiple proteins, with the transcription factor
15 hypoxia-inducible factor-1 α (HIF-1 α), which controls the expression of multiple
16 genes. A series of dual-target derivatives was designed and synthesized, and
17 the lead compound 12e was evaluated against panobinostat and sorafenib.
18 12e exerted multiple anti-breast cancer effects: it was antiproliferative,
19 antimetastatic and antiangiogenic, and it reversed tumour immunosuppression
20 and immune escape. Its antiproliferative effect was comparable to that of
21 panobinostat, its suppression of lung metastasis was comparable to that of
22 sorafenib, and its antiangiogenic effect exceeded that of sorafenib.

1 Mechanistically, dual inhibition of HDAC6 and HIF-1 α produced a novel
2 anti-breast cancer mechanism in which multiple signalling pathways and the
3 downstream functional proteins of both targets were synergistically reinforced.
4 The design goal of a multifunctional dual-target antitumour agent was
5 achieved: HDAC6/HIF-1 α is a viable target combination for drug discovery,
6 and 12e is a candidate drug for TNBC.

7 **Keywords:** HDAC6/HIF-1 α dual inhibitor; Triple-negative breast cancer;
8 Multi-functional antitumour; Immunomodulation

9

10 **Introduction**

11 Triple-negative breast cancer (TNBC) remains one of the most
12 challenging malignancies to treat due to its lack of expression of oestrogen
13 receptor (ER), progesterone receptor (PR), and human epidermal growth
14 factor receptor 2 (HER2). Consequently, TNBC is characterized by high
15 invasiveness, metastasis potential and recurrence rate. Traditional treatment
16 relies mainly on chemotherapy; however, the development of drug resistance
17 significantly limits the sustainability of therapeutic effects and patient survival
18 rates. Thus, diverse treatment strategies for TNBC are urgently needed.

19 In recent clinical trials, emerging therapies such as poly(ADP-ribose)
20 polymerase (PARP) inhibitors, immune checkpoint inhibitors, Trop2-targeting
21 antibody–drug conjugates (ADCs), and multitarget inhibitors have
22 demonstrated certain therapeutic benefits. These therapies significantly

1 prolong progression-free survival (PFS) and overall survival (OS) in specific
2 patient subgroups¹⁻⁶. However, the profound intra-tumoral heterogeneity and
3 complex molecular mechanisms of TNBC remain major obstacles to
4 advancing therapeutic outcomes. Therefore, targeting the complex tumour
5 microenvironment (TME), developing multifunctional antitumour drugs with
6 synergistic effects, reversing drug resistance during TNBC progression, and
7 enhancing immune reactivity are critical challenges in current research.

8 In recent years, cocktail therapy combining multiple drugs has achieved
9 encouraging clinical results; however, the combination of multiple agents also
10 increases limitations due to cumulative toxicity. This highlights the urgent need
11 to design and synthesize the multifunctional antitumour drugs that can exert
12 synergistic or combined effects through multiple pathways, achieving
13 therapeutic outcomes comparable to those of cocktail therapy. Here, we report
14 the construction of HDAC6/HIF-1 α targets and the drug design, synthesis,
15 efficacy, and underlying molecular mechanisms of the candidate dual-target
16 inhibitor 12e against HDAC6 and HIF-1 α .

17 **Results**

18 **Construction and design of an HDAC6/HIF-1 α dual-target drug**

19 On the basis of the clinical success of “cocktail therapy”, we aimed to
20 achieve multifunctional antitumour effects, including antiproliferative,
21 antimetastatic, antiangiogenic and immune microenvironment remodelling
22

1 effects, through dual-target synergistic/combined enhancement. We selected
2 genes that are highly expressed in solid tumour breast cancer, such as histone
3 deacetylase 6 and its regulated transcription factor HIF-1 α , as a dual-target
4 combination. For a literature analysis and the mechanism of synergistic
5 enhancement, please refer to S2.1.

6 In general, compounds that directly bind to the target site, which can
7 effectively exert inhibitory effects, are selected for inhibitor drug design.
8 Structure and function studies of targets have shown that, for the protein
9 domains of targets with mainly a single function, the designed compounds
10 have achieved the expected effects. Although HIF-1 α is a transcription factor
11 that can regulate more than 60 genes⁷, the multigene regulatory mechanism
12 has not yet been clarified by structural biology studies. Therefore, direct
13 binding to a target not only effectively inhibits VEGF and its downstream
14 pathways but also has unpredictable effects on other genes regulated by
15 HIF-1 α , resulting in side effects. However, HIF-1 α inhibitors have been under
16 investigation for more than two decades, and more than a dozen inhibitors
17 have entered clinical trials. However, to date, no HIF-1 α inhibitor has been
18 marketed as a drug (only HIF-2 α agonists have been marketed). The results of
19 clinical trials have shown that the toxicity and side effects are significant, and
20 the inhibitory effect on HIF-1 α has not reached the expected level.

21 Our research group has carried out derivative synthesis studies of HIF-1 α
22 inhibitors from natural sources. The alkaloid derivatives 11g and ION-31a are

1 HIF-1 α inhibitors that bind to HSP90, reduce protein stability, and
2 downregulate the mRNA level of HIF-1 α ⁸⁻¹⁰, thereby downregulating the
3 expression level of HIF-1 α . The pharmacological characteristics of the
4 abovementioned derivatives include strong inhibitory effects on VEGF but
5 weak cytotoxicity [8-10], which is different from the characteristics of HIF-1 α
6 inhibitors that have failed clinical trials and are mainly cytotoxic. Therefore, if a
7 previous research method involving HIF-1 α inhibitors is adopted, the
8 structure–activity relationship method of cell phenotype and chemical structure
9 can be used, and the HDAC6 target can be introduced at the same time to
10 enhance the antitumour proliferation effect; thus, it is possible to avoid the risk
11 of clinical failure as shown by many HIF-1 α inhibitors and achieve the
12 synergistic development of dual targets.

13 In addition, the HDAC family is divided into four types and has multiple
14 subtypes. Compared with selective inhibitors, nonselective inhibitors generally
15 have greater toxicity and side effects. Encouragingly, unlike HIF-1 α inhibitors,
16 several pan-HDACS inhibitors have already become marketed drugs^{11,12}.
17 Furthermore, since the launch of panobinostat, selective HDAC inhibitors have
18 been researched for more than a decade. Only four selective inhibitors are
19 currently in phase I–II clinical trials, and no drugs have been marketed yet,
20 indicating the high difficulty of this research.

21 Moreover, the distribution selectivity of HDAC6 and the localization of its
22 active substrates suggest that the derivative 12e mainly acts on HDAC6.

1 HDAC6 is the only member of the HDAC family that is localized mainly in the
2 cytoplasm¹³. It mainly targets proteins in the cytoplasm by directly
3 deacetylating microtubules, cortical actin, and heat shock protein 90 (HSP90).
4 In contrast to other subtypes, 12e acts on HDAC6, which is its main function.
5 We performed cell phenotype verification experiments on breast cancer cells
6 overexpressing HDAC6.

7 To ensure the successful drug design of a dual-target inhibitor, we
8 conducted comprehensive and multilevel screening and efficacy evaluation,
9 studied the molecular mechanism of action, and performed common
10 pharmacological and cell biology molecular mechanism verification
11 experiments. We overexpressed HDAC6 and HIF-1 α agonists and designed
12 cell phenotype experiments corresponding to each function to determine
13 whether the lead compound function corresponds to the relevant function of
14 the target and whether functional targeting is achieved. However, because the
15 drug was not designed to be selective for HDAC6, the toxicity may have been
16 increased. We performed a preliminary safety investigation.

17 As shown in Figure 1, the derivative design was based on the essential
18 pharmacodynamic structure of the HIF-1 α inhibitor 11g; the ionone skeleton
19 was used as the cap group (abscisic acid with the same chiral properties at
20 position 6), and the couroloyl group of the HDAC pharmacophore and the
21 binding group hydroxamic acid were used as the right of the dual-target design
22 template. The intermediate link chain connects different HIF-1 α

pharmacophiles, as confirmed by previous studies at the “R” position (Figure 1); it constitutes a series of derivatives.

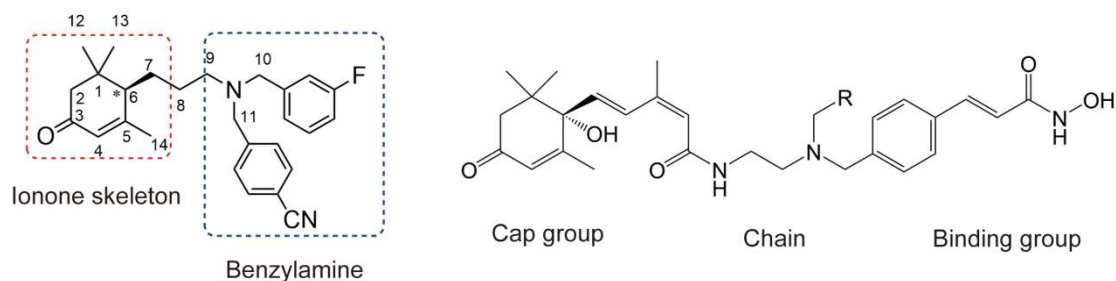
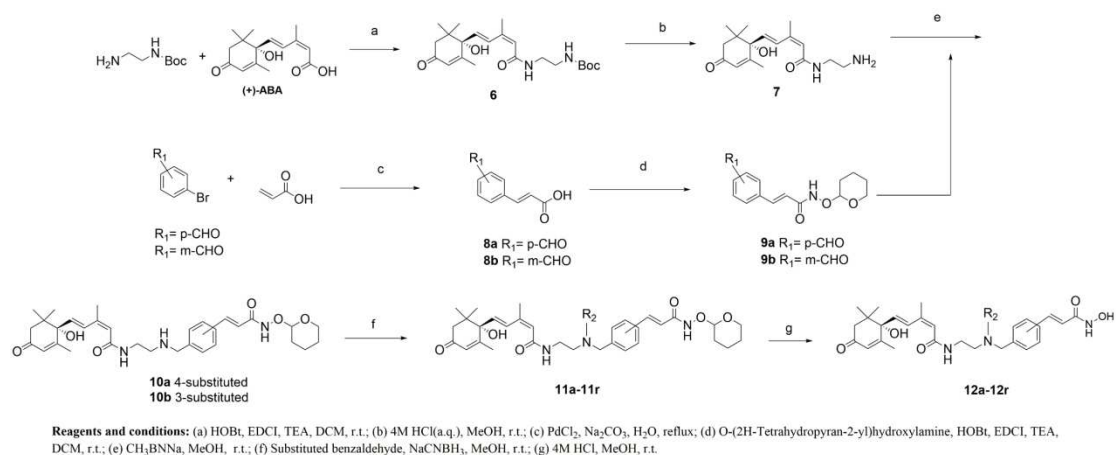


Figure 1. Structures of 11g and a series of derivatives

Derivative synthesis

The design of the synthetic route is shown in Figure 2. In this paper, N-Boc amide 6 was prepared by amide condensation using naturally extracted and purified dextrorotatory abscisic acid (optically pure, consistent with the standard substance of Sigma Company after optical rotation analysis) as the raw material. After the Boc-protecting group of this amide was removed, free primary amine 7 was obtained. Bromobenzaldehyde undergoes a Heck reaction with acrylic acid to yield formylcinnamic acid 8a or 8b, after which THP-protected hydroxamic acid 9a or 9b was prepared through an amide condensation reaction. Compounds 7 and 9a–9b undergo reductive amination to form secondary amines 10a–10b, which were then derivatized with various substituted benzaldehydes to yield derivatives 11a–11r. Finally, the protecting group THP was removed to obtain the target compounds of hydroxamic acid, 12a–12r (S2.3 and Figure S1-S18).

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2
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4



NO.	R ₂	Benzene substituted position	NO.	R ₂	Benzene substituted position	NO.	R ₂	Benzene substituted position
12a		4-substituted	12g		4-substituted	12m		3-substituted
12b		4-substituted	12h		4-substituted	12n		3-substituted
12c		4-substituted	12i		4-substituted	12o		3-substituted
12d		4-substituted	12j		4-substituted	12p		3-substituted
12e		4-substituted	12k		3-substituted	12q		3-substituted
12f		4-substituted	12l		3-substituted	12r		3-substituted

5 **Figure 2. Synthetic routes of derivatives and their chemical structures.**

6

7 **Comprehensive screening of derivatives and structure–activity**

8 **relationships**

9 Since the epigenetic regulatory factor HDAC6 regulates the acetylation

1 levels of multiple proteins and the transcription factor HIF-1 α regulates the
2 transcription of multiple genes, combining target screening with multifunctional
3 cell phenotype screening is necessary to ensure target inhibition and the
4 expected multifunctional vision. HDAC6 activity was determined by
5 fluorescence enzyme inhibition activity combined with cytotoxicity screening.
6 HIF-1 α and its functions were further screened using two indicators of HIF-1 α
7 expression level and cell migration inhibitory activity that our research group
8 had previously used. Furthermore, through a comprehensive analysis of
9 various data, derivatives with stronger comprehensive activity were selected
10 for the study of drug efficacy and molecular mechanisms.

11 On the basis of the comprehensive screening results of the targets and
12 different cell phenotypes (Tables 1-3; Figure S19), we found that derivative 12e
13 has stronger comprehensive activity, and the structure–activity relationships
14 are as follows:

15 (1) The “cap” group with a 3-carbonyl-6R-ionone “skeleton is not only an
16 essential group for the activity of HIF-1 α but can also serve as a cap group for
17 HDAC6, possessing a necessary group for dual-target inhibition.

18 (2) The “benzylamine” structure is the pharmacodynamic group inhibited
19 by HIF-1 α , and the “Y” structure is connected to meet the coordination
20 requirements with HDAC6 “Zn”.

21 (3) Coumaryl hydroxamic acid is the pharmacodynamic binding group of
22 HDAC6, on the basis of this structure, the activity of the para-position

substituents of the benzene ring in the derivative “Y” structure is greater than that in the meta position, among which the para-methoxybenzyl substituent (12e) has the strongest activity.

(4) A molecular mechanism study of lead compound 12e revealed that its anti-breast cancer proliferation effect stems from the inhibition of HDAC6, while its anti-breast cancer metastasis, angiogenesis and immune microenvironment remodelling effects result from the inhibition of HIF-1 α . The synergistic effect of HIF-1 α and HDAC6 (Figure 9) is in line with the theoretical expectation that the introduction of HDAC6 in target construction can enhance anti-proliferation and exert synergistic/combined effects, demonstrating the feasibility of the dual-target combination.

Table 1. Inhibitory activities (IC₅₀) of derivatives against HDAC6 (n = 3).

Compounds	IC ₅₀ (μM)	Compounds	IC ₅₀ (μM)
LBH589	0.015 ± 0.003	12j	0.614 ± 0.014
12a	1.052	12k	0.335 ± 0.026
12b	0.211 ± 0.019	12l	0.415 ± 0.038
12c	0.289 ± 0.027	12m	0.556 ± 0.051
12d	0.455 ± 0.033	12n	0.139 ± 0.009
12e	0.106 ± 0.008	12o	0.557 ± 0.047
12f	0.897 ± 0.077	12p	0.281 ± 0.022
12g	0.454 ± 0.037	12q	0.216 ± 0.018
12h	0.552 ± 0.044	12r	0.227 ± 0.023
12i	0.163 ± 0.010		

Table 2. Inhibitory effects of derivatives on the proliferation of different cancer cells (n = 3).

Compounds	IC ₅₀ (μM)
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	MDA-MB-231	HCT116	BxPC-3
12a	3.86 ± 0.23	12.72 ± 0.77	9.14 ± 0.511
12b	6.14 ± 0.41	2.27 ± 0.17	3.12 ± 0.243
12c	2.94 ± 0.22	15.07 ± 0.74	8.11 ± 0.332
12d	> 20.00	> 20.00	> 20.00
12e	1.85 ± 0.11	3.90 ± 0.36	6.48 ± 0.347
12f	17.75 ± 0.59	> 20.00	> 20.00
12g	2.97 ± 0.14	4.69 ± 0.34	75.39 ± 0.277
12h	> 20.00	> 20.00	> 20.00
12i	> 20.00	> 20.00	> 20.00
12j	19.24 ± 1.03	> 20.00	> 20.00
12k	13.14 ± 0.87	16.74 ± 0.86	> 20.00
12l	13.87 ± 0.93	17.33 ± 0.79	19.47 ± 1.311
12m	17.33 ± 0.99	> 20.00	> 20.00.
12n	6.32 ± 0.41	5.07 ± 0.45	8.24 ± 0.402
12o	10.22 ± 0.67	17.44 ± 1.11	> 20.00
12p	> 20.00	> 20.00	> 20.00
12q	17.9 ± 1.33	19.43 ± 1.21	> 20.00
12r	> 20.00	> 20.00	> 20.00

Table 3. Inhibitory effects of derivatives on the migration of MDA-MB-231 cells (n = 3).

Compounds	IC ₅₀ (μM)	Compounds	IC ₅₀ (μM)	Compounds	IC ₅₀ (μM)
12a	3.89 ± 0.23	12g	2.39 ± 0.14	12m	13.76 ± 0.99
12b	4.66 ± 0.27	12h	> 20.00	12n	3.92 ± 0.16
12c	3.62 ± 0.16	12i	> 20.00	12o	8.94 ± 0.42
12d	17.90 ± 0.93	12j	16.30 ± 0.28	12p	> 20.00
12e	2.87 ± 0.11	12k	11.22 ± 0.54	12q	14.77 ± 0.65
12f	13.70 ± 0.75	12l	10.61 ± 0.51	12r	19.83 ± 1.03

Derivative 12e inhibits migration, adhesion, and invasion in MDA-MB-231

and 4T1 cells and inhibits migration and tube formation in HUVECs

The occurrence, development and metastasis of tumours involve the proliferation of tumour cells. After the formation of the primary lesion, the tumour cells shed and undergo migratory movement, adhere to the microvessels, secrete proteases to invade the matrix and enter the blood vessels/lymphatic vessels, travelling along the blood circulation to the distal end and forming metastatic foci. Metastatic lesions proliferate, generating new blood vessels to provide nutrients for the metastatic lesions, and the tumour grows rapidly. This study shows that derivative 12e dose-dependently inhibited the proliferation, migration, adhesion and invasion of the human breast cancer cell line MDA-MB-231 and the murine breast cancer cell line 4T1. Furthermore, the migration and lumen formation of human umbilical vein endothelial cells (HUVECs) were inhibited in a dose-dependent manner, and angiogenesis was suppressed. The positive control drugs panobinostat and sorafenib also demonstrated inhibitory activity in various ways. The chemotherapy drug panobinostat showed strong antiproliferative activity. In terms of other aspects, owing to the many cell deaths in the high-dose group, the treatment window was narrow. Sorafenib is an antiangiogenic drug that has good antimigration, adhesion, invasion and angiogenic activities, but its active concentration is greater than that of 12e (Figure S20-24).

Derivative 12e inhibits the proliferation of MDA-MB-231 cells by inducing

1 **G2/M** phase arrest and triggering intrinsic and extrinsic apoptotic pathways

2 To evaluate the multifunctional antitumour efficacy of the candidate
3 compound, we first assessed the antiproliferative activity of 12e against murine
4 4T1 and human MDA-MB-231 cells. As shown in Figure S25, 12e inhibited the
5 viability of both cell lines in a concentration-dependent manner. The IC₅₀
6 values of 12e were $2.51 \pm 0.01 \mu\text{M}$ for 4T1 cells and $1.85 \pm 0.08 \mu\text{M}$ for
7 MDA-MB-231 cells. Notably, compared with the clinical drug sorafenib, 12e
8 exhibited superior antiproliferative potency. To elucidate the mechanism of
9 growth inhibition, we performed flow cytometry analysis. Cell cycle analysis
10 revealed that compared with the control treatment, 12e treatment significantly
11 induced G2/M phase arrest in MDA-MB-231 cells (Figure S26A). Furthermore,
12 Annexin V-FITC/PI staining demonstrated that 12e promoted apoptosis in a
13 dose-dependent manner, with the apoptosis rate increasing significantly from
14 3.69% (control) to 46.65% at 10 μM (Figure S26B).

15 We next investigated the molecular mechanisms driving these phenotypes
16 using western blotting (Figure S27). Treatment with an HDAC6 inhibitor, 12e,
17 dose-dependently increased the levels of acetylated α -tubulin (Ac- α -tubulin)
18 and acetylated HSP90 (Ac-HSP90), confirming effective target engagement
19 inside the cells.

20 The accumulation of Ac- α -tubulin disrupts microtubule dynamics, thereby
21 leading to the observed mitotic arrest. Moreover, hyperacetylated α -tubulin
22 dissociates from voltage-dependent anion channel (VDAC) on the outer

1 mitochondrial membrane. Consistent with these findings, we observed
2 increased free VDAC protein levels following 12e treatment (Figure S27),
3 which is known to increase mitochondrial permeability and trigger the release
4 of cytochrome c, activating the intrinsic apoptotic pathway. In parallel, the
5 acetylation of HSP90 impairs its chaperone function, leading to the
6 degradation of its client proteins. We observed a dose-dependent decrease in
7 the expression of FLIP_L (c-FLIP), a key antiapoptotic regulator and HSP90
8 client protein (Figure S27). The downregulation of FLIP_L expression sensitizes
9 cells to the extrinsic apoptotic pathway^{14,15}. Collectively, these results indicate
10 that 12e exerts its potent antiproliferative effects by simultaneously blocking
11 mitosis and activating both intrinsic and extrinsic apoptotic pathways through
12 the modulation of HDAC6 substrates.

13

14 **Derivative 12e suppresses TNBC metastasis and angiogenesis *in vitro* by**
15 **blocking HIF-1 α /VEGF/VEGFR2 signalling**

16 Given the high metastatic potential of TNBC, we investigated the effects of
17 12e on key metastatic steps, including migration, adhesion, and invasion. In
18 wound-healing assays, 12e significantly suppressed the migration of
19 MDA-MB-231 and 4T1 cells (Figures S20–S21). Consistent with these findings,
20 12e markedly inhibited the adhesion of both MDA-MB-231 and 4T1 cells to the
21 extracellular matrix (Figure S22) and significantly reduced cell invasion ability
22 in Transwell assays (Figure S23).

1 Since angiogenesis is critical for tumour expansion and metastasis, we
2 further assessed the effect of 12e on HUVECs. As shown in Figure S24, 12e
3 potently inhibited both the migration and tube formation of HUVECs.
4 Quantitative analysis indicated that 12e effectively reduced the number of
5 branch points and the tube length, demonstrating antiangiogenic activity
6 comparable to or better than that of sorafenib at the tested concentrations.

7 To elucidate the molecular mechanism underlying these antimetastatic
8 and antiangiogenic effects, we investigated the HIF-1 α /VEGF/VEGFR2
9 signalling axis. 12e treatment dose-dependently downregulated HIF-1 α protein
10 expression in MDA-MB-231 cells under hypoxic conditions (Figure S28A).
11 Consequently, the secretion of VEGF, a major downstream target of HIF-1 α ,
12 was markedly suppressed. ELISA results confirmed that 12e decreased both
13 the intracellular and extracellular VEGF levels under normoxic and hypoxic
14 conditions (Figure S28B-28C). Notably, the inhibitory effect of 12e on HIF-1 α
15 expression was comparable to that of the positive control panobinostat.

16 We next examined the downstream signalling pathways driven by VEGF. In
17 MDA-MB-231 cells, 12e dose-dependently inhibited the phosphorylation of
18 VEGFR2 and its downstream effectors, Akt and ERK1/2 (Figure S28D).
19 Similarly, in HUVECs stimulated with exogenous VEGF, 12e effectively
20 suppressed the VEGF-induced phosphorylation of VEGFR2, Akt, and ERK1/2
21 (Figure S28E). Collectively, these results suggest that 12e exerts its
22 multifunctional antitumour effects by dual targeting of HDAC6 and HIF-1 α ,

1 thereby blocking the VEGF/VEGFR2 signalling axis to suppress TNBC
2 metastasis and angiogenesis.

3 4 **Derivative 12e modulates tumour metabolism and immune checkpoint** 5 **expression by inhibiting the HIF-1 α /glycolysis/PD-L1 axis**

6 Hypoxia is a hallmark of the TNBC microenvironment that drives
7 metabolic reprogramming and immune escape. To investigate the
8 immunometabolic effects of 12e, we measured glycolytic activity and PD-L1
9 expression in MDA-MB-231 cells under hypoxic conditions. As shown in Figure
10 S29B, hypoxia significantly elevated intracellular ATP and lactic acid levels,
11 indicating enhanced glycolysis. Treatment with 12e effectively reversed this
12 metabolic shift, inducing a concentration-dependent reduction in both lactic
13 acid and ATP production.

14 To elucidate the underlying mechanism, we examined the expression of
15 key glycolytic enzymes downstream of HIF-1 α . Treatment with 12e markedly
16 decreased the protein levels of HK2 and PKM2 (Figure S29A), confirming that
17 12e suppresses aerobic glycolysis by blocking the HIF-1 α -mediated
18 transcriptional upregulation of these rate-limiting enzymes.

19 Furthermore, high lactate levels and HIF-1 α stabilization upregulate the
20 expression of the immune checkpoint protein PD-L1, thereby promoting T-cell
21 inactivation^{16,17}. Consistent with the inhibition of the HIF-1 α pathway, 12e
22 treatment significantly suppressed the hypoxia-induced upregulation of PD-L1

1 expression (Figure S29A). Collectively, these findings suggest that 12e may
2 reverse tumour immune escape by simultaneously dampening
3 immunosuppressive glycolysis and downregulating PD-L1 expression via the
4 HIF-1 α /HK2/PKM2 signalling axis.

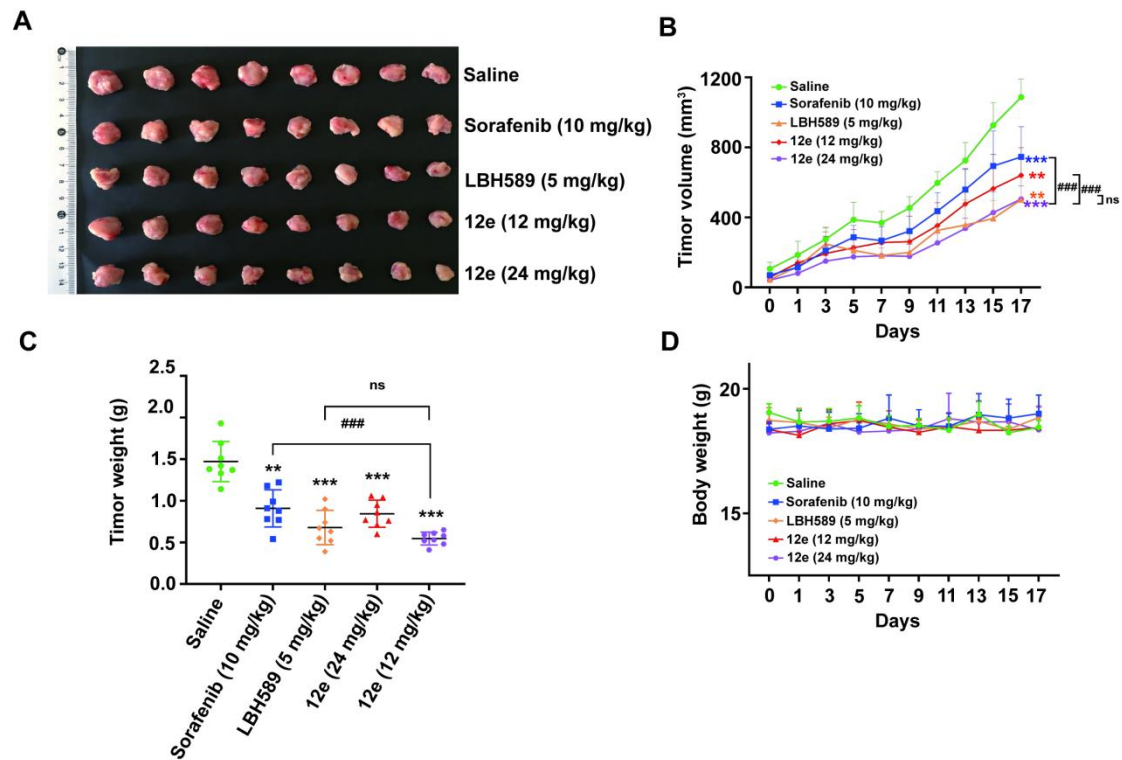
5
6 **Derivative 12e suppresses tumour growth, lung metastasis, and**
7 **angiogenesis and reverses immunosuppression *in vivo***

8 ***In vivo antiproliferative efficacy of 12e in a tumour-bearing mouse model***

9 To evaluate the *in vivo* therapeutic efficacy of the candidate compound, we
10 established a mouse model of breast cancer. Throughout the treatment
11 regimen, no significant difference in body weight was observed between the
12 12e-treated groups and the saline control group, indicating that the derivative
13 possesses a favourable safety profile with no overt toxicity (Figure 3D). In
14 contrast, analysis of tumour volume over time revealed that 12e treatment
15 significantly suppressed primary tumour progression (Figure 3B). Compared
16 with the saline group, both the low-dose (12 mg/kg) and high-dose (24 mg/kg)
17 12e groups exhibited statistically significant reductions in tumour volume. By
18 the end of the study, the tumour inhibition rates were 41.03% for the low-dose
19 group and 53.49% for the high-dose group.

20 These findings are consistent with the terminal tumour weight analysis
21 (Figure 3C) and the gross morphology of excised tumours (Figure 3A), as well
22 as the trend observed in small animal fluorescence imaging (Figure 4C).

1 Notably, the antitumour efficacy of high-dose 12e was comparable to that of
 2 the positive control panobinostat (LBH589), with no statistically significant
 3 difference observed between these two groups (Figure 3B–C).



4
 5 **Figure 3. *In vivo* antitumour effects of 12e, sorafenib and LBH589.**

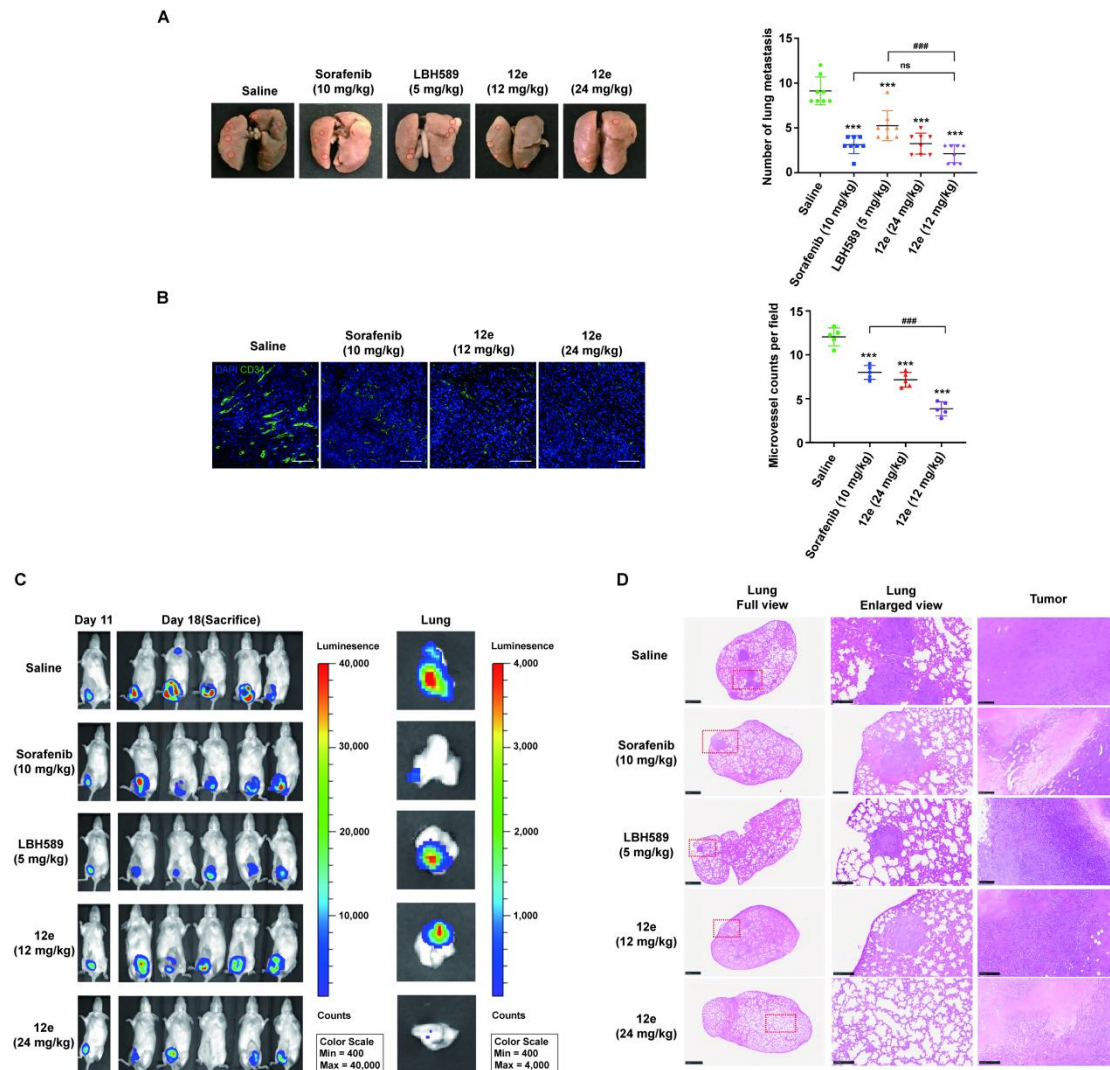
6 (A) Images of tumours from each group. (B) Curves of the tumour volume over
 7 time. The data were analysed using two-way ANOVA followed by Tukey's
 8 multiple comparison test. (C) Statistics of tumour weight. Data are presented
 9 as the means $\pm$ SDs ($n=8$) and were analysed using two-sample, two-tailed
 10 unpaired Student's t test. (D) Curves of mouse body weight over time.
 11 Compared with the saline group, ns indicates not significant, $*P < 0.05$, $**P <$
 12 0.01 and $***P < 0.001$; compared with the low-dose 12e, LBH589 or sorafenib
 13 group, ns indicates not significant, $\#P < 0.05$, $##P < 0.01$ and $###P < 0.001$.

12e inhibits lung metastasis and suppresses tumour angiogenesis in vivo

Lung metastasis is a frequent and lethal complication of TNBC progression. We evaluated the antimetastatic efficacy of 12e by quantifying pulmonary nodules in tumour-bearing mice. The saline control group exhibited a high metastatic burden, with an average of 9 ± 2 nodules per lung. In contrast, 12e treatment significantly reduced the number of metastatic nodules in a dose-dependent manner (low dose: 3 ± 1 ; high dose: 2 ± 1), resulting in inhibition rates of 64.38% and 76.71%, respectively (Figure 4A). Comparative analysis revealed that the antimetastatic efficacy of high-dose 12e was comparable to that of sorafenib (3 ± 2 nodules; inhibition rate 65.75%) but significantly superior to that of panobinostat (5 ± 2 nodules; inhibition rate 42.47%). Histological examination via H&E staining further corroborated these findings. Lung tissues from the saline group showed extensive infiltration of tumour cells characterized by hyperchromatic, enlarged nuclei and a dense cellular arrangement, accompanied by a marked loss of alveolar architecture. Conversely, the 12e-treated groups displayed preserved lung morphology with minimal tumour infiltration, similar to the effects observed in the sorafenib group (Figure 4D).

Given that angiogenesis is a prerequisite for tumour expansion and metastasis, we assessed the microvessel density (MVD) in tumour tissues using CD34 immunofluorescence staining. As shown in Figure 4B, compared

1 with saline treatment, 12e treatment significantly decreased the MVD in a
 2 dose-dependent manner. Notably, the antiangiogenic activity of high-dose 12e
 3 was significantly stronger than that of the standard antiangiogenic agent
 4 sorafenib, suggesting that 12e effectively disrupted the tumour vascular supply.



5
 6 **Figure 4. Inhibitory effects of 12e on lung metastasis and tumour**
 7 **angiogenesis *in vivo*.**

8 (A) Representative images of lung tissues from each group (left) and statistical
 9 analysis of the number of pulmonary metastatic nodules (right). Red dashed
 10 circles indicate metastatic nodules. (B) Representative immunofluorescence

1 images of CD34 staining in tumour tissues (left) and statistical analysis of the
2 microvessel density (right). Green fluorescence indicates CD34-positive
3 microvessels, and blue fluorescence indicates DAPI-stained nuclei. (C) *In vivo*
4 bioluminescence imaging of tumour-bearing mice at Days 11 and 18 (left) and
5 *ex vivo* bioluminescence imaging of excised lungs (right). The colour scale
6 indicates the intensity of luminescence. (D) Representative H&E staining
7 images of lung tissues. The right panels show enlarged views of the red boxed
8 areas in the left panels. Data are presented as the means $\pm$ SDs from three
9 biological replicates and were analysed using two-sample, two-tailed, unpaired
10 Student's *t* test. Compared with the saline group, ns indicates not significant,
11 $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$; compared with the LBH589 or
12 sorafenib group, ns indicates not significant, $\#P < 0.05$, $\##P < 0.01$ and $###P <$
13 0.001.

14

15 ***Derivative 12e reverses tumour immunosuppression in vivo***

16 The TME of TNBC severely limits the efficacy of immune surveillance. To
17 investigate whether 12e could reverse this immunosuppression, we analysed
18 the infiltration of key immune cell populations in tumour tissues using flow
19 cytometry.

20 First, we examined the innate immune compartment. Treatment with 12e
21 dose-dependently increased the proportion of F4/80⁺ macrophages. In the
22 high-dose 12e group, the population of F4/80⁺ macrophages reached $20.58 \pm$

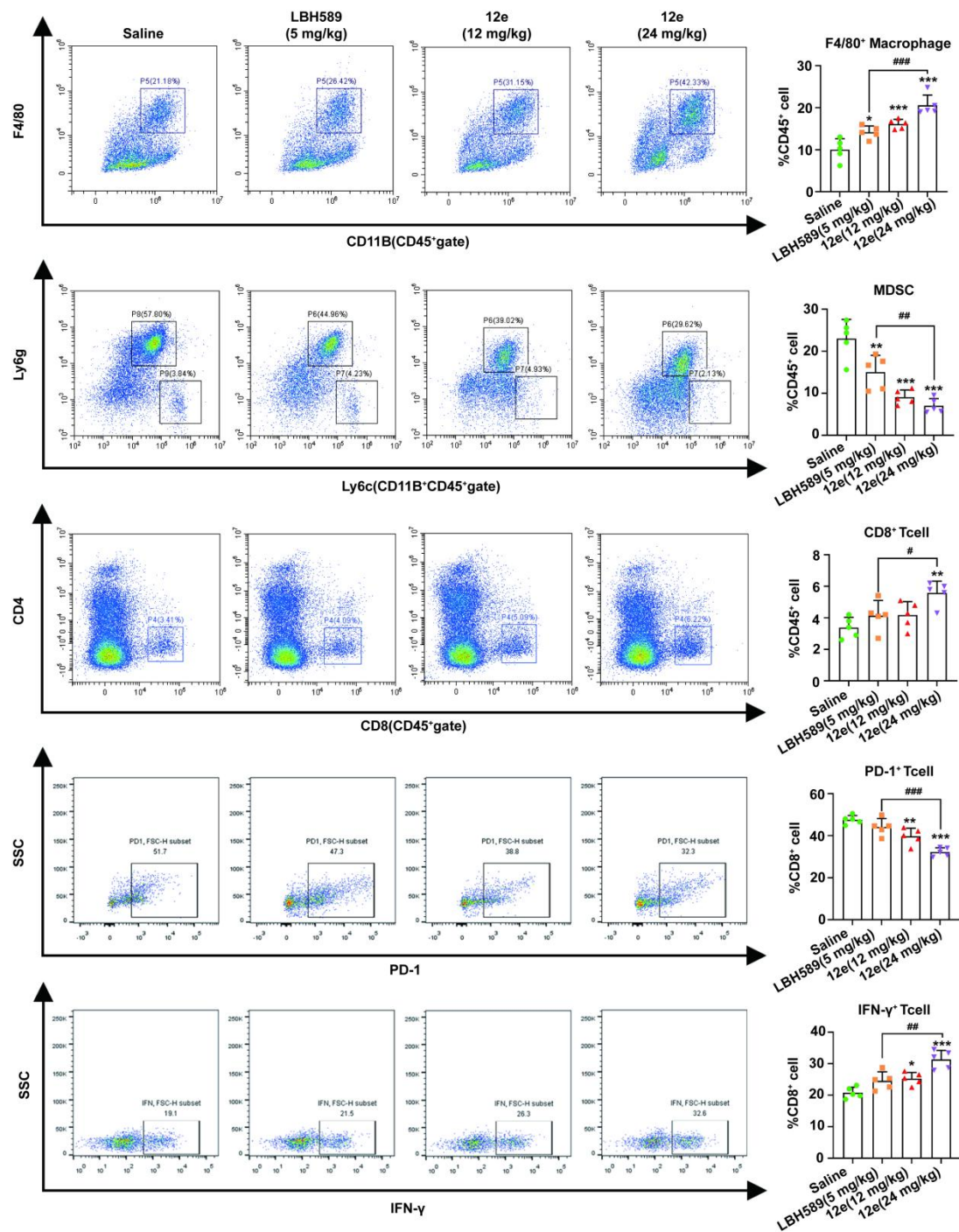
2.17%, which was approximately twofold greater than that in the saline group and significantly greater than that in the panobinostat group ($14.16 \pm 1.34\%$) (Figure 5). Concurrently, 12e treatment significantly reduced the infiltration of myeloid-derived suppressor cells (MDSCs). The proportion of MDSCs decreased from $26.02 \pm 4.51\%$ in the saline group to $7.10 \pm 1.72\%$ in the high-dose 12e group, a reduction that was significantly more pronounced than that observed with panobinostat.

Next, we assessed the adaptive immune response, focusing on CD8⁺ cytotoxic T cells. 12e treatment promoted the infiltration of CD8⁺ T cells into the tumour tissue. The percentage of CD8⁺ T cells increased from $3.38 \pm 0.65\%$ in the saline group to $5.58 \pm 0.75\%$ in the high-dose 12e group, whereas the percentage in the panobinostat group did not significantly differ from that in the saline control group. Importantly, 12e not only increased the abundance of CD8⁺ T cells but also enhanced their functional status. The percentage of activated IFN- γ ⁺ CD8⁺ T cells significantly increased from $20.78 \pm 1.71\%$ to $31.32 \pm 2.81\%$. In contrast, the percentage of exhausted PD-1⁺ CD8⁺ T cells significantly decreased from $47.58 \pm 2.06\%$ to $32.22 \pm 2.08\%$ in response to 12e treatment. Panobinostat did not significantly affect IFN- γ or PD-1 expression on CD8⁺ T cells compared with the control.

Collectively, these results demonstrate that 12e effectively reverses tumour immunosuppression by reducing MDSC accumulation and enhancing both the infiltration and activation of cytotoxic T cells, thereby shifting the TME

1 towards an immune-active phenotype.

2



3

4 **Figure 5. Effects of derivative 12e on the tumour microenvironment (TME)**

5 **in tumour-bearing mice.**

6 (A) Representative flow cytometry plots (left) and quantitative analysis (right)

of the proportions of F4/80⁺ macrophages, MDSCs, CD8⁺ T cells, PD-1⁺ CD8⁺ T cells, and IFN- γ ⁺ CD8⁺ T cells in tumour tissues. Data are presented as the means $\pm$ SDs from three biological replicates and were analysed using two-sample, two-tailed, unpaired Student's *t* test. Compared with the saline group, ns indicates not significant, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001; compared with the LBH589 group, ns indicates not significant, #*P* < 0.05, ##*P* < 0.01 and ###*P* < 0.001.

Validation of the molecular mechanisms underlying the multifunctional antitumour efficacy of 12e *in vivo*

Derivative 12e suppresses tumour growth in vivo by modulating the HDAC6/Ac- α -tubulin/Ac-HSP90 signalling axis

To corroborate the mechanism of action *in vivo*, we evaluated the expression of key proteins involved in HDAC6 signalling in tumour tissues via western blot analysis. As presented in Figure 6, compared with saline treatment, treatment with 12e elicited robust, dose-dependent upregulation of the expression of the HDAC6 substrates Ac- α -tubulin and Ac-HSP90. This accumulation of acetylated substrates confirms effective HDAC6 inhibition within the TME. Furthermore, 12e treatment significantly increased the expression of VDAC while concurrently downregulating FLIP_L (Figure 9). These findings are highly consistent with our *in vitro* observations, providing strong evidence that 12e inhibits TNBC proliferation *in vivo* by synergistically

inducing mitotic arrest and activating both intrinsic and extrinsic apoptotic cascades through HDAC6 inhibition.

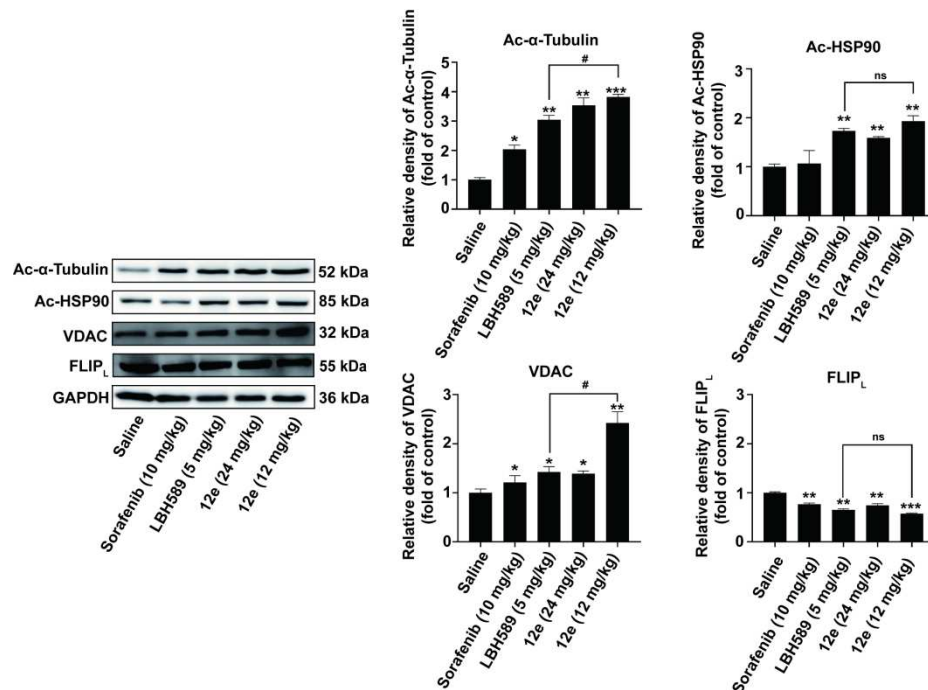


Figure 6. Effects of derivative 12e on the protein expression of Ac-α-tubulin, Ac-HSP90, VDAC, and FLIPL in tumour tissues.

Representative western blot bands (left) and quantitative analysis (right) of the protein levels of Ac-α-tubulin, Ac-HSP90, VDAC, and FLIPL. Data are presented as the means $\pm$ SDs from three biological replicates and were analysed using two-sample, two-tailed, unpaired Student's *t* test. Compared with the saline group, ns indicates not significant, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001; compared with the LBH589 group, ns indicates not significant, #*P* < 0.05, ##*P* < 0.01 and ###*P* < 0.001.

Derivative 12e attenuates tumour angiogenesis and metastasis in vivo

1 ***via blockade of the HIF-1 α /VEGF/VEGFR2 signalling pathway***

2 To further substantiate that the observed inhibition of lung metastasis and
3 angiogenesis *in vivo* stems from the targeted disruption of the HIF-1 α pathway,
4 we analysed the activity of the VEGF signalling axis in tumour tissues. As
5 quantified by ELISA, treatment with 12e elicited a profound, dose-dependent
6 reduction in VEGF levels in both tumour lysates and serum (Figure S30),
7 confirming its systemic ability to suppress the secretion of this critical
8 angiogenic factor. Consistent with these findings, western blot analysis of
9 tumour homogenates revealed that 12e significantly downregulated HIF-1 α
10 protein expression (Figure 7). Consequently, the activation of downstream
11 angiogenic signal transduction was effectively abrogated, as evidenced by the
12 marked suppression of the phosphorylation of VEGFR2 and its key effectors,
13 Akt and ERK1/2 (Figure 7). Collectively, these data align with our *in vitro*
14 findings, providing conclusive evidence that 12e exerts its antimetastatic and
15 antiangiogenic effects by dismantling the HIF-1 α /VEGF/VEGFR2 signalling
16 axis (Figure 9).

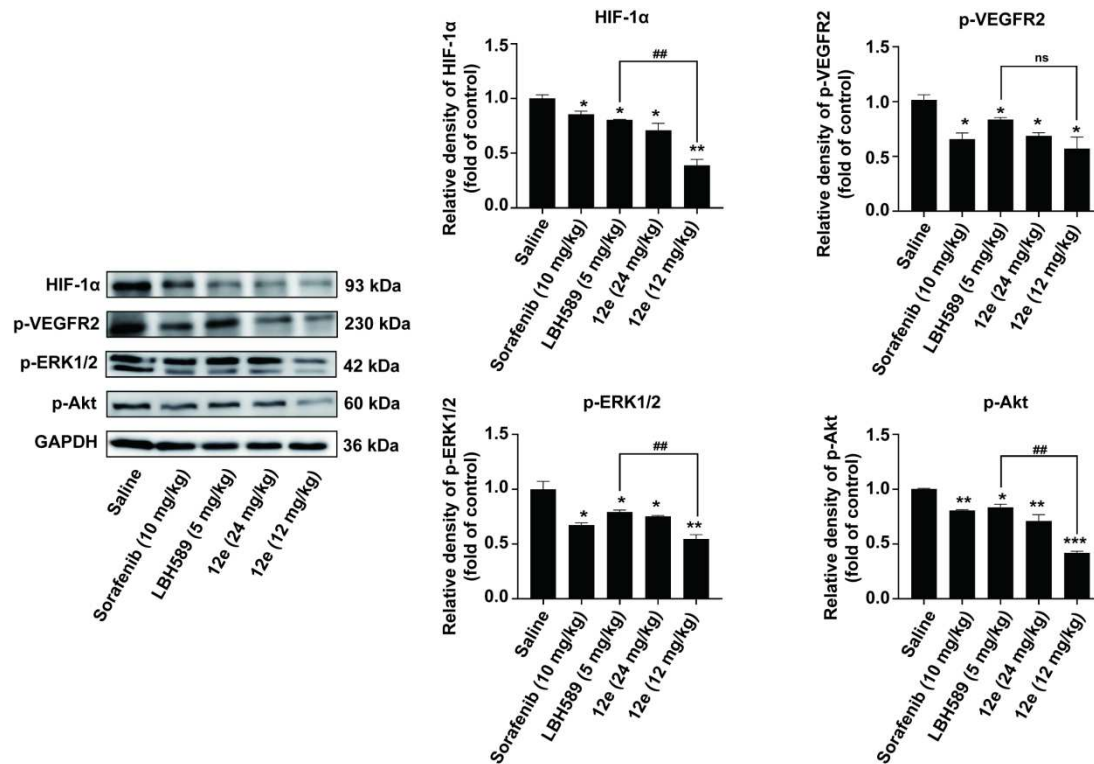


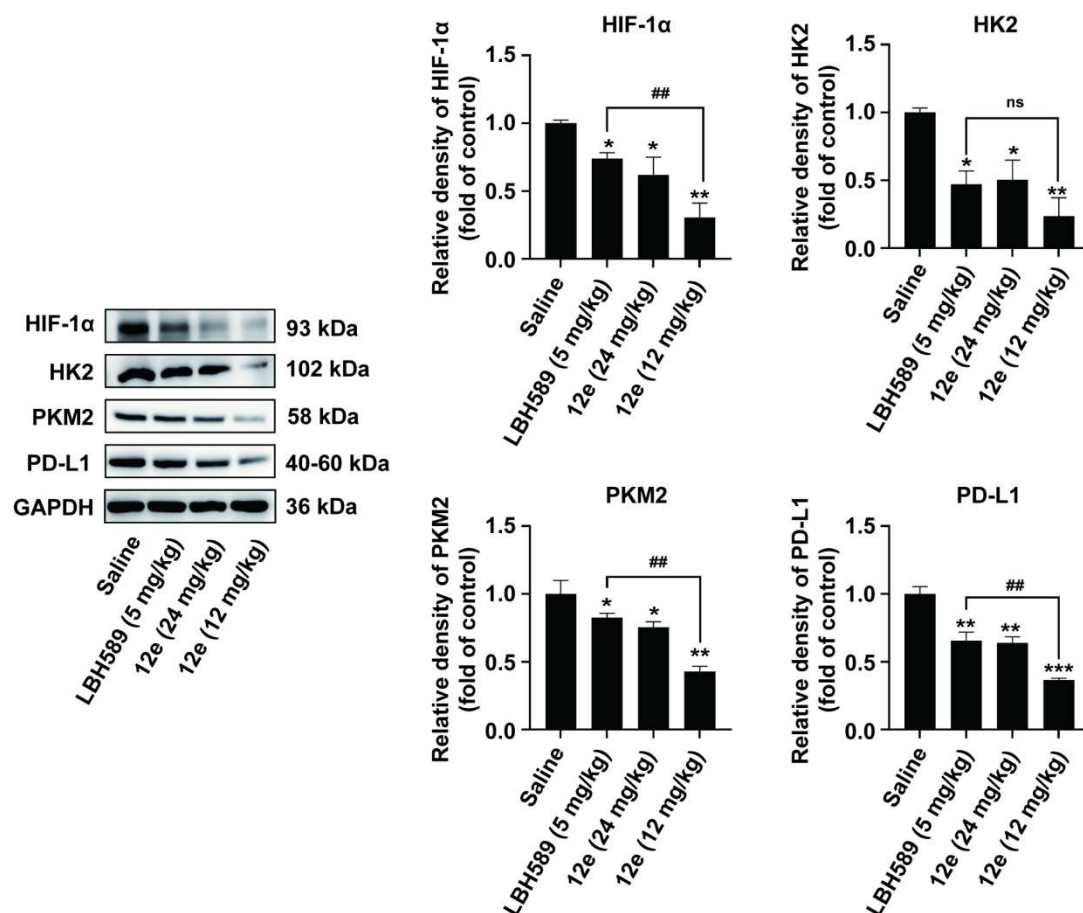
Figure 7. Effects of derivative 12e on the HIF-1α/VEGF/VEGFR2 signalling pathway in tumour tissues.

Representative western blot bands (left) and quantitative analysis (right) of HIF-1α, p-VEGFR2, p-ERK1/2 and p-Akt protein levels. Data are presented as the means $\pm$ SDs from three biological replicates and were analysed using two-sample, two-tailed, unpaired Student's *t* test. Compared with the saline group, ns indicates not significant, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001; compared with the LBH589 group, ns indicates not significant, #*P* < 0.05, ##*P* < 0.01 and ###*P* < 0.001.

Derivative 12e reverses tumour immunosuppression and immune escape in vivo by remodelling the glycolytic microenvironment

To determine whether the observed immune reactivation stems from

1 metabolic modulation, we examined the effect of 12e on the glycolytic status of
 2 tumour-bearing mice. Consistent with our *in vitro* findings, compared with
 3 saline treatment, 12e treatment elicited a significant, dose-dependent
 4 reduction in the levels of lactic acid and ATP in both serum and tumour tissues
 5 (Figure S31). Mechanistically, western blot analysis of tumour homogenates
 6 confirmed that 12e effectively suppressed the protein expression of HIF-1 α
 7 and its downstream rate-limiting glycolytic enzymes, HK2 and PKM2 (Figure 8).
 8 These data demonstrate that 12e potently inhibits aerobic glycolysis *in vivo*,
 9 thereby restricting the energy supply and reducing metabolic byproducts within
 10 the tumour.



11
 12 **Figure 8. Effects of derivative 12e on the expression of glycolysis-related**

proteins and PD-L1 in tumour tissues.

Representative western blot bands (left) and quantitative analysis (right) of HIF-1 α , HK2, PKM2, and PD-L1 protein levels. Data are presented as the means $\pm$ SDs from three biological replicates and were analysed using two-sample, two-tailed, unpaired Student's *t* test. Compared with the saline group, ns indicates not significant, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001; compared with the LBH589 group, ns indicates not significant, #*P* < 0.05, ##*P* < 0.01 and ###*P* < 0.001.

Metabolic competition between tumour cells and immune cells is a critical driver of immunosuppression. Tumour cells, characterized by aberrant glucose metabolism, rapidly consume glucose and produce excessive lactate through glycolysis¹⁸. This glucose deprivation creates a harsh metabolic environment that functionally impairs effector T cells and proinflammatory M1-like macrophages, which rely heavily on glucose for activation^{19,20}. Furthermore, glycolysis is integral to redox homeostasis and the suppressive function of MDSCs²¹, whereas lactate accumulation triggers the upregulation of PD-L1 expression on tumour cells²².

In the present study, the saline group exhibited a distinct immunosuppressive phenotype. However, 12e treatment effectively reversed this state by reshaping the metabolic microenvironment. As a result of HIF-1 α /glycolysis inhibition, 12e dose-dependently downregulated PD-L1

1 protein expression in tumour tissues (Figure 8). This metabolic reprogramming
2 was accompanied by a profound shift in the immune landscape. As detailed in
3 Section 2.8.3, 12e treatment significantly increased the infiltration of M1-like
4 macrophages and activated cytotoxic T cells while concurrently reducing the
5 populations of MDSCs and exhausted T cells.

6 To further verify the restoration of antitumour immunity, we assessed the
7 levels of key immunostimulatory cytokines and cytotoxic mediators. Compared
8 with saline treatment, 12e treatment induced robust, dose-dependent
9 increases in the serum and tissue levels of TNF- α , IFN- γ , perforin, and
10 granzyme B (Figure S32). These findings indicate that 12e successfully
11 transforms the “cold”, immunosuppressive TME into a “hot”, immune-active
12 state.

13 Collectively, these results suggest that 12e exerts synergistic
14 immunomodulatory effects through the dual targeting of HDAC6 and HIF-1 α .
15 By dismantling the HIF-1 α -mediated glycolytic pathway, 12e remodels the
16 tumour metabolic and immune microenvironment, thereby reversing immune
17 escape. The comprehensive molecular mechanism underlying the
18 multifunctional antitumour efficacy of derivative 12e is summarized in Figure 9.

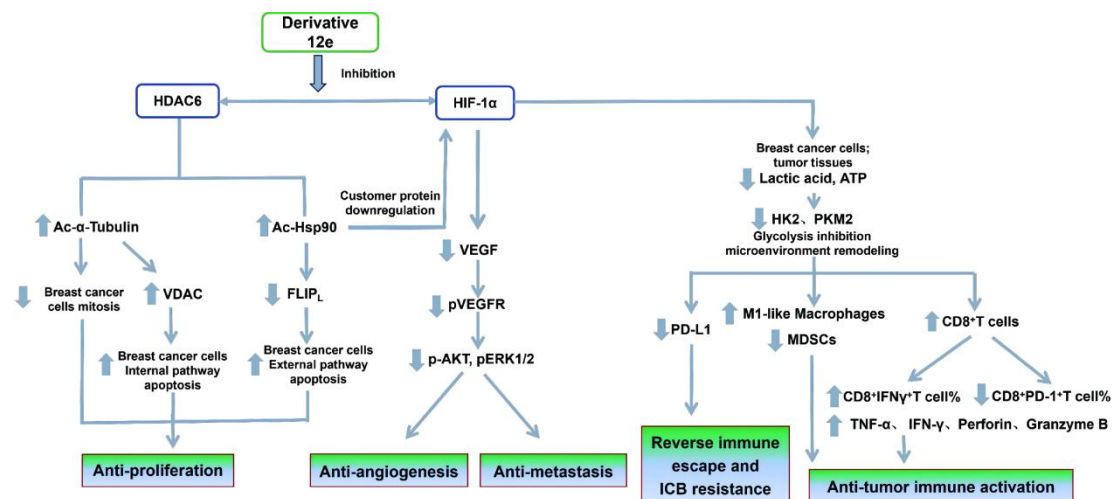


Figure 9. Schematic representation of the multifunctional antitumour mechanism of derivative 12e in TNBC.

12e acts as a dual-target inhibitor of HDAC6 and HIF-1 α , exerting synergistic effects: (1) Anti-proliferative effects via induction of G2/M arrest (Ac- α -Tubulin) and apoptosis (Ac-HSP90/FLIP_L/VDAC); (2) anti-metastatic and anti-angiogenic effects via blockade of the HIF-1 α /VEGF/VEGFR2 axis; and (3) reversal of immunosuppression via metabolic remodelling (downregulation of HK2, PKM2, lactate acid and ATP) and PD-L1 suppression, promoting the infiltration and activation of cytotoxic CD8⁺ T cells and M1-like macrophages.

Validation of the dual-targeting mechanism of 12e via HDAC6 overexpression and HIF-1 α activation

To rigorously confirm that the multifaceted antitumour efficacy of 12e is strictly mediated through the dual inhibition of HDAC6 and HIF-1 α , we performed mechanistic rescue experiments using HDAC6-overexpressing (HDAC6-OE) cells and the HIF-1 α agonist DMOG.

1 First, to identify the driver of cytotoxicity, we assessed the viability of
2 MDA-MB-231 cells. Both 12e and the HDAC inhibitor panobinostat significantly
3 suppressed the proliferation of vector-transfected control cells. However, this
4 antiproliferative effect was markedly attenuated in HDAC6-OE cells (Figure
5 S33), confirming that HDAC6 inhibition is the primary mechanism underlying
6 12e-induced cytotoxicity. Notably, the HIF-1 α agonist DMOG alone did not
7 affect cell viability, nor did it interfere with the growth-inhibitory effects of 12e in
8 this context.

9 Next, we investigated the specific contribution of HIF-1 α to the
10 noncytotoxic functions of 12e. While 12e significantly inhibited MDA-MB-231
11 cell migration (Figure S34) and HUVEC tube formation (Figure S35), these
12 inhibitory effects were effectively reversed by cotreatment with DMOG.
13 Similarly, the ability of 12e to suppress glycolytic metabolism was abrogated in
14 the presence of DMOG (Figure S36). These results provide compelling
15 evidence that 12e suppresses metastasis, angiogenesis, and glycolytic
16 metabolic reprogramming primarily by blocking the HIF-1 α signalling axis.
17 Furthermore, the results of the wound healing assay of HDAC6-OE
18 MDA-MB-231 cells indicated that, compared with that of the control group, the
19 ability of panobinostat to inhibit the migration of HDAC6-OE MDA-MB-231
20 cells disappeared. Although the ability of 12e to inhibit cell migration was
21 weakened compared with that of the control group, the difference was
22 significant ($***P < 0.001$; Figure S37). Moreover, in the 12e+DMOG group,

1 after HIF-1 α agonist intervention, the ability of 12e to inhibit the migration of
2 HDAC6-OE breast cancer cells was blocked, indicating that 12e also inhibits
3 HIF-1 α through pathways other than HDAC6/HSP90. Compound 12e achieves
4 synergistic/combined multifunctional anti-breast cancer effects through
5 dual-target inhibition.

6 In summary, these experiments elucidate the distinct yet cooperative roles
7 of the two targets: 12e inhibits breast cancer proliferation by targeting HDAC6
8 while simultaneously suppressing metastasis and angiogenesis and reversing
9 immunosuppressive glycolysis through HIF-1 α inhibition.

10 **Comparison with positive control drugs**

11 As a marketed drug, the HDAC6 inhibitor panobinostat also demonstrated
12 the multifunctional pharmacological effects of HDAC6 inhibitors. However,
13 among the clinical indications of marketed drugs, panobinostat, vorinostat and
14 romedixin are used for the clinical treatment of peripheral T-cell lymphoma,
15 cutaneous T-cell lymphoma and multiple myeloma. Chidamide is used to treat
16 peripheral T-cell lymphoma and breast cancer²³⁻²⁵. The multifunctional
17 pharmacological effects of inhibiting HDAC6, in addition to its ability to inhibit
18 tumour proliferation, have not been the focus of any other clinically significant
19 applications. In this study, the anti-breast cancer proliferation effect of the
20 high-dose 12e group was comparable to that of panobinostat, and its anti-lung
21 metastasis, angiogenesis, tumour immunosuppression reversal and immune
22 escape effects were stronger than those of panobinostat. On the other hand,

1 sorafenib, a VEGFR inhibitor, is a marketed antiangiogenic drug. The
2 anti-breast cancer lung metastasis effect of high-dose 12e was comparable to
3 that of sorafenib, and its anti-angiogenic effect was stronger than that of
4 sorafenib.

5 **Preliminary investigation of drug safety**

6 A comparison of the cytotoxicity of each derivative against the breast
7 cancer cell line MDA-MB-231 and the normal human breast endothelial cell
8 line MCF-10A (Table S1) indicated that, compared with their effects on breast
9 cancer cells, the derivatives were less cytotoxic to normal human breast
10 endothelial cells. Among them, the IC₅₀ of derivative 12e against MCF-10A
11 cells was 16.79 μ M, which was approximately 9 times that of 12e against
12 MDA-MB-231 cells. Moreover, on the basis of the empirical analysis of
13 pharmacokinetic studies, a blood drug concentration of 16 μ M is generally
14 impossible to achieve because of the large molecular weight of 12e. Therefore,
15 12e is not toxic to normal cells.

16 Compared with those in the normal saline group, H&E staining (100 $\times$) of
17 heart, liver, spleen and kidney tissues in the panobinostat group, sorafenib
18 group, and low-dose and high-dose 12e groups did not significantly differ in
19 terms of the cell and tissue morphology (Figure S38). These findings indicate
20 that the lows and high doses of 12e and panobinostat and sorafenib did not
21 damage various organs.

22 After administration, the serum levels of alanine aminotransferase (ALT),

1 aspartate transaminase (AST), UREA, and creatinine (creatinine, CRE-BD) in
2 4T1 breast orthotopic transplanted tumour model mice are shown in Figure
3 S39. Compared with those in the normal saline group, ALT and AST levels in
4 the panobinostat group, the sorafenib group, and the low-dose and high-dose
5 12e groups did not significantly differ ($P > 0.05$), indicating that the dosage and
6 administration frequency of all the treatments had no effect on the liver
7 function of the mice. Compared with those in the normal saline group, the
8 UREA and CRE-BD indicators did not significantly differ among the four
9 groups ($P > 0.05$), indicating that the dosage and administration frequency did
10 not affect renal function in any of the groups.

11

12 **Discussion**

13 In general, compounds that directly bind to the target site, which can
14 effectively exert inhibitory effects, are selected for inhibitor drug design.
15 Studies on the structure and function of targets have shown that for the protein
16 domains of targets with mainly a single function, the designed compounds
17 have achieved the expected effects. However, HIF-1 α is a transcription factor
18 that can regulate over 60 genes, and HDAC6 is an epigenetic regulatory factor
19 that can regulate the acetylation of multiple functional proteins.

20 There are many subtypes of HDAC6, and selective drug design is needed.
21 For such dual-target designs with inherent multifunctional regulation, it is
22 unnecessary to reflect all the functions of each target; rather, the design should

1 be limited to multifunctional drugs that prevent tumour occurrence and
2 development. However, the multigene regulation of HIF-1 α and the regulatory
3 mechanisms of multiple functional HDAC6 proteins have not been clarified in
4 structural biology studies. Therefore, we adopted a structure–activity approach
5 combining the cell phenotype and chemical structure. Using our experience
6 with the binding group of HDAC6 and hydroxamic acid, and combined with the
7 structure–activity relationship knowledge accumulated by our group over many
8 years of research on HIF-1 α inhibitors, we designed a dual-target drug
9 corresponding to the functional efficacy of both pathways.

10 With respect to the design of selective HDAC6 inhibitors, unlike HIF-1 α
11 inhibitors, which currently have no marketed drugs, multiple pan-HDAC
12 inhibitors have already become marketed drugs. Moreover, because of the
13 distribution selectivity of HDAC6 (the only subtype in the HDAC family that is
14 distributed mainly in the cytoplasm) and its substrate α -tubulin (proliferation
15 related), the selectivity of HSP90 (HIF-1 α related) can be inferred, and in
16 contrast to other subtypes, the main pharmacological effect of the derivative
17 12e is on HDAC6. This finding is also supported by the results of cell
18 phenotype verification experiments in breast cancer cells overexpressing
19 HDAC6. Of course, 12e can also affect other subtypes and may increase
20 toxicity and side effects; thus, we have performed a preliminary safety
21 investigation.

22 To determine whether a dual-target drug design is successful, the first

1 step is to evaluate whether the efficacy and intensity have reached the design
2 goals. The results of this study indicate that the derivative 12e achieved the
3 expected multifunctional efficacy in suppressing breast cancer proliferation,
4 metastasis, angiogenesis and immune microenvironment remodelling. Its
5 antiproliferative effect is comparable to that of panobinostat, its anti-breast
6 cancer lung metastasis effect is comparable to that of sorafenib, and its
7 antiangiogenesis effect is stronger than that of sorafenib.

8 Second, it is necessary to determine whether the multifunctional efficacy
9 of the lead compound that is designed and synthesized specifically
10 corresponds to the related functions of the two targets, that is, functional
11 targeting. We employed pharmacological validation methods to demonstrate
12 the correspondence between the relevant pharmacological effects and the
13 targets through phenotypic experiments involving HDAC6-overexpressing cell
14 lines and cells treated with HIF-1 α agonists (section 2.10).

15 Furthermore, to examine potential toxicity and side effects, we conducted
16 a preliminary safety investigation. The cytotoxic effects of the derivatives on
17 MDA-MB-231 breast cancer cells and normal human breast epithelial cells
18 (MCF10A) were investigated. Additionally, blood biochemical analyses of the
19 HE pathology of various organs and liver and kidney functions of the
20 experimental mice in each group were performed. The results revealed that at
21 the test dose, compared with the blank control group, there was no effect on
22 the various organs and liver and kidney functions of the experimental animals

1 in each group.

2 In conclusion, although the derivative 12e is not a selective HDAC6
3 inhibitor and physically binds to HIF-1 α , it meets the following three
4 requirements: multifunctional efficacy and efficacy intensity, exclusive
5 correspondence between each efficacy and dual target function, and
6 preliminary safety.

7 In this paper, a series of derivatives were designed and synthesized, and
8 derivative 12e had the strongest activity. Through in vitro and in vivo studies on
9 the pharmacodynamics and molecular mechanisms, the multifunctional
10 anti-breast cancer mechanism of the HDAC6-HIF-1 α inhibitor 12e was clarified.
11 As shown in Figure 9, 12e promotes the apoptosis of breast cancer cells
12 through two pathways, namely, HDAC6/ α -Ac-tubulin/VDAC (①) and
13 HDAC6/Ac-HSP90/FLIPL (②), and inhibits mitosis through
14 HDAC6/Ac-tubulin/spindle depolymerization (③). Furthermore, through the
15 synergistic inhibition of the HIF-1 α /glycolytic pathway by
16 HDAC6/Ac-HSP90/HIF-1 α and 12e itself, the energy supply (④) is inhibited,
17 and the HIF-1 α /VEGF/angiogenesis pathway inhibits the nutrient supply (⑤).
18 In addition, HIF-1 α /glycolytic/immune microenvironment remodelling activates
19 the immune system to kill tumour cells (⑥) and reverse immune escape,
20 thereby increasing the relative expression of killer T cells (⑦). When the
21 effective blood drug concentration is reached, the synergistic and combined
22 effects of the above seven aspects, which are multiplied stepwise, form a

1 powerful comprehensive antiproliferative effect. Second,
2 HDAC6/Ac-HSP90/HIF-1 α and 12e itself synergistically inhibit the
3 HIF-1 α /VEGF/VEGFR2/Akt and Erk1/2 pathways, resulting in antiangiogenic
4 and antimetastatic effects. Finally, through the combined synergistic effects of
5 suppressing proliferation, metastasis, angiogenesis, and immune
6 microenvironment remodelling, the development of breast cancer is
7 comprehensively blocked.

9 **Conclusions**

10 On the basis of the concept of multifunctionality and
11 synergistic/combo enhancement, in this study, for the first time, a
12 dual-target combination of the epigenetic regulatory factor HDAC6 that
13 regulates the acetylation of multiple proteins and the transcription factor
14 HIF-1 α , which regulates the transcription of multiple genes, was constructed.
15 Through drug design and synthesis, derivative 12e has been shown to have
16 multiple anti-breast cancer effects, including anti-proliferative, anti-metastatic,
17 and anti-angiogenic effects and the ability to reverse tumour
18 immunosuppression and immune escape. The antiproliferative effect of 12e
19 was comparable to that of panobinostat. Its anti-breast cancer lung metastasis
20 effect was comparable to that of sorafenib. Its antiangiogenic effect was
21 stronger than that of sorafenib. It has successfully achieved the design goal of
22 a multifunctional antitumour dual-target agent with potential clinical

significance and can be a candidate drug for the treatment of triple-negative breast cancer.

The molecular mechanism results indicate that the derivative 12e, by inhibiting HDAC6 and HIF-1 α , induces the formation of a novel anti-breast cancer mechanism involving the synergistic enhancement of multiple signalling pathways and combined multifunctional enhancement through the downstream functional proteins of the dual targets. This multiple and stepwise superposition amplifies the degree of multifunctional efficacy, achieving significant clinical effects and confirming that this dual-target combination is feasible and can be used as a research target combination for drugs.

Methods

Chemical reagents and instruments

All commercially available starting materials and solvents are of reagent grade and used without further purification (Bide Pharmatech Ltd., Shanghai, Shanghai). Column chromatography was performed using a 300–400 mesh or 200–300 mesh silica gel. ^1H and ^{13}C NMR spectra were recorded with the Bruker advance III 400 MHz NMR spectrometer. High-resolution mass spectra were obtained from acquity UPLC/QTOF premier mass spectrometer. HPLC spectrum was recorded for all data on an Agilent Technologies 1260 series LC system. All compounds are >95% pure by HPLC. HPLC trace and chromatographic conditions of 12e were shown in Figure S40 and Table S2.

1 **Materials of bioassay**

2 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was
3 purchased from Macklin Biochemical Co., Ltd (Shanghai, China). BCA protein
4 assay kit was obtained from Invitrogen, ThermoFisher Scientific Inc. (Waltham,
5 MA, USA). VEGF (ab259412) and IFN- γ (ab259377) were purchased from
6 Abcam (Cambridge, MA, USA). Matrigel Matrix was provided by Corning, Inc.
7 (Corning, NY, USA). RIPA lysis buffer, 4% Paraformaldehyde (PFA), dimethyl
8 sulfoxide (DMSO) and Tween-80 were acquired from Solarbio
9 Science&Technology Co., Ltd. (Beijing, China).

10 Antibodies: β -Actin (13E5) (#4970), FLIP (D5J1E) (#56343), VDAC
11 (D73D12) (#4661), Phospho-VEGF Receptor 2 (Tyr1175) (19A10) (#2478),
12 HDAC6 (D2E5) (#7558), Alpha Tubulin (Acetyl-Lys40) (#5335), and
13 Phospho-AKT (Ser473) (D9E) XP® (#4060) Antibody were purchased from
14 Cell Signaling Technology, Inc. (Danvers, MA, USA). ERK1 (phospho T202 +
15 Y204) + ERK2 (phospho T185 + Y187) (ab278538) Antibody was purchased
16 from Abcam, Inc. (Cambridge, UK). GAPDH (B8016), HIF-1 Alpha (RM4217),
17 and HSP 90 (Acetyl Lys292/284) (BD-PK0029) Antibody were purchased from
18 Biodragon, Inc. (Suzhou, China). CD45 (#570291 #559884), CD8 (#564422),
19 PD-1 (#562584), Tim-3 (#568796), CD3 (#564379), CD4 (#563790), CD25
20 (#553075), CD20 (#759202), NK1.1 (#563096), CD11b (#567469), Ly6G
21 (#740157), Ly6C (#568954), F4/80 (#569237), CD163 (#155309), CD80
22 (#553769), CD11c (#565586), TNF (#554419), and IFN (#563376) Antibodies

were purchased from BD Biosciences (San Jose, CA, USA). GranzymeB (#25-8898-82) Antibody was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Cell culture

Human mammary carcinoma cell line MDA-MB-231 and mouse mammary carcinoma cell line 4T1 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Primary human umbilical vein endothelial cells (HUVECs) were derived from the umbilical cord endothelium. MDA-MB-231 cells were cultured in L-15 medium (ThermoFisher Scientific Inc. Waltham, MA, USA) containing 10% FBS and 1% penicillin/streptomycin. 4T1 cells were maintained in DMEM (high glucose) medium (ThermoFisher Scientific Inc. Waltham, MA, USA) with 10% FBS and 1% antibiotic solution. HUVECs were cultured in Endothelial Cell Medium (ScienCell, San Diego, CA, USA) containing 5% FBS, 1% endothelial cell growth supplements, and 1% antibiotic solution. The verification of HDAC6 overexpression in MDA-MB-231 cells was shown in Figure S41.

Animals

Female BALB/c mice were supplied by SPF (Beijing) Biotechnology Co., Ltd. (Beijing, China) and were acclimatized for 7 days prior to experimentation. Animals were allowed free access to a standard diet and water under maintained conditions of 25 °C and 50% relative humidity.

All care and handling of animals was performed with the approval of the

Tianjin Medical University Institutional Animal Care and Use Committee, approval number: TMUaMEC 2021001. Additionally, this study was carried out following the “3R” rule (“replacement”, “refinement”, “reduction”). Animals were anesthetized with an intraperitoneal injection of 50 mg/kg 1% pentobarbital sodium solution and sacrificed via cervical dislocation.

Cell viability assay

To investigate Effects of derivatives on cell viability, MDA-MB-231, 4T1 and HUVECs were seeded into 96-well plates at a density of 1×10^3 cells/well for 24 h. Then cells were treated with different concentrations of the derivatives for 72 h. MTT (0.5 mg/mL) was added to each well, and cells were incubated for an additional 4 h. The optical density (OD) value of each well was read at 490 nm using a microplate reader (BioTek Synergy HTX, BioTek Instruments, Inc., Winooski, VT, USA). The medium containing 0.1% DMSO was considered as control.

Wound healing assay

The effect of derivatives on cell migration was determined by a scratch assay. MDA-MB-231 and 4T1 cells were seeded into a 6-well plate and grown to full confluency. Then a clean wound was created by scratching across the centre of the wells using a 200 μ L pipette tip. Cell debris was removed, and the medium was refreshed with 0.1% DMSO, different concentrations of derivative and incubated for 24 h. The wound was photographed at 0 h and 24 h using an inverted microscope (Motic AE2000, Kowloon, China) in three random fields.

1 The widths of the wounds were measured using ImageJ 1.51j8 software
2 (National Institutes of Health, Bethesda, MD, USA).

3 **Invasion assay**

4 Invasion of cells was measured in Matrigel (BD, Franklin Lakes, NJ, USA)
5 -coated transwell inserts (Costar, Manassas, VA, USA) containing
6 polycarbonate filters with 8 µm pores. The inserts were coated with 50 µL of
7 Matrigel matrix according to the manufacturer's recommendations. 5×10^4
8 cells in 100 µL of cell suspension with various concentrations of derivative
9 were plated in the upper chamber, whereas 750 µL of medium with 10% fetal
10 bovine serum were added to lower well. After incubating for 24 h, cells that
11 migrated to the lower surface of the membrane were fixed and stained. For
12 each membrane, three random fields were counted at 10× magnification.
13 Relative invasion rate (%) = (number of migrated cells in sample/number of
14 migrated cells in control) × 100%.

15

16 **Adhesion assay**

17 with modifications. The 96-well plates (BD Biosciences, Bedford, MA, USA)
18 were coated with fibronectin (20 µg/mL) at 4°C overnight and then blocked in
19 BSA (1%) for 1 h. MDA-MB-231 cells were exposed to different concentrations
20 of compound for 24 h before seeding. Target cells were suspended in medium.
21 Cells ($5 \times 10^4 \text{ mL}^{-1}$) were seeded to fibronectin coated plates and then
22 incubated for 1 h at 37°C. Nonadherent cells were removed by gentle washing

1 with PBS. Then, crystal violet staining assay was employed to analyze the
2 adhesion ability of cells. The OD value was measured using microplate reader
3 at 570 nm. Relative adhesion rate (%) = (OD value of sample/ OD value of
4 control) × 100%.

5 **Endothelial network formation assay**

6 To investigate the effect of 12e on tube formation, each well of a 96-well
7 plate was coated with growth factor-reduced reconstituted basement
8 membrane matrix (Matrigel, catalogue no. 354230, Corning, NY, USA) and
9 incubated for 1 h at 37 °C for solidification. HUVECs (1×10^4) in the medium
10 containing VEGF (20 ng/mL) were seeded in plates and added with different
11 concentrations of compound. Then cells were incubated for 12 h to allow for
12 tube formation. The formation of tube-like structures was captured using an
13 inverted microscope and quantified using ImageJ 1.51j8 software.

14 **Lactic acid and ATP test**

15 To construct the hypoxic MDA-MB-231 cell model, cells were incubated
16 for 24 h in culture dishes for adherence and pre-starved for 12 h with L15
17 starvation medium (containing only 1% FBS), and then the medium was
18 replaced with fresh starvation medium containing different concentrations of
19 12e. Thereafter, the cells were placed into an anoxic culture cassette, which
20 was filled with high-purity nitrogen (purity of nitrogen, > 99.999%), sealed, and
21 cultured in an incubator for 6 h. Then the quantification of extracellular lactic
22 acid and intracellular ATP were determined using the Lactic Acid (L-LA)

Content Assay Kit and ATP Content Assay Kit supplied by Sangon Biotech (Shanghai) Co., Ltd., respectively. The culture medium or cell lysate were collected for measurement according to the manufacturer's instructions.

Western blotting

Total proteins from cells or tissues were extracted in ice-cold RIPA buffer supplemented with 1% PMSF (Solarbio, Beijing, China), and protein concentrations were determined using the BCA protein assay (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). Equal protein samples were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk for 1 h at room temperature and then incubated overnight with primary antibodies at 4°C. The next day, the membranes were washed with TBST and incubated with secondary antibodies for 1 h. After rinsing with TBST, the bands were visualized using enhanced chemiluminescence on a UVITEC Nine Alliance Q9 system (UVITEC Ltd., Cambridge, UK), and greyscale analysis was performed using ImageJ 1.51j8 software (National Institutes of Health, Bethesda, MD, USA).

Evaluation of antitumor effect and biosafety in orthotopic breast cancer model

For preparing the 4T1 tumor models, 5×10^5 4T1 cells were orthotopically injected into the abdominal mammary fat pad. After one week, the tumor-bearing mice were randomly divided into three groups (n = 6 per group):

(i) Control (physiological saline, qod), (ii) 10 mg/kg Sorafenib (dissolved in physiological saline containing 0.5% Tween-80 and 1% DMSO, qod), (iii) 5 mg/kg Panobinostat (dissolved in physiological saline containing 0.5% Tween-80 and 1% DMSO, qod), (iv) 12 mg/kg 12e (dissolved in physiological saline containing 0.5% Tween-80 and 1% DMSO, qod), (v) 24 mg/kg 12e (dissolved in physiological saline containing 0.5% Tween-80 and 1% DMSO, qod), and . During the experiment, each group was administered with the treatment for 17 days via intraperitoneal injection, and the body weight was monitored and recorded every 2 days. The tumors were excised after euthanizing the animals at the end of the experiment for photographs, and other organs along with the tumor tissues, for H&E staining. Beckman Coulter Chemistry Analyzer AU5800 Series (Beckman Coulter, Inc., Brea, CA, USA) was used to analyze the hematological and biochemical characteristics of blood samples.

Histopathology analysis

Tissues were fixed in PFA and embedded in paraffin, and then 5 µm-thick serial sections were cut from the tissue blocks. After deparaffinisation and rehydration, sections were stained with H&E (Solarbio Science & Technology Co., Ltd., Beijing, China) and used to assess pathological changes. For vascular assessment, immunofluorescence staining was performed using the CD34 antibody (Abcam ab315802) following the manufacturer's instructions for the OPAL 7-COLOR MANUAL IHC KIT (Akoya Biosciences, Marlborough,

MA, USA).

Enzyme-linked immunosorbent assay (ELISA)

Tumors and serum of mice were collected at the end of the experiment. An equal amount of tumor tissues were taken and suspended in an equal volume of PBS, homogenized and then centrifuged. The supernatant was collected for detecting the levels of VEGF, ATP, Lactic acid, TNF- α and IFN- γ , Perforin, and Granzyme B according to the manufacturer's instructions (CUSABIO TECHNOLOGY LLC, Houston, TX, USA).

Flow cytometry analysis

Tissues underwent enzymatic digestion to generate single-cell suspensions, with the aid of a mixed enzyme solution composed of collagenase, hyaluronidase, and DNase I. The resulting cell suspensions were supplemented with anti-Fc receptor antibody (BioXcell BE0307) and FVS (BD 565388), followed by a 15-min incubation period on ice. Thereafter, cells were subjected to primary antibody staining in BD Horizon Brilliant Stain Buffer Plus, with the reaction maintained on ice for 30 min. For intracellular staining assays, cells were first stimulated for 4 hours with LeukoAct Cctl supplemented with GolgiPlug (BD 550583). Subsequently, successive surface and intracellular staining procedures were implemented using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD 554714). All cell samples were ultimately analyzed on a Beckman Coulter CytoFLEX (Beckman Coulter, Inc., Brea, CA, USA)

Construction of HDAC6-overexpressing stable cell lines

Human HDAC6 cDNA (NM_001321225.2) was cloned into the pLV3-CMV-CopGFP-Pure expression vector (pLV3-HDAC6). The empty pLV3-CMV-CopGFP-Pure vector served as the negative control. Lentiviral vectors encoding human HDAC6 were produced by polyethylenimine (PEI)-mediated co-transfection of HEK293T cells with the recombinant transfer plasmid (pLV3-HDAC6) and lentiviral packaging plasmids. Viral supernatants were harvested 48–72 h post-transfection, concentrated via polyethylene glycol (PEG) 8000 precipitation followed by ultracentrifugation, then titrated on HEK293T cells using a serial dilution assay to determine viral titer. For transduction of MDA-231 cells, cells were seeded in 6-well plates and cultured until reaching 30% confluency. At this confluency, viral particles (multiplicity of infection [MOI] = 5–10) suspended in complete medium supplemented with 8 µg/mL polybrene were added to each well. The plates were then centrifuged at 1,200 ×g for 2 h to enhance transduction efficiency. 48 hours post-transduction, cells were passaged into fresh complete medium containing 2 µg/mL puromycin for selection of stably transduced cells, and this selection pressure was maintained for 5–7 days to eliminate untransfected cells. The surviving cells were expanded in culture, and the overexpression of HDAC6 was validated by qPCR and Western blotting.

Statistical analysis

GraphPad Prism 9.00 (GraphPad Software Inc., San Diego, CA, USA)

1 was used to conduct the statistical analyses. All the data are presented as the
2 mean $\pm$ standard error of the mean (SEM) from three independent biological
3 replicates. Comparisons between two groups were performed by unpaired
4 one-tailed and two-tailed Student's *t*-tests with Welch's correction. To compare
5 multiple groups, one-way analysis of variance (ANOVA) followed by Bonferroni
6 correction was used. Statistical significance was defined as *P* values less than
7 0.05 (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

8

9 **Abbreviations:** ADCs, antibody-drug conjugates; Akt, protein kinase B; ATCC,
10 american type culture collection; ATP, adenosine triphosphate; ELISA,
11 enzyme-linked immunosorbent assay; ER, estrogen receptor; ERK1/2,
12 extracellular regulated protein kinases 1/2; FLIP_L, FLICE-like inhibitory protein
13 long-form; HDAC6, histone deacetylase 6; HER2, human epidermal growth
14 factor receptor 2; HIF-1 α , hypoxia-inducible factor-1 α ; HK2, hexokinase 2;
15 HPLC, high performance liquid chromatographyl; HSP90, heat shock protein
16 90; HUVECs, human umbilical vein endothelial cells; H&E, hematoxylin and
17 eosin; MDSCs, myeloid-derived suppressor cells; MVD, microvessel density;
18 NMR, nuclear magnetic resonance; OD, optical density; OS, overall survival;
19 PARP, poly(ADP-ribose) polymerase; PD-1, programmed cell death protein;
20 PD-L1, programmed death-ligand 1; PFS, progression-free survival; PKM2,
21 pyruvate kinase M2; PR, progesterone receptor; TME, tumor
22 microenvironment; TNBC, triple-negative breast cancer; VDAC,

voltage-dependent anion channel; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; ZBG, zinc-binding group.

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CRedit authorship contribution statement

Hongquan Duan and Zhenyu Zhao: conceptualization, writing-review and editing, supervision, funding acquisition. **Xiaochuan Duan, Zekun Li and Xinyao Liu:** data curation, investigation, methodology. **Yimiao Li, Xiao Jiang and Lu Liu:** data curation, investigation. **Ying Chen and Nan Qin:** formal analysis, project administration.

Data availability

1 Data, analytic methods, and study materials will be made available to other
2 researchers upon reasonable request.

4 **Declaration of competing interest**

5 The authors declare that they have no known competing financial interests or
6 personal relationships that could have appeared to influence the work reported
7 in this paper.

9 **Ethical statement**

10 All care and handling of animals was performed with the approval of the Tianjin
11 Medical University Institutional Animal Care and Use Committee, approval
12 number: TMUaMEC 2021001.

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