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Synthesis and biological evaluation of novel asymmetric naphthalene diimide derivatives as anticancer agents depending on ROS generation†

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Naphthalenetetracarboxylic diimide (NDI) is widely used as a photoelectric material in the field of medicine. A series of asymmetric naphthalene diimide derivatives were synthesized and evaluated for their anticancer properties by various experimental assays. As the representative compound, **3c** exerted significantly greater inhibitory effects on hepatoma cells SMMC-7721 and Hep G2 with an IC₅₀ value of 1.48 ± 0.43 μM and 1.70 ± 0.53 μM, respectively, than normal hepatocytes QSG-7701 with an IC₅₀ value of 7.11 ± 0.08 μM. Treatment with compound **3c** (3 μM) for 48 h resulted in apoptosis of SMMC-7721 cells and Hep G2 cells with 52.1% and 67.8% apoptotic cells, respectively. Compound **3c** induced autophagy and suppressed the migration of hepatoma cells in a concentration-dependent manner, resulting from the generation of reactive oxygen species (ROS). Based on its biological ability, compound **3c** was considered as a potent anticancer agent.

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Introduction

Hepatocellular carcinoma (HCC) is currently the third most common cause of death by cancer, and the death rate of HCC has been increasing in the past few years.¹ More importantly, at present, there are only a few specific drugs available for the treatment of HCC. Therefore, it is of utmost urgency to explore new drugs for HCC therapy.

Naphthalenetetracarboxylic diimide (NDI), characterized by its aromatic planar structure, exhibits physiological activity, particularly in the optic system, and is widely used as a photoelectric material in the field of medicine.^{2–4} Recently, several NDI scaffolds have been reported as anticancer agents, exhibiting double-stranded or G-quadruplex DNA binding ability.^{5,6} The nature and size of the side-chain terminal groups in NDI, which provide electrostatic interactions, are important factors in its binding selectivity.^{6,7} Meanwhile, several polyamine-modified NDI conjugates showed more potent inhibition of cancer cells than amonafide both *in vitro* and *in vivo*.⁸ However, the synthetic route of polyamine is long, which is not efficient in terms of atom economy.

Some asymmetric derivatives modified by short amino chains also exhibited potent anticancer effects. In the present study, in order to discover new anticancer agents with a better pharmacological and toxicological profile than that of previously reported NDI-polyamine conjugates, a series of new NDI conjugates were synthesized by conjugating NDI with another short terminal amine groups. The effects of the target compounds on growth, apoptosis, autophagy and migration of hepatoma cells, and the related mechanical research were investigated.

Results and discussion

There are two routes for the synthesis of asymmetric derivatives **3a–i** and **4a–c**. One is a one-pot reaction, which involves the direct condensation of the starting material naphthalene-1,4,5,8-tetracarboxylic acid (**1**) with *n*-butylamine and another

Scheme 1 Synthesis of the target compounds **3a–i**. Reagents and conditions: (a) *n*-butylamine, H₂O, aq. KOH, reflux; (b) R₁NH₂, DMF, reflux.

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Scheme 2 Synthesis of the target compounds **4a–c**. Reagents and conditions: (a) R_2NH_2 , 3-amino-1-propanol, EtOH/toluene, reflux.

amine to obtain a mixture. The resulting mixture obtained from this route is difficult to purify. The other route involves a two-step reaction, in which *n*-butylamine and another amine are reacted successively with the starting material. Despite being long, this route produces easily-purified target compounds with a much higher yield than the first route. Since the reaction between **1** and 3-amino-1-propanol was not successful, we finally employed the first route for the synthesis of the target compounds. The synthesis routes are shown in Schemes 1 and 2. The structures of all the target compounds were confirmed by 1H NMR, ^{13}C NMR, ESI-MS, and elemental analysis.

The cytotoxicity of the compounds was determined by their half maximal inhibitory concentration (IC_{50}), which is a

unit expressing the effectiveness of a compound in inhibiting certain biological or biochemical functions. The IC_{50} values of the target compounds against HepG2, SMMC-7721, MDA-MB-231, MCF-7, and HCT-116 are summarized in Table 1. These compounds exhibited potent cytotoxic activity against the tested cell lines with different IC_{50} values. The compounds containing a propyl alcohol substitute showed the most potent activity. Several compounds containing *n*-butyl also exhibited significant inhibitory activity against the cancer cells, while the other *n*-butyl compounds (**3e–i**) showed no inhibitory activity due to their poor solubility. Therefore, the inhibitory activity of the compound was significantly influenced by the substituent group.

Compound **3c**, the structure of which is shown in Fig. 1A, exhibited the most potent activity against hepatoma cells. It proved to be about 5-fold more potent than amonafide, which was reported in our previous study.⁹ Furthermore, **3c** suppressed the growth of SMMC-7721 and Hep G2 cells in a time- and concentration-dependent manner, as shown in Fig. 1B. The IC_{50} values of **3c** against the cancer cells for 24 h were $4.29 \pm 0.03 \mu M$ and $5.38 \pm 0.83 \mu M$ for SMMC-7721 and Hep G2 cells, respectively. In order to determine the effect of **3c** on non-cancer cells after 48 h of treatment, we further performed MTT assays using human normal hepatocytes QSG-7701.

Table 1 The inhibitory activities of the target compounds toward tumor cells

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Cpds.	R _A	R _B	IC ₅₀ (μM)				
			HepG2	SMMC-7721	MDA-MB-231	MCF-7	HCT-116
Amonafide			9.45 ± 1.29	9.37 ± 0.82	6.62 ± 0.66	12.92 ± 4.15	6.46 ± 0.53
3a			35.87 ± 5.70	37.94 ± 7.60	>50	>50	36.33 ± 5.84
3b			17.01 ± 4.76	35.09 ± 1.98	15.08 ± 2.46	12.62 ± 2.32	9.22 ± 1.45
3c			1.48 ± 0.43	1.70 ± 0.53	5.79 ± 0.64	5.15 ± 1.01	14.71 ± 2.65
3d			30.63 ± 7.46	25.38 ± 1.88	18.25 ± 1.37	21.39 ± 1.82	32.69 ± 0.09
3e			>50	>50	>50	>50	16.32 ± 0.67
3f			>50	>50	>50	>50	32.34 ± 2.57
3g			>50	>50	>50	>50	>50
3h			45.23 ± 3.78	36.41 ± 4.22	>50	39.64 ± 5.16	28.19 ± 2.35
3i			>50	>50	>50	>50	45.87 ± 4.29
4a			5.96 ± 1.79	4.44 ± 1.63	6.21 ± 1.24	3.36 ± 0.95	5.29 ± 2.06
4b			4.14 ± 0.03	3.80 ± 0.61	5.39 ± 1.15	4.78 ± 1.01	8.29 ± 0.97
4c			3.08 ± 1.26	2.7 ± 0.25	7.64 ± 2.13	6.98 ± 1.35	4.35 ± 1.97

Fig. 1 Compound **3c** inhibited the proliferation and changed the cell morphology of hepatoma cells. (A) The chemical structure of compound **3c**. (B) SMMC-7721 cells and Hep G2 cells were treated with compound **3c** for 24 h and 48 h, respectively. The inhibition rate was examined through MTT assays. (C) SMMC-7721, Hep G2 and QSG-7701 cells were treated with compound **3c** for 48 h, and the cell viability was detected by the MTT assay. (D) Representative images of the cell morphology after **3c** treatment for 24 h (20 $\times$).

As shown in Fig. 1C, the IC_{50} of **3c** for QSG-7701 cells was $7.11 \pm 0.08 \mu\text{M}$, which was much higher than that for hepatoma cells, implying that **3c** selectively killed the cancer cells. Moreover, after **3c** treatment, a round cell shape, a reduction in cell volume, and a shrunken cytoplasm of hepatoma cells were observed, as shown in Fig. 1D. Therefore, **3c** was selected for further investigation.

Evident cellular damage and detachment of cells from the culture substrate were observed in the presence of **3c**, implying that **3c** might induce apoptosis. To investigate the effect of **3c** on apoptosis, SMMC-7721 and Hep G2 cells were stained with acridine orange/ethidium bromide (AO/EB). Analysis of cell death is displayed in Fig. 2A; the decreased intensity of green fluorescence and the increased intensity of red fluorescence suggested that **3c** induced the death of SMMC-7721 and Hep G2 cells in a concentration-dependent manner. To confirm **3c**-induced apoptosis, SMMC-7721 and Hep G2 cells were also stained with Annexin V/PI and examined by flow cytometry. As shown in Fig. 2B, the population of apoptotic cells was upregulated with increasing concentration of **3c**.

To explore the molecular mechanism of **3c**-induced apoptosis, a western blot assay was carried out. PARP-1 is a mediator of apoptosis, and activation of PARP-1 is a hallmark of apoptosis.¹⁰ **3c** treatment significantly triggered the expression of cleaved PARP-1 in both hepatoma cells (Fig. 2C). As a proto-oncogene, Bcl-2 plays a key role in mitochondrial apoptotic pathways.¹¹ As shown in Fig. 2C, **3c** treatment weakened the expression of Bcl-2 protein. Apoptosis induction by caspase 8 regulates mitochondrial dysfunction in the Fas-mediated pathway,^{12,13} and the activation of caspase 8 was observed in both hepatoma cells after **3c** treatment. The tu-

Fig. 2 Compound **3c** induced apoptosis of hepatoma cells. (A) After **3c** treatment for 24 h, hepatoma cells were stained using AO/EB staining (20 $\times$). The fluorescence intensity was detected by HCS. (B) The apoptosis of hepatoma cells induced by **3c** was detected using Annexin V-FITC/PI staining by flow cytometry. (C) Hepatoma cells were treated with compound **3c** for 24 h. The relative expression of p53, PARP-1, caspase-8 and Bcl-2 was detected by western blot analysis.

mor suppressor p53 has a direct apoptotic effect on the mitochondria, and its activation induces cell cycle arrest and apoptosis through various pathways.¹⁴ As shown in Fig. 2C, **3c** treatment upregulated p53 expression, indicating that **3c** induced apoptosis *via* the p53 pathway.

We observed extensive vacuolization in SMMC-7721 and Hep G2 cells after **3c** treatment (Fig. 3A), which was further confirmed by using transmission electron microscopy in SMMC-7721 cells (Fig. 3B). Meanwhile, we also found that **3c** treatment triggered the formation of autophagosomes in SMMC-7721 cells (Fig. 3B). Therefore, we postulated that **3c** promoted autophagy in hepatoma cells. The fluorescent dye monodansylcadaverine (MDC) is considered as a specific marker for autophagic vacuoles, and it is used to detect the induction of autophagy.¹⁵ As shown in Fig. 3C, the fluorescence intensity of MDC in SMMC-7721 and Hep G2 cells increased by the addition of **3c**. The elevated fluorescence intensity of MDC demonstrated that **3c** triggered autophagy.

Light chain 3 (LC3), a kind of microtubule-associated protein, is a specific marker for autophagy initiation.¹⁶ Western blot analysis revealed that LC3-II levels were significantly

Fig. 3 Compound 3c induced autophagy. (A) Hepatoma cells were treated with compound 3c for 12 h, and photographs were taken (20 $\times$). (B) Representative transmission electron microscopy images after SMMC-7721 cells were treated with compound 3c (2 μ M) for 12 h. (C) The fluorescence intensity of MDC was detected by HCS after hepatoma cells were treated with compound 3c for 24 h. (D) The relative expression of LC3, BECN1 and p62 was measured by western blot analysis. (E) 3-MA partially reversed the inhibitory effect of compound 3c on the cell viability of hepatoma cells.

increased in a concentration-dependent manner in hepatoma cells treated with 3c (Fig. 3D). Beclin 1 (BECN1) is a key autophagy-promoting factor that plays a central role in the regulation of cell death and autophagy.¹⁷ In 3c-treated hepatoma cells, the expression of BECN1 was enhanced (Fig. 3D). The selective autophagy substrate p62 is degraded by autophagy.¹⁸ The accumulation and depletion of p62, which indicated autophagy induction and inhibition, respectively, are often used as markers in autophagic flux investigations.¹⁹ After 3c treatment, a decrease of p62 expression was observed in hepatoma cells (Fig. 3D). In addition, cells treated with 3c for 48 h in the presence of 3-methyladenine (3-MA), an autophagy inhibitor, showed slightly higher viability than cells treated with 3c alone (Fig. 3E). Taken together, these results indicated that 3c treatment induced autophagy and promoted autophagic flux in hepatoma cells.

Among many elements that regulate apoptosis or autophagy, the redox environment of the cell is generally believed to be extremely important in mediating either apoptosis or autophagy.^{20,21} Hence, we evaluated the relative intracellular levels of reactive oxygen species (ROS) using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). As shown

in Fig. 4A, the intensity of fluorescence was obviously increased in 3c-treated hepatoma cells. Moreover, treating hepatoma cells with the antioxidant α -tocopherol (TOC) inhibited the increase of fluorescence intensity in response to 3c (Fig. 4B). These results showed that the increased intracellular levels of ROS in SMMC-7721 and Hep G2 cells were induced by 3c.

To determine the functional role of ROS in cell growth, apoptosis and autophagy in hepatoma cells treated with 3c, we pretreated the cells with TOC for 1 h before the cells were treated with 3c. SMMC-7721 and Hep G2 cells pretreated with TOC were significantly more resistant to 3c than non-pretreated cells, showing a marked increase in viability (Fig. 5A), morphological changes (Fig. 5B), and reduction in the number of apoptotic cells (Fig. 5C). Meanwhile, pretreatment with TOC reversed the upregulation of p53, cleaved PARP-1 and LC3-II and the downregulation of p62 induced by compound 3c (Fig. 5D and E), which further confirmed the critical role of ROS in 3c-induced apoptosis and autophagy.

Metastasis, a condition where cancer cells leave their primary cancer site and establish a secondary site in a distant organ, is a complex process. The prognosis of hepatoma is poor mainly due to its high risk of metastasis.^{22,23} Thus, various anti-metastatic agents have been extensively explored. Migration of cancer cells to a secondary organ site is a prominent event in the metastatic cascade.²⁴ To investigate the effect of 3c on cancer cell migration *in vitro*, we firstly performed the scratch wound healing assay. As shown in Fig. 6A, SMMC-7721 and Hep G2 cells displayed a concentration-dependent loss of migration ability after exposure to 3c. The inhibitory effect of 3c on cell migration was further validated by the transwell migration assay (Fig. 6B). Western blot analysis proved that the loss of the cell's migration ability resulted from the 3c-induced decrease in the expression of β -catenin and phospho-Akt (Ser473) (Fig. 6C).

Fig. 4 Compound 3c promoted ROS generation in hepatoma cells. (A) Hepatoma cells were treated with compound 3c for 24 h. The variations of intracellular ROS levels were examined by HCS (20 $\times$). (B) The variations of intracellular ROS levels in 3c-treated SMMC-7721 cells and Hep G2 cells in the presence of α -tocopherol (TOC).

Fig. 5 Compound 3c inhibited proliferation and triggered apoptosis and autophagy depending on ROS. (A) Cell viability of hepatoma cells treated with 3c after pretreatment with TOC was determined by the MTT assay. (B) TOC reduced the changes of cell morphology of hepatoma cells induced by 3c (20×). (C) After pretreatment with TOC, the apoptosis of hepatoma cells induced by 3c was examined by flow cytometry. (D) The expression of p53 and PARP-1 in 3c-treated hepatoma cells in the presence of TOC. (E) The expression of LC3 and p62 in 3c-treated hepatoma cells in the presence of TOC.

Fig. 6 Compound 3c suppressed the migration of hepatoma cells depending on ROS. (A) The statistical results of migrated cells from the scratch wound healing assay. Hepatoma cells were treated with 3c for the indicated time. (B) Representative images and statistical results of migrated cells from the transwell migration assay. (C) The expression of β-catenin and p-Akt (Ser473) and β-actin in hepatoma cells. (D) TOC pretreatment reduced the inhibitory effects of 3c on cell migration of hepatoma cells. (E) The expression of β-catenin in 3c-treated hepatoma cells after pretreatment with TOC.

Previous studies revealed that ROS induced by antitumor agents mediated the migration and invasion of cancer cells,

implying the important role of ROS in cancer therapy.^{25,26} In line with this data, pretreatment of hepatoma cells with TOC

restored their ability to migrate which was inhibited by **3c** (Fig. 6D). Pretreatment with TOC also weakened the inhibitory effects of **3c** on β -catenin expression (Fig. 6E), indicating that ROS were responsible for the inhibitory effect of **3c** on hepatoma cell migration.

Conclusions

In the present study, we synthesized a series of novel asymmetric naphthalene diimide derivatives and investigated their anticancer activities. The anticancer effects of these compounds were significantly influenced by their substituent groups. Among these derivatives, compound **3c** displayed a stronger inhibitory effect on hepatoma cells SMMC-7721 and Hep G2 than on non-cancer cells QSG-7701, suggesting that compound **3c** selectively killed hepatoma cells. Compound **3c** induced ROS generation, which was reversed by TOC. In **3c**-treated hepatoma cells, the expressions of cleaved PARP, caspase 8, and p53 were upregulated, whereas Bcl-2 expression was downregulated, resulting in the apoptosis of hepatoma cells. The upregulation of LC3-II and Beclin 1, as well as the downregulation of p62 protein, implied that compound **3c** induced autophagy and autophagy flux. The decreased levels of β -catenin and p-Akt (Ser473) were responsible for the loss of migration activities in **3c**-treated hepatoma cells. More importantly, we found that the activity of compound **3c** in cancer cells depended on the generation of ROS. These data provided evidence that compound **3c** was a promising lead compound for further investigation as an anticancer agent with a ROS generation-inducing mechanism.

Experimental

Chemistry

Melting points (mp) were determined using an X-6 hot stage microscope and were not corrected. ^1H and ^{13}C NMR spectra were recorded using TMS as the internal standard in CDCl_3 with a Bruker BioSpin GmbH spectrometer at 300 MHz and 75 MHz, respectively. MS spectra were recorded on a Shimadzu LCMS-2010A instrument with an ESI mass selective detector. Elemental analyses were performed on a Gmbe Vario EL elemental instrument. Flash column chromatography was performed with silica gel (200–300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd.

Synthesis of intermediate 2

The synthesis of naphthalene diimide derivatives was carried out according to a published procedure.²⁷ The starting material **1** (2.0 g, 7.46 mmol) was dissolved in 350 mL water and mixed with 35 mL 1 M aqueous KOH solution. This mixture was vigorously stirred and heated until almost all the compound was dissolved. The pH of the solution was acidified to 6.4 by adding 1 M phosphoric acid. To this solution, *n*-butylamine (0.74 mL, 7.46 mmol) was added and its pH was readjusted to 6.4 with phosphoric acid. The mixture was heated to reflux overnight, cooled to room temperature and

filtered. The filtrate was acidified using 5 mL acetic acid, creating a solid precipitate, which was then filtered and vacuum-dried to generate a pale yellow solid, namely, intermediate **2** (1.74 g, yield 73%). MS (ESI⁺): m/z : 324.2 $[\text{M} + \text{H}]^+$.

General procedures for the preparation of target compounds 3a–i. Intermediate **2** (0.5 g, 1.55 mmol) was dissolved in DMF (10 mL). Various amines (1.7 mmol) were added to the solution while being stirred, and the mixture was heated to reflux for 10 h. The solvent was vacuum-evaporated, and the crude residue was dissolved in CH_2Cl_2 (50 mL) and washed with water and brine. The organic layer was collected and dried over Mg_2SO_4 , whereas the solvent was vacuum-evaporated. The crude residue was purified by silica gel column chromatography with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (50:1) to obtain the target compounds, namely **3a–i**.

3a, light pink solid, yield: 27.4%. ^1H NMR (300 MHz, CDCl_3) δ : 8.80 (s, 4H, Ar-H), 8.75 (dd, $J = 4.8$, 1.5 Hz, 1H, Ar-H), 8.61 (d, $J = 2.1$ Hz, 1H, Ar-H), 7.73–7.69 (m, 1H, Ar-H), 7.58–7.48 (m, 1H, Ar-H), 4.29–4.13 (m, 2H, $1 \times \text{CH}_2$), 1.88–1.65 (m, 2H, $1 \times \text{CH}_2$), 1.59–1.31 (m, 2H, $1 \times \text{CH}_2$), 1.00 (t, $J = 7.2$ Hz, 3H, $1 \times \text{CH}_3$). ^{13}C NMR (75 MHz, CDCl_3) δ : 162.84, 162.65, 150.03, 149.60, 136.41, 131.61, 131.37, 131.05, 127.24, 127.08, 126.86, 126.26, 124.03, 40.87, 30.15, 20.35, 13.81. ESI-MS m/z : 400.18 $[\text{M} + 1]^+$. Anal. calcd. for $\text{C}_{23}\text{H}_{17}\text{N}_3\text{O}_4$: C 69.17%, H 4.29%, N 10.52%; found: C 69.42%, H 4.21%, N 10.47%.

3b, light pink solid, yield: 26.0%. ^1H NMR (300 MHz, CDCl_3) δ : 9.01 (d, $J = 4.8$, 2H, Ar-H), 8.81 (s, 4H, Ar-H), 7.5 (t, $J = 4.8$, 1H, Ar-H), 4.25–4.19 (m, 2H, $1 \times \text{CH}_2$), 1.78–1.70 (m, 2H, $1 \times \text{CH}_2$), 1.51–1.43 (m, 2H, $1 \times \text{CH}_2$), 1.00 (t, $J = 7.2$, 3H, $1 \times \text{CH}_3$). ^{13}C NMR (75 MHz, CDCl_3) δ : 162.71, 162.58, 159.72, 131.33, 131.03, 127.29, 126.34, 121.49, 40.85, 30.15, 20.35, 13.82. ESI-MS m/z : 401.17 $[\text{M} + 1]^+$.

3c, yellow solid, yield: 37.0%. ^1H NMR (300 MHz, CDCl_3) δ : 8.75 (s, 4H, Ar-H), 4.26 (t, $J = 7.5$ Hz, 2H, $1 \times \text{CH}_2$), 4.20 (t, $J = 7.5$ Hz, 2H, $1 \times \text{CH}_2$), 2.43 (t, $J = 7.2$ Hz, 2H, $1 \times \text{CH}_2$), 2.23 (s, 6H, $2 \times \text{CH}_3$), 1.91 (m, 2H, $1 \times \text{CH}_2$), 1.78–1.70 (m, 2H, $1 \times \text{CH}_2$), 1.51–1.40 (m, 2H, $1 \times \text{CH}_2$), 0.99 (t, $J = 7.2$ Hz, 3H, $1 \times \text{CH}_3$). ^{13}C NMR (75 MHz, CDCl_3) δ : 162.86, 130.94, 126.64, 126.62, 57.18, 45.37, 40.76, 39.34, 30.16, 25.96, 20.34, 13.82. ESI-MS m/z : 408.24 $[\text{M} + 1]^+$.

3d, dark yellow solid, yield: 39.0%. ^1H NMR (300 MHz, CDCl_3) δ : 8.73 (d, $J = 7.5$ Hz, 4H, Ar-H), 4.27 (t, $J = 7.2$ Hz, 2H, $1 \times \text{CH}_2$), 4.18 (t, $J = 7.2$ Hz, 2H, $1 \times \text{CH}_2$), 3.51 (t, $J = 4.8$ Hz, 4H, $2 \times \text{CH}_2$), 2.53–2.47 (m, 2H, $1 \times \text{CH}_2$), 2.40 (t, $J = 4.8$ Hz, 4H, $2 \times \text{CH}_2$), 1.98–1.89 (m, 2H, $1 \times \text{CH}_2$), 1.77–1.67 (m, 2H, $1 \times \text{CH}_2$), 1.51–1.38 (m, 2H, $1 \times \text{CH}_2$), 1.00 (t, $J = 7.2$ Hz, 3H, $1 \times \text{CH}_3$). ^{13}C NMR (75 MHz, CDCl_3) δ : 162.92, 162.78, 130.92, 130.87, 126.66, 126.64, 126.58, 66.90, 56.45, 53.54, 40.76, 39.43, 30.14, 24.36, 20.34, 13.82. ESI-MS m/z : 450.26 $[\text{M} + 1]^+$. Anal. calcd. for $\text{C}_{25}\text{H}_{27}\text{N}_3\text{O}_5$: C 66.80%, H 6.05%, N 9.35%; found: C 66.91%, H 6.08%, N 9.21%.

3e, light pink solid, yield: 30.0%. ^1H NMR (300 MHz, CDCl_3) δ : 8.73–8.66 (m, 4H, Ar-H), 4.16 (t, $J = 7.5$ Hz, 2H, $1 \times \text{CH}_2$), 2.97–2.81 (m, 1H, $1 \times \text{CH}$), 1.73–1.67 (m, 2H, $1 \times \text{CH}_2$), 1.48–1.40 (m, 2H, $1 \times \text{CH}_2$), 1.32–1.24 (m, 2H, $1 \times \text{CH}_2$), 0.97

(t , $J = 7.2$ Hz, 3H, $1 \times \text{CH}_3$), 0.91–0.87 (m, 2H, $1 \times \text{CH}_2$). ^{13}C NMR (75 MHz, CDCl_3) δ : 163.91, 162.78, 130.91, 130.75, 126.91, 126.49, 40.75, 30.14, 24.74, 20.34, 13.81, 8.56. ESI-MS m/z : 377.20 $[\text{M} + 1]^+$. Anal. calcd. for $\text{C}_{22}\text{H}_{20}\text{N}_2\text{O}_4$: C 70.20%, H 5.36%, N 7.44%; found: C 70.00%, H 5.45%, N 7.29%.

3f, dark red solid, yield: 53.0%. ^1H NMR (300 MHz, CDCl_3) δ : 8.75 (s, 4H, Ar-H), 4.20 (t, $J = 7.5$ Hz, 2H, $1 \times \text{CH}_2$), 4.10 (d, $J = 7.2$ Hz, 2H, $1 \times \text{CH}_2$), 1.78–1.67 (m, 2H, $1 \times \text{CH}_2$), 1.51–1.42 (m, 2H, $1 \times \text{CH}_2$), 1.37–1.32 (m, 1H, $1 \times \text{CH}$), 0.99 (t, $J = 7.2$ Hz, 3H, $1 \times \text{CH}_3$), 0.53–0.49 (m, 4H, $2 \times \text{CH}_2$). ^{13}C NMR (75 MHz, CDCl_3) δ : 163.10, 162.86, 130.96, 130.93, 126.70, 126.68, 126.61, 45.26, 40.76, 30.15, 20.35, 13.82, 10.19, 4.01. ESI-MS m/z : 365.22 $[\text{M} + 1]^+$.

3g, pink solid, yield: 37.5%. ^1H NMR (300 MHz, CDCl_3) δ : 8.74 (s, 4H, Ar-H), 4.24–4.12 (m, 4H, $2 \times \text{CH}_2$), 1.81–1.67 (m, 4H, $2 \times \text{CH}_2$), 1.53–1.35 (m, 2H, $1 \times \text{CH}_2$), 1.02 (t, $J = 6.6$ Hz, 3H, $1 \times \text{CH}_3$), 0.98 (t, $J = 6.3$ Hz, 3H, $1 \times \text{CH}_3$). ^{13}C NMR (75 MHz, CDCl_3) δ : 162.85, 130.95, 130.93, 126.61, 126.60, 42.42, 40.75, 30.15, 21.37, 20.34, 13.82, 11.49. ESI-MS m/z : 405.22 $[\text{M} + 1]^+$.

3h, dark red solid, yield: 37%. ^1H NMR (300 MHz, CDCl_3) δ : 8.74 (s, 4H, Ar-H), 4.19 (t, $J = 7.5$ Hz, 2H, $1 \times \text{CH}_2$), 4.06 (d, $J = 7.5$ Hz, 2H, $1 \times \text{CH}_2$), 2.28–2.18 (m, 1H, $1 \times \text{CH}$), 1.75–1.65 (m, 2H, $1 \times \text{CH}_2$), 1.51–1.38 (m, 2H, $1 \times \text{CH}_2$), 1.01–0.95 (m, 9H, $3 \times \text{CH}_3$). ^{13}C NMR (75 MHz, CDCl_3) δ : 163.15, 162.85, 131.04, 130.93, 126.61, 126.54, 47.62, 40.75, 30.15, 27.40, 20.34, 20.27, 13.82. ESI-MS m/z : 363.20 $[\text{M} + 1]^+$. Anal. calcd. for $\text{C}_{21}\text{H}_{18}\text{N}_2\text{O}_4$: C 69.60%, H 5.01%, N 7.73%; found: C 69.82%, H 4.96%, N 7.73%.

3i, dark pink solid, yield: 35.0%. ^1H NMR (300 MHz, CDCl_3) δ : 8.76–8.67 (m, 4H, Ar-H), 5.06–4.96 (m, 1H, $1 \times \text{CH}$), 4.19 (t, $J = 7.5$ Hz, 2H, $1 \times \text{CH}_2$), 2.57–2.15 (m, 2H, $1 \times \text{CH}_2$), 1.91 (d, $J = 13.2$ Hz, 2H, $1 \times \text{CH}_2$), 1.77–1.69 (m, 4H, $2 \times \text{CH}_2$), 1.49–1.41 (m, 4H, $2 \times \text{CH}_2$), 1.34–1.24 (m, 2H, $1 \times \text{CH}_2$), 0.98 (t, $J = 7.2$ Hz, 3H, $1 \times \text{CH}_3$). ^{13}C NMR (75 MHz, CDCl_3) δ : 163.22, 162.90, 130.92, 130.82, 127.17, 126.64, 126.53, 126.31, 54.46, 40.73, 30.15, 29.07, 26.47, 25.33, 20.34, 13.82. ESI-MS m/z : 379.20 $[\text{M} + 1]^+$. Anal. calcd. for $\text{C}_{22}\text{H}_{22}\text{N}_2\text{O}_4$: C 69.83%, H 5.86%, N 7.40%; found: C 70.13%, H 5.86%, N 7.40%.

General procedures for the preparation of target compounds 4a–c

The starting material **1** (1.07 g, 4.0 mmol) was dissolved in DMF (10 mL). Subsequently, 3-amino-1-propanol (0.3 g, 4.0 mmol) and various amines (4.0 mmol) were added to the solution while being stirred. The mixture was heated to reflux for 10 h, cooled to room temperature, and filtered. The filtrate was put in ice water to allow precipitation. The resulting cake was washed with water, dried, and then purified by silica gel column chromatography with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (50:1) to obtain the target compounds, namely, **4a–c**.

4a, yellow solid, yield: 33.1%. ^1H NMR (300 MHz, CDCl_3) δ : 8.73 (s, 4H, Ar-H), 4.35 (t, $J = 6.6$ Hz, 2H, $1 \times \text{CH}_2$), 4.24 (t, $J = 7.5$ Hz, 2H, $1 \times \text{CH}_2$), 3.63 (t, $J = 5.7$ Hz, 2H, $1 \times \text{CH}_2$), 2.76 (brs, 1H, –OH), 2.43 (t, $J = 7.2$ Hz, 2H, $1 \times \text{CH}_2$), 2.22 (s, 6H, 2

$\times \text{CH}_3$), 2.04–1.96 (m, 2H, $1 \times \text{CH}_2$), 1.93–1.85 (m, 2H, $1 \times \text{CH}_2$). ^{13}C NMR (75 MHz, CDCl_3) δ : 163.38, 162.70, 131.22, 130.91, 126.81, 126.18, 59.17, 57.12, 45.33, 39.34, 37.58, 30.92, 25.91. ESI-MS m/z : 410.21 $[\text{M} + 1]^+$. Anal. calcd. for $\text{C}_{22}\text{H}_{23}\text{N}_3\text{O}_5 \cdot 0.2 \text{H}_2\text{O}$: C 63.97%, H 5.71%, N 10.17%; found: C 63.61%, H 5.57%, N 9.89%.

4b, pink solid, yield: 22.5%. ^1H NMR (300 MHz, CDCl_3) δ : 8.76 (s, 4H, Ar-H), 4.36 (t, $J = 6.0$ Hz, 2H, $1 \times \text{CH}_2$), 4.05 (d, $J = 6.9$ Hz, 2H, $1 \times \text{CH}_2$), 3.62 (t, $J = 5.7$ Hz, 2H, $1 \times \text{CH}_2$), 2.27–2.18 (m, 1H, $1 \times \text{CH}$), 2.04–1.96 (m, 2H, $1 \times \text{CH}_2$), 1.70 (brs, 1H, –OH), 0.99 (s, 3H, $1 \times \text{CH}_3$), 0.97 (s, 3H, $1 \times \text{CH}_3$). ^{13}C NMR (75 MHz, CDCl_3) δ : 163.48, 163.04, 131.27, 131.07, 126.84, 126.72, 126.20, 59.15, 47.67, 37.51, 30.94, 27.41, 20.26. ESI-MS m/z : 381.38 $[\text{M} + 1]^+$. Anal. calcd. for $\text{C}_{21}\text{H}_{20}\text{N}_2\text{O}_5$: C 66.31%, H 5.30%, N 7.36%; found: C 66.70%, H 5.38%, N 7.34%.

4c, light yellow solid, yield: 38.5%. ^1H NMR (300 MHz, CDCl_3) δ : 8.77 (s, 4H, Ar-H), 4.38 (t, $J = 6.0$ Hz, 2H, $1 \times \text{CH}_2$), 4.32 (t, $J = 7.2$ Hz, 2H, $1 \times \text{CH}_2$), 3.65 (s, 6H, $3 \times \text{CH}_2$), 2.63 (s, 6H, $3 \times \text{CH}_2$), 2.16–1.89 (m, 4H, $2 \times \text{CH}_2$). ^{13}C NMR (75 MHz, CDCl_3) δ : 163.43, 163.35, 162.90, 162.82, 131.28, 130.91, 126.88, 126.66, 126.47, 126.25, 66.91, 59.15, 56.44, 53.53, 39.49, 37.53, 30.90, 24.33. ESI-MS m/z : 452.21 $[\text{M} + 1]^+$. Anal. calcd. for $\text{C}_{24}\text{H}_{25}\text{N}_3\text{O}_6 \cdot 0.25 \text{H}_2\text{O}$: C 63.22%, H 5.64%, N 9.22%; found: C 63.15%, H 5.29%, N 8.94%.

Cells lines and culture

Hep G2 cells, SMMC-7721 cells, MDA-MB 231 cells, MCF-7 cells and HCT-116 cells were cultured in RPMI 1640 medium (Gibco, Life Technologies) supplemented with 10% FBS and 1% penicillin-streptomycin (Solarbio, China) at 37 °C in a humidified atmosphere with 5% CO_2 . All the cell lines were purchased from American Type Culture Collection (MD, USA).

Cell viability assay

Cells (4500 cells per well) were seeded in 96-well plates. After incubation for 24 h, the cells were treated with different concentrations of the compounds. The cells were treated with the compounds for the indicated time. The cells were subsequently cultured in the presence of 1 mg mL^{-1} MTT reagent for 4 h. Supernatants in the plates were discarded and 100 μL DMSO was added to the plates. The OD value was determined using a microtiter plate reader.

Apoptosis detection

For the detection of apoptosis, acridine orange/ethidium bromide (AO/EB) staining and Annexin V/PI staining were performed as previously described.^{9,28} For AO/EB staining, SMMC-7721 and Hep G2 cells were seeded into a 96-well plate and treated with **3c** for 24 h. The cells were washed twice with phosphate buffer saline (PBS) and stained with 5 $\mu\text{g mL}^{-1}$ AO/EB and Hoechst 33342. The fluorescence intensity was detected using high content screening (HCS). For Annexin V/PI staining, cells were grown in 6-well plates and

treated with **3c** for 24 h. The cells were collected, washed twice with PBS, stained with Annexin V/PI, and examined for apoptosis by flow cytometry (BD Biosciences, USA).

Detection of ROS levels in cells

Hep G2 and SMMC-7721 cells (12 000 cells per well) were cultured in a 96-well plate and were exposed to **3c** for 24 h. After treatment, the cells were washed twice with serum-free medium and stained with 10 μ M DCFH-DA for 15 min at 37 °C. The cells' fluorescence intensity was detected by HCS (ThermoFisher, USA).

Western blot

Cells were collected after treatment, washed with PBS twice and mixed with a lysis buffer. The protein in the mixture was quantified using the BCA Protein Quantification kit (Beyotime Biotechnology), and the protein concentration in the samples was determined. The protein expression of each sample was detected as previously described. Primary antibodies against the following proteins were used: β -actin, p53, caspase 8, PARP-1, Bcl-2, p62, LC3, BECN1, phospho-Akt (S473) and β -catenin.

Migration assay

The effect of compound **3c** on cell migration was measured by the scratch wound healing assay and transwell migration assay according to a previous study.²⁹ In the scratch wound healing assay, SMMC-7721 and Hep G2 cells were plated in a 24-well plate. After the cells adhered to the plate, a 10 μ L tip was used to draw a straight line across the cell monolayer, creating a gap, and the cells were subsequently treated with **3c** for 24 h. Photographs of the migrated cell in the gap were taken using an inverted microscope, and the migration rate of the cells was calculated. In the transwell migration assay, 1×10^5 cells in 100 μ L serum-free medium were seeded on the upper chamber, and 600 μ L RPMI 1640 containing various concentrations of **3c** with 10% FBS was added to the lower chamber. After incubation, the transwell membrane was fixed with 4% paraformaldehyde for 20 min. The cells on the upper side of the membrane were gently removed, and the migrated cells on the lower side were stained with 0.2% crystal violet. Photographs were taken and the migrated cells were counted.

MDC staining

Hep G2 and SMMC-7721 cells were cultured in a 96-well plate and treated with **3c** at different concentrations for 24 h. Afterwards, the cells were washed twice with PBS and stained with 50 μ M MDC for 30 min at 37 °C. The cells' fluorescence intensity was detected by HCS.

Transmission electron microscopy assay

SMMC-7721 cells were plated in a 10 cm diameter cell culture dish and treated with **3c** for 12 h. The cells were then fixed

with 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4 °C, and washed 3 times with cacodylate buffer. The cells were further fixed with 1% osmium tetroxide dissolved in 0.1 M cacodylate buffer for 1 h at room temperature. Next, 70 nm sections were cut using a Reichert Ultracut S ultramicrotome (Leica, Heidelberg, Germany). The sections were subsequently stained with 4% uranyl acetate for 10 min and Reynold's lead citrate for 1.5 min. The sections were imaged at 80 kV using a JEOL 1200 EX transmission electron microscope (Leica, Heidelberg, Germany).

Data analysis

All the data are presented as the mean $\pm$ SD and analyzed using Student's *t*-test or analysis of variance (ANOVA) followed by a *q*-test. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 were considered to be statistically significant.

Conflicts of interest

The authors declare no competing interest.

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