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Novel naphthalimide–amino acid conjugates with flexible leucine moiety as side chain: Design, synthesis and potential antitumor activity

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ABSTRACT

A series of novel naphthalimide derivatives with flexible leucine side chains were designed and synthesized. Their antitumor activities were evaluated against HeLa, A549, P388, HL-60, MCF-7, HCT-8 and A375 cancer cell lines in vitro. The preliminary results showed that most of the derivatives had moderate antitumor activities with the IC_{50} values of 10^{-6}–10^{-5} M. More importantly, compounds **8a–c** exhibited exclusive antitumor activities against MCF-7 cell line. The interaction between compound **8b** and BSA was also investigated. DNA binding experiments showed that these derivatives behaved as DNA intercalating agents. This work provided a novel class of naphthalimide-based lead compounds with exclusive antitumor activities against MCF-7 cell line for further optimization.

1. Introduction

The design of highly efficient antitumor agents is of significant importance in organic and medicinal chemistry. Naphthalimide-based anticancer drugs constitute an indispensable part in the development of anticancer drugs. Since the first lead compound, Amonafide (Fig. 1), was discovered by Braña et al.,[1,2] plenty of naphthalimide derivatives were synthesized and their antitumor activities were evaluated upon a variety of murine and human tumor cell lines.[3–7] However, most of them were abandoned because of poor therapeutic index and unpredictable toxicity.[5,8–10] From then on, much attention was paid to the modification of naphthalimide skeleton. The modified derivatives were characterized by fused multi-aromatic ring system and one or two flexible basic side chains.[6,7,11–15] Although the significant results have been obtained in this way, there were some problems on the poor solubility and complicated synthesis of the derivatives. It was necessary for us to attempt alternative structural modification of naphthalimide.

In recent years, various drug–amino acid conjugates were reported, such as anthraquinone (Fig. 1),[16,17] doxorubicin,[18,19] metal complex,[20–25] nitrosourea/nitrogen mustard,[26] vinblastin,[27] hydroxymethylacylfulvene,[28] benzothiazole,[29] imidazotetrazines,[30] methionine-enkephalin,[31] camptothecins,[32] bile acid,[33] and 9-hydroxyellipticinium (Fig. 1)[34]. The promising results were obtained in this way. It was well known that amino acids were essential starting materials for cell growth and building blocks for protein synthesis. Furthermore, amino acids could also improve the cell uptake of antitumor agents.[25] Therefore, a possible strategy for increasing the transport of lipophilic compounds of biological interest across cell membrane, was the conjugation with amino acids.[34] This inspired us to assume that naphthalimide conjugated with flexible leucine moiety as side chain might have some improved or different biological activity.

As shown in Figure 2, the naphthalimide scaffold was utilized as key prototype structural unit, and the leucine, arylamine and aliphatic amine functional groups were conjugated to the naphthalene ring. The amino substituents introduced to the position-6 of naphthalimide ring, contrary to Amonafide, would be difficult to be acetylated[35] and the side effects might be reduced. The introduction of arylamine to leucine moiety might result in favorable solubility and pharmacologic profiles.[36,37] Furthermore, the introduction of thio group, known to facilitate the interaction of naphthyl heterocycles with DNA,[38] might lead to a concomitant increase in the cytotoxicity against tumor cell lines. Therefore, the 6-amino substituted benzo[*de*]isochromene-1,3-dione-leucine conjugates **7** and **8** were prepared and their antitumor activities were evaluated against a variety of cancer cell lines. The preliminary results showed that most of the compounds had moderate antitumor activities with the IC_{50} values of 10^{-6}–10^{-5} M. More importantly, compounds **8a–c** exhibited the exclusive antitumor activities against MCF-7 cell line. As all knew that serum albumin, the most abundant protein in plasma, functioned in the binding and transportation of various ligands such as fatty acids, hormones, and drugs.[39] The distribution, free concentration, and metabolism of these ligands strongly depended on their binding properties with serum albumin.[40,41] Therefore, the interaction between compound **8b** and bovine serum albumin (BSA) has been

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investigated in order to understand its unique antitumor property. Their spectra and calf thymus DNA (ct-DNA) binding properties were also studied.

2. Results and discussion

2.1. Synthesis and spectra data

The synthetic routes of the designed compounds *N*-(substituted aryl)-2-(6-(2-(dimethylamino) ethylamino)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-4-methylpentanamide **7a–c** and *N*-(substituted aryl)-2-(1,3-dioxo-6-(2-(phenylthio)ethylamino)-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-4-methylpentanamide **8a–c** were shown in Scheme 1. 6-Nitrobenzo[*de*]isochromene-1,3-dione **1**[42] was treated with L-leucine methyl ester hydrochloride in EtOH to afford intermediate **2**. The following nucleophilic substitution reaction between 4-nitronaphthalimide and various aliphatic amines led to the substrates **3** and **4**, which were subjected to hydrolysis to obtain the key intermediates **5** and **6**, respectively. The subsequent condensation reaction of **5** and **6** with various arylamines afforded the target compounds **7a–c** and **8a–c** with moderate yields of 55–65%, respectively. The structures of all the newly synthesized compounds were well identified by ^{1}H NMR, ^{13}C NMR, HRMS, EI and IR spectra.

The UV–vis and fluorescent data of the target compounds were measured and shown in Table 1. It was found that the compounds with different substituents had slight difference on absorption but significant effects on emission. The absorption and emission maxima of these compounds were around 436 nm and 525 nm, respectively, while the fluorescence quantum yields of these compounds were dramatically changed with different substituents. This phenomenon could be well explained by photo-induced electron transfer (PET) effects.[43]

2.2. Cytotoxic evaluation in vitro

The in vitro antitumor activities of the target compounds were evaluated by examining their cytotoxic effects using sulforhodamine B (SRB) assay[45] against A549 (human lung cancer cell line) and MTT tetrazolium dye assay[46] against HeLa (human cervical carcinoma cell line), P388 (murine leukemia cell line), HL-60 (Human promyelocytic leukemia cell line), MCF-7 (Human caucasian breast adenocarcinoma cell line), HCT-8 (human ileocecal adenocarcinoma cell line) and A375 (human melanoma cell line), respectively. The IC_{50} represented the drug concentration (μM) required to inhibit cell growth by 50% and the results were summarized in Table 2.

From Table 2, it could be seen that the conjugates showed moderate cytotoxicities against tested cancer cell lines except for HCT-8. Compounds **7a**, **7b** and **7c** demonstrated the highest cytotoxicities against MCF-7, A375 and A375 cell lines with IC_{50} values of 8.64, 6.51 and 6.60 μM, respectively. More importantly, compounds **8a–c** showed the exclusive cytotoxicities against MCF-7 with IC_{50} values of 24.55, 9.45 and 12.56 μM, respectively. Compounds **8a–c** with phenylthioethylamino substituent instead of dimethylaminoethylamino substituent at the position-6 of naphthalimide ring, showed a striking contrast compared to **7a–c**, indicating that the position-6 of naphthalimide was a crucial active site. The introduction of appropriate substituents to the position-6 of naphthalimide could obviously improve the antitumor selectivity. Moreover, the cytotoxicities of compounds **8a–c** were weaker than those of **7a–c** against cancer cell lines. The reason was possibly that the nitrogen atoms at amino side chain of **7a–c** could be protonated to different extent under physiological pH, and form hydrogen bond with DNA bases, which might contribute to their cytotoxicities.[47–49] Additionally, we found that the antitumor activities of the target compounds were influenced by the arylamino substituents on the leucine moiety. When the substituents were *o*-dianisidine and sulfanilamide groups, relatively favorable antitumor activities were obtained. It was well known that antitumor activity of one compound was determined by many factors including cell membrane crossing ability, protein binding and transportation, DNA binding ability, drug metabolism etc., once these factors were effectively balanced, higher antitumor potency was possible.[13] Therefore, manipulation of naphthalimide–leucine conjugates to achieve a appreciable properties for potent antitumor drugs, seemed to be closely dependent on structures and conformation of the side chain substituents.

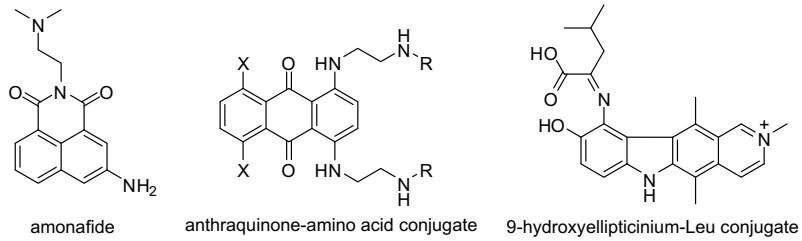


Figure 1. The structures of some representative antitumor compounds.

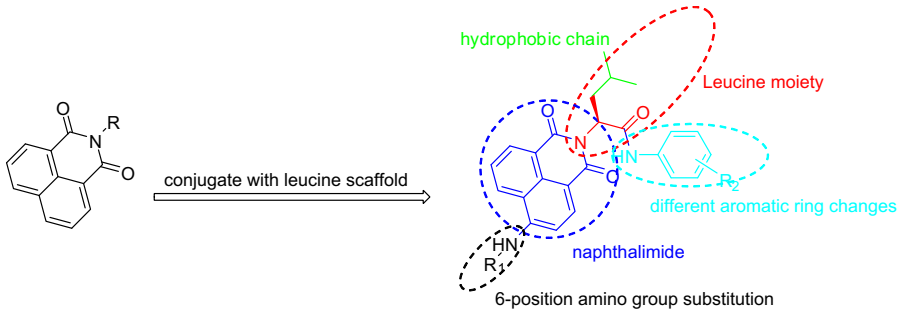
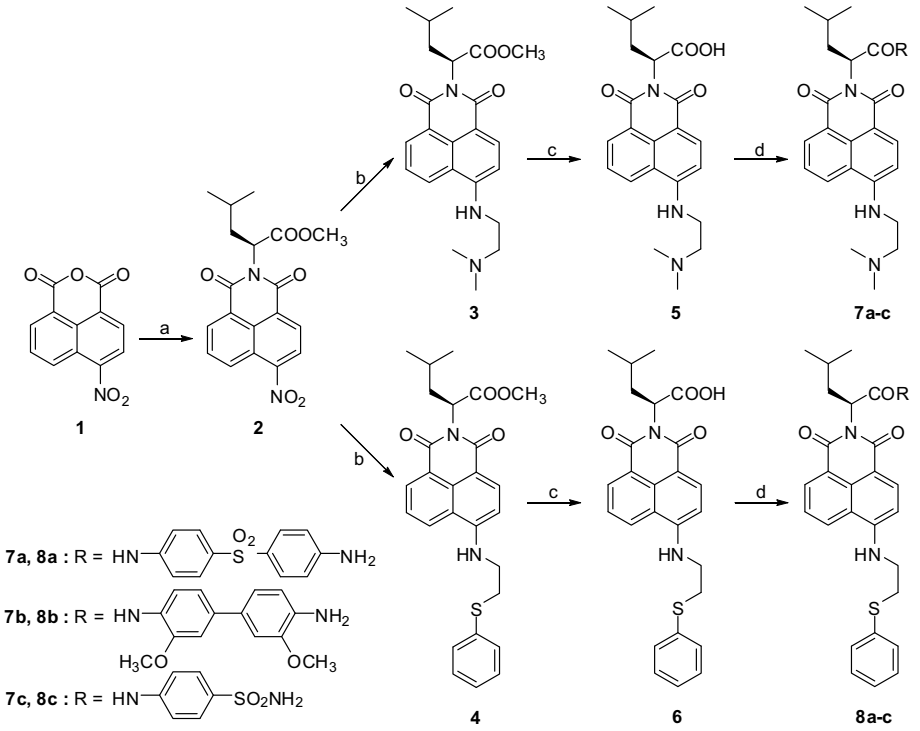


Figure 2. The design strategy of naphthalimide–leucine conjugates.



Scheme 1. Reagents and conditions: (a) L-leucine methyl ester hydrochloride, EtOH, Et_3N, reflux 1 h, 90% yield; (b) corresponding amine, DMF, rt, 24 h, 60% yield; (c) $LiOH{\cdot}H_2O$, THF–MeOH–H_2O, 50 °C overnight, 96% yield; (d) corresponding arylamine, $CHCl_3$, EDCI, DMAP, rt, 48 h, 55–65% yield.

Table 1
Absorption and emission data[a,b] of naphthalimide–leucine conjugates

Compound	UV λ_{max} (log ε)	FL λ_{max} (Φ)
7a	436 (4.31)	527 (0.0151)
7b	437 (4.31)	525 (0.0073)
7c	436 (4.28)	524 (0.0126)
8a	436 (4.35)	530 (0.2820)
8b	440 (4.33)	529 (0.0230)
8c	434 (3.87)	533 (0.2600)

[a] In absolute ethanol.
[b] With quinine sulfate in 0.1 mol/L sulfuric acid as quantum yield standard (Φ = 0.58).[44]

Table 2
Cytotoxicity of naphthalimide–leucine conjugates against HeLa, A549, P388, HL-60, MCF-7, HCT-8 and A375 cell lines

	Cytotoxicity (IC_{50} μM)						
	HeLa[a]	A549[b]	P388[a]	HL-60[a]	MCF-7[a]	HCT-8[a]	A375[a]
7a	>50	17.20	>50	>50	8.64	>50	>50
7b	6.74	25.08	7.67	22.79	15.02	>50	6.51
7c	8.09	>50	7.23	17.83	8.65	>50	6.60
8a	>50	>50	>50	>50	24.55	>50	>50
8b	>50	>50	>50	>50	9.45	>50	>50
8c	>50	>50	>50	>50	12.56	>50	>50

[a] Cytotoxicity (CTX) against cancer cell line was measured by microculture tetrazolium–formazan method after 48 h.[46]
[b] CTX against cancer cell line was measured by sulforhodamine B dye-staining method after 72 h.[45]

2.3. DNA binding properties

The affinities of the target compounds for ct-DNA were determined by spectroscopic technique[50–53] and viscosity measurement.[11,49] The fluorescence, UV–vis spectra and viscosity measurement of representative compound **7c** were illustrated in Figure 3A, B and Figure 4, respectively.

It was obvious that both absorption and emission of compound **7c** exhibited dramatic changes with increasing ct-DNA concentration (Fig. 3). From the analyses of the relationship between the fluorescence intensities and ct-DNA concentrations by nonlinear curve fitting methods,[50–53] these significant fluorescence changes made the association steady constants of **7c** with ct-DNA available with $4.92 \times 10^4\ M^{-1}$; Also, absorption spectra demonstrated slight bathochromic shifts (1–3 nm) and strong hypochromicities (12.8–57.6%). Furthermore, the increase in viscosity of ct-DNA solution was observed versus the increase in concentration of **7c** (Fig. 4), indicating that the lengthening, unwinding and stiffening of the helix which increased the viscosity of the solution, resulted from the intercalation of compound **7c** into ct-DNA.[11,49] These collectively demonstrated that the derivatives behaved as DNA intercalating agents. Derivatives that strongly binding ct-DNA, were high cytotoxic agents.[49] (data not listed). The roles of leucine moiety in the DNA binding were well under way.

2.4. BSA binding properties

The interaction between compounds **8a–c** and BSA have been investigated in order to understand their unique antitumor properties. The UV–vis and fluorescence spectra of representative compound **8b** were illustrated in Figure 5A and B.

As shown in Figure 5, slight increase in absorption and significant increase in emission occurred with increasing BSA concentration. The variations of absorbance and emission intensity were 14.9% and 676%, respectively. The dramatic increase of fluorescence intensity showed that the albumin proteins introduced motional restriction on the drug molecule in the proteinous environments[54] and such a 'rigidification' decreased the vibrational–rotational processes that coupled the excited and ground states, thereby decreasing the radiationless decay rate and increasing

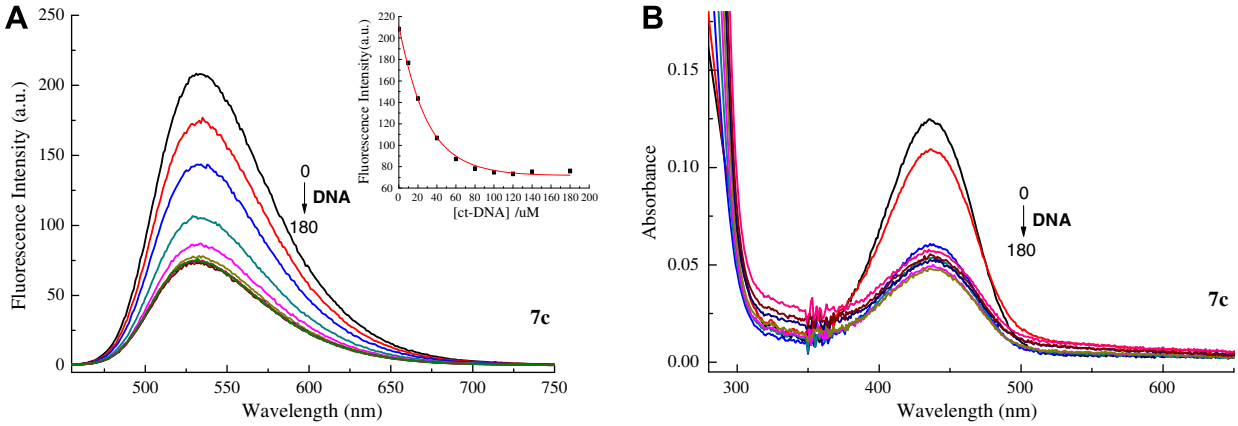


Figure 3. Titration curves of compound **7c** (10 μM) in 20 mM Tris–HCl buffer (pH 7.5, 25 °C) containing different concentrations of ct-DNA from 0 to 180 μM. (A) Fluorescence emission spectra. Inset: fluorescence intensity as a function of ct-DNA concentration. (B) UV–vis absorption spectra.

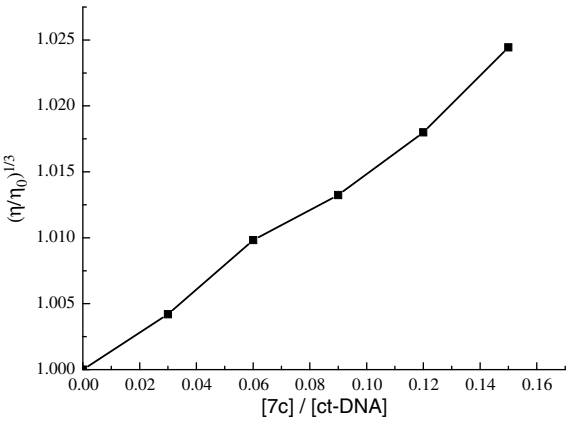


Figure 4. Effects of increasing amount of compound **7**c on the relative viscosities of ct-DNA at 25 (± 0.1) °C. [ct-DNA] = 100 μM in 20 mM Tris–HCl buffer (pH 7.5). η and η_0 represented the viscosity of ct-DNA in the presence and absence of compound **7c**, respectively.

the fluorescence quantum yield of the protein–bound molecule.[55,56] Also, the slight increase of absorbance proved the moderate interaction between naphthalimide ring and BSA. Therefore, these spectral changes were caused by compound–BSA complex formation, in which compound **8b** was strongly bound at the hydrophobic positions of the BSA supported by the hydrophobic interaction.[57] It was obvious that hydrophobic substituents of compounds **8a–c** proved to be a key structural unit for their binding abilities to BSA, which might provide some suggestions on the understanding of their exclusive antitumor selectivity against MCF-7 cell line. Detailed interaction mode, mechanism and interaction–antitumor activity relationships were still under way.

3. Conclusion

In summary, a series of novel naphthalimide derivatives with flexible leucine side chains were designed and synthesized, and their antitumor activities were evaluated against a variety of cancer cell lines in vitro. The preliminary results showed that most of the derivatives had moderate antitumor activities. More importantly, compounds **8a–c** exhibited the exclusive antitumor activities against MCF-7 cell line. The interaction between compound **8b** and BSA might provide some suggestions on the understanding of their exclusive antitumor selectivity against MCF-7 cell line. DNA binding studies showed that these derivatives behaved as DNA intercalating agents. The present work demonstrated that assembling the biological active unit of naphthalimide and leucine might be able to result in a novel class of lead compounds with potential antitumor activities. This was the first report about the naphthalimide–leucine conjugates and their exclusive antitumor activities against MCF-7 cell line as we have known. Further structural optimization and structure–antitumor activity relationships

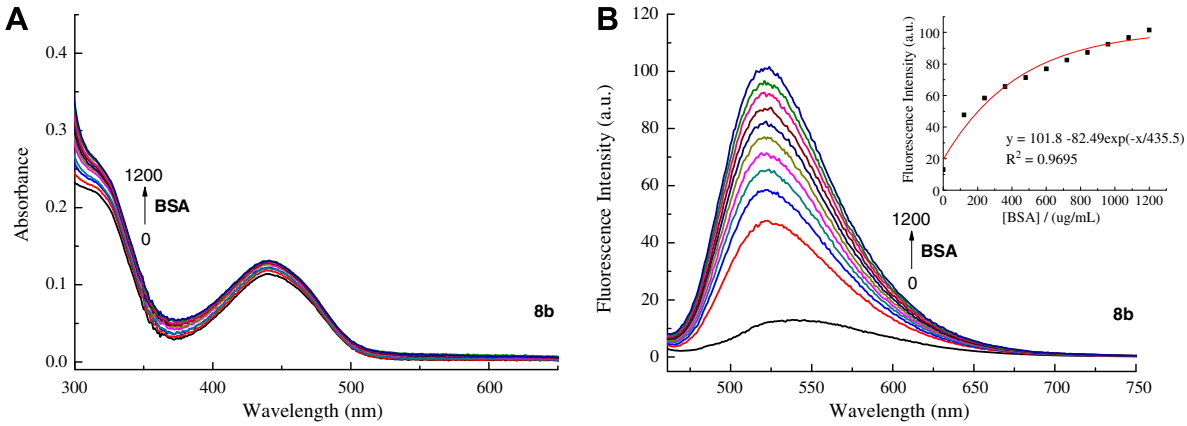


Figure 5. Titration curves of compound **8b** (10 μM) in 20 mM HEPES buffer (pH 7.2, 25 °C) containing different concentrations of BSA from 0 to 1200 μg/mL. (A) UV–vis absorption spectra, (B) fluorescence emission spectra. Inset: fluorescence intensity as a function of BSA concentration.

about the designed naphthalimide–leucine conjugates were under the way.

4. Experimental

4.1. Materials and methods

All reagents were of the commercial quality and were used without purification. ^{1}H and ^{13}C NMR were obtained with a Bruker AV-400 spectrometer with chemical shifts reported as ppm (in $CDCl_3$/DMSO-d_6, TMS as internal standard). IR spectra were obtained using a Perkin–Elmer 2000 FTIR instrument. High-resolution mass spectra (HRMS) were obtained on a HPLC-Q-Tof MS (Micro) spectrometer. Melting points were determined by an X-6 micro-melting point apparatus and uncorrected. Absorption spectra were determined on PGENERAL TU-1901UV–VIS spectrophotometer. Fluorescence spectroscopic studies were performed with a Hitachi F-4500. Column chromatography was performed using silica gel 200–300 mesh.

4.2. DNA binding experiments

4.2.1. Preparation of ct-DNA solution

Ct-DNA (highly polymerized) was purchased from Sigma Aldrich. Solutions of ct-DNA in 10 mM Tris–HCl, 1 mM EDTA buffer (pH 7.5) gave a ratio of UV absorbance at 260 nm and 280 nm of 1.8–1.9:1, indicating that the ct-DNA was sufficiently free of protein. The concentration of ct-DNA was determined spectrophotometrically assuming the molar absorption was 6600 M^{-1} cm^{-1} (260 nm).[58] The solution was stored at 4 °C and used after no more than four days. Doubly distilled water was used to prepare the buffer solution.

4.2.2. DNA titration experiments

The concentrations of compounds and ct-DNA were 10 μM and 0–180 μM, respectively, in 20 mM Tris–HCl, 50 mM NaCl buffer (pH 7.5) and DMSO (1% by volume). The solution in a final volume was 10 mL, which was used for spectrophotometric titration experiments. An equilibrium period of 6 h for constant oscillation at 25 °C in the dark of the mixed solution was allowed before recording each spectrum. The association constants (K_a) were derived according to the equation $I = I_0 + \{(I_\infty - I_0)/2[Q]_0\}$. $\{([DNA]_0 + [Q]_0 + 1/K_a) - \{([DNA]_0 + [Q]_0 + 1/K_a)^2 - 4[DNA]_0[Q]_0\}^{1/2}\}$, wherein I_0, I, and I_∞ represented the fluorescence intensities of compounds alone, the sample, and DNA totally bound, respectively. $[DNA]_0$ and $[Q]_0$ were the initial analytical concentrations of ct-DNA and the agents, respectively.[50]

4.2.3. Viscosity measurements

Viscosity measurements were carried out using an Ubbelodhe viscometer at 25 (±0.1) °C in thermostated bath. Flow times were measured with a digital stopwatch and each sample was measured three times and an average flow time was calculated. Data were presented as $(\eta/\eta_0)^{1/3}$ versus the ratio of the concentration of compound to that of ct-DNA, where η and η_0 represented the viscosity of ct-DNA solution in the presence and absence of compound **7c**, respectively.

4.3. BSA binding experiments

BSA (lyophilized powder) was purchased from Sigma Aldrich. The stock solution was prepared at concentration of 40 mg/mL in 20 mM HEPES (*N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulphonic acid]) buffer (pH 7.2, 25 °C) and stored at 4 °C and used after no more than four days for equilibrium. The concentrations of compounds and BSA were 10 μM and 0–1200 μg/mL, respectively, in 20 mM HEPES buffer (pH 7.2) and DMSO (1% by volume). The solution in a final volume was 10 mL, which was used for spectrophotometric titration experiments. An equilibrium period of 15 min for constant oscillation at 25 °C in the dark of the mixed solution was allowed before recording each spectrum.

4.4. Synthesis

4.4.1. Methyl 4-methyl-2-(6-nitro-1,3-dioxo-1*H*-benzo[*de*] isoquinolin-2(3*H*)-yl)pentanoate (2)

Compound **1** (300 mg, 1.234 mmol), prepared by using the previously reported method,[42] was dissolved in EtOH (5 mL), then L-leucine methyl ester hydrochloride (269 mg, 1.480 mmol) and Et_3N (150 mg, 1.480 mmol) were added. The solution was stirred and refluxed under nitrogen for 60 min, and then cooled and concentrated under vacuum. The residue was subjected to column chromatography on silica gel by using CH_2Cl_2/CH_3OH 60:1 (v/v) as eluent, affording **2** (411 mg, 1.111 mmol, 90%) as orange-yellow solid. mp 105.2–107.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.863 (d, J = 8.8 Hz, 1H), 8.753 (d, J = 7.2 Hz, 1H), 8.707 (d, J = 8.0 Hz, 1H), 8.420 (d, J = 8.0 Hz, 1H), 8.012 (t, J = 8.0 Hz, 1H), 5.774 (q, J_1 = 4.0 Hz, J_2 = 8.8 Hz, 1H), 3.732 (s, 3H), 2.282–2.214 (m, 1H), 2.150–2.082 (m, 1H), 1.584 (s, br, 1H), 1.014 (d, J = 6.0 Hz, 3H), 0.933 (d, J = 6.0 Hz, 3H); MS (EI+) calcd for $C_{19}H_{18}N_2O_6$ [M^+] 370.4, found 370.1.

4.4.2. General procedure for the preparation of methyl 2-(6-(2-substituted amino)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-4-methylpentanoate

Compound **2** (200 mg, 0.540 mmol) was dissolved in DMF (2 mL), the corresponding amine (5.400 mmol) was added. The solution was stirred at room temperature under nitrogen for 24 h, and then concentrated under vacuum. The residue was subjected to column chromatography on silica gel by using CH_2Cl_2/CH_3OH 40:1 (v/v) as eluent, affording the corresponding products as orange solid.

4.4.2.1. Methyl 2-(6-(2-(dimethylamino)ethylamino)-1,3-dioxo-1*H*-benzo[de]isoquinolin-2(3H)-yl)-4-methylpentanoate (3).

Yield: 60%. mp 57.5–59.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.576 (d, J = 7.2 Hz, 1H), 8.461 (d, J = 8.4 Hz, 1H), 8.179 (d, J = 8.4 Hz, 1H), 7.633 (t, J = 8.4 Hz, 1H), 6.678 (d, J = 8.4 Hz, 1H), 6.372 (s, br, 1H), 5.798 (q, J_1 = 4.8 Hz, J_2 = 9.2 Hz, 1H), 3.703 (s, 3H), 3.396 (q, J_1 = 4.8 Hz, J_2 = 11.2 Hz, 2H), 2.750 (t, J = 6.0 Hz, 2H), 2.350 (s, 6H), 2.258–2.187 (m, 1H), 2.142–2.071 (m, 1H), 1.633–1.532 (m, 1H), 1.011 (d, J = 6.4 Hz, 3H), 0.916 (d, J = 6.8 Hz, 3H); MS (EI+) calcd for $C_{23}H_{29}N_3O_4$ [M^+] 411.5, found 412.2.

4.4.2.2. Methyl 2-(1,3-dioxo-6-(2-(phenylthio)ethylamino)-1*H*-benzo[de]isoquinolin-2(3H)-yl)-4-methylpentanoate (4).

Yield: 60%. mp 67.6–69.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.689 (s, 1H), 8.566 (s, 1H), 8.131 (s, 1H), 7.741 (s, 1H), 7.652 (s, 2H), 7.519–7.471 (m, 4H), 6.775 (s, 1H), 5.999 (s, 1H), 3.920 (s, 3H), 3.807 (s, 2H), 3.532 (s, 2H), 2.394–2.313 (m, 2H), 1.781 (s, 1H), 1.199 (s, 3H), 1.105 (s, 3H); MS (EI+) calcd for $C_{27}H_{28}N_2O_4S$ [M^+] 476.6, found 476.2.

4.4.3. General procedure for the preparation of methyl 2-(6-(2-substituted amino)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-4-methylpentanoic acid

Compound **3/4** (0.540 mmol) was dissolved in THF/MeOH/H_2O 4:1:1 (v/v/v), then $LiOH{\cdot}H_2O$ (45 mg, 1.080 mmol) was added. The solution was heated at 50 °C overnight and concentrated to half of the volume, neutralized with 1 M HCl. The precipitate was collected by filtration and dried in vacuum to give the desired product (0.518 mmol, 96%) as orange solid.

4.4.3.1. 2-(6-(2-(Dimethylamino)ethylamino)-1,3-dioxo-1*H*-benzo[de]isoquinolin-2(3H)-yl)-4-methylpentanoic acid (5). Yield: 96%. mp 205.5–207.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.738 (d, J = 8.4 Hz, 1H), 8.442 (d, J = 7.2 Hz, 1H), 8.278 (d, J = 8.4 Hz, 1H), 7.861 (s, br, 1H), 7.703 (t, J = 8.0 Hz, 1H), 6.859 (d, J = 8.8 Hz, 1H), 5.547 (d, J = 6.8 Hz, 1H), 3.623 (d, J = 5.2 Hz, 2H), 2.957 (s, br, 2H), 2.491 (s, 6H), 2.010 (t, J = 6.8 Hz, 2H), 1.449–1.384 (m, 1H), 0.903 (d, J = 6.4 Hz, 3H), 0.819 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.27, 164.05, 163.17, 150.99, 135.06, 131.61, 129.95, 129.53, 125.00, 122.02, 120.70, 108.12, 104.70, 56.25, 51.41, 44.45, 38.18, 25.46, 23.53, 22.43; MS (EI+) calcd for $C_{22}H_{27}N_3O_4$ [M^+] 397.5, found 398.2.

4.4.3.2. 2-(1,3-Dioxo-6-(2-(phenylthio)ethylamino)-1*H*-benzo[de]isoquinolin-2(3H)-4-methylpentanoic acid (6). Yield: 96%. mp 206.0–208.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.542 (s, br, 1H), 8.650 (d, J = 8.4 Hz, 1H), 8.443 (d, J = 7.2 Hz, 1H), 8.235 (d, J = 8.8 Hz, 1H), 7.989 (t, J = 5.2 Hz, 1H), 7.705 (t, J = 8.0 Hz, 1H), 7.408 (d, J = 8.0 Hz, 2H), 7.317 (t, J = 7.6 Hz, 2H), 7.193 (t, J = 7.2 Hz, 1H), 6.759 (d, J = 8.8 Hz, 1H), 5.552 (t, J = 6.8 Hz, 1H), 3.636 (q, J = 6.4 Hz, 2H), 3.332 (m, 2H), 2.004 (t, J = 7.2 Hz, 2H), 1.451–1.384 (m, 1H), 0.903 (d, J = 6.8 Hz, 3H), 0.819 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.13, 164.02, 163.13, 150.87, 135.93, 135.06, 131.62, 130.00, 129.59, 129.30, 129.12, 126.48, 125.01, 122.01, 120.60, 108.02, 104.57, 51.29, 42.71, 31.38, 25.42, 23.52, 22.42; MS (EI+) calcd for $C_{26}H_{26}N_2O_4S$ [M^+] 462.6, found 463.1.

4.4.4. General procedure for the preparation of *N*-(substituted aryl)-2-(6-(2-(dimethylamino)ethylamino)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-4-methylpentanamide (7a–c)

To a solution of compound **5** (50 mg, 0.126 mmol) in $CHCl_3$ (2 mL), the corresponding substituted arylamine (0.126 mmol), EDCI (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride) (48 mg, 0.252 mmol) and DMAP (4-dimethylaminopyridine) (31 mg, 0.252 mmol) were added. The solution was stirred at room temperature under nitrogen for 48 h and concentrated. The residue was purified on silica gel column chromatography by using CH_2Cl_2/CH_3OH 30:1 (v/v) as the eluent, affording **7a–c** as orange/khaki solid. Yield 55–65%.

4.4.4.1. *N*-(4-(4-Aminophenylsulfonyl)phenyl)-2-(6-(2-(dimethylamino)ethylamino)-1,3-dioxo-1*H*-benzo[de]isoquinolin-2(3H)-yl)-4-methylpentanamide (7a). Yield: 65%. Viscous orange solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.837 (s, 1H), 8.667 (d, J = 8.4 Hz, 1H), 8.429 (d, J = 7.2 Hz, 1H), 8.249 (d, J = 8.8 Hz, 1H), 7.942 (s, 1H), 7.721–7.655 (m, 5H), 7.471 (d, J = 8.4 Hz, 2H), 6.803 (d, J = 8.8 Hz, 1H), 6.577 (d, J = 8.8 Hz, 2H), 6.093 (s, 2H), 5.595 (q, J_1 = 5.2 Hz, J_2 = 8.8 Hz, 1H), 3.480 (dd, J_1 = 6.0 Hz, J_2 = 12 Hz, 2H), 2.575 (t, J = 6.8 Hz, 2H), 2.220 (s, 6H), 2.185–2.151 (m, 1H), 1.887–1.817 (m, 1H), 1.465–1.398 (m, 1H), 0.914 (d, J = 6.4 Hz, 3H), 0.824 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.58, 164.48, 163.43, 153.82, 151.08, 143.37, 137.38, 134.97, 131.37, 130.27, 129.57, 128.99, 127.80, 126.54, 124.81, 122.74, 120.57, 120.45, 113.39, 108.34, 104.32, 57.36, 52.79, 45.79, 41.44, 38.02, 25.29, 23.60, 22.63; FTIR (KBr, cm^{-1}): 3355, 2948, 2859, 1680, 1647, 1580, 1535, 1450, 1357, 1290, 1242, 1142, 1101, 830, 771, 682, 548; HRMS (ES+) calcd for $C_{34}H_{37}N_5O_5SNa$ ([M+Na])$^+$ 650.2413, found 650.2392.

4.4.4.2. *N*-(4′-Amino-3,3′-dimethoxybiphenyl-4-yl)-2-(6-(2-(dimethylamino)ethylamino)-1,3-dioxo-1*H*-benzo[de]isoquinolin-2(3H)-yl)-4-methylpentanamide (7b). Yield: 55%. Viscous khaki solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.547 (d, J = 7.2 Hz, 1H), 8.434–8.402 (m, 2H), 8.314 (s, 1H), 8.231 (d, J = 8.4 Hz, 1H), 8.023 (s, 1H), 7.593 (t, J = 8.0 Hz, 1H), 7.113 (dd, J_1 = 1.6 Hz, J_2 = 8.4 Hz, 1H), 6.995–6.974 (m, 3H), 6.748 (d, J = 8.4 Hz, 1H), 6.624 (d, J = 8.4 Hz, 1H), 6.491 (s, 1H), 5.952 (dd, J_1 = 6.0 Hz, J_2 = 8.8 Hz, 1H), 3.911 (s, 3H), 3.791 (s, 3H), 3.394 (dd, J_1 = 4.8 Hz, J_2 = 10.4 Hz, 2H), 2.764 (t, J = 6.0 Hz, 2H), 2.361 (s, 6H), 2.322–2.215 (m, 2H), 1.687–1.620 (m, 1H), 1.053 (d, J = 6.8 Hz, 3H), 0.989 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.33, 164.72, 164.04, 149.99, 148.25, 147.48, 137.23, 135.54, 135.09, 131.79, 131.63, 130.00, 127.03, 126.33, 124.67, 122.57, 120.33, 120.06, 119.57, 119.19, 115.03, 109.49, 109.30, 108.45, 104.40, 67.96, 56.84, 55.93, 55.60, 54.06, 44.95, 40.17, 37.84, 25.79, 23.23, 22.33; FTIR (KBr, cm^{-1}): 3355, 2955, 2859, 1680, 1647, 1576, 1535, 1509, 1450, 1357, 1238, 1112, 1031, 774; HRMS (ES+) calcd for $C_{36}H_{41}N_5O_5Na$ ([M+Na])$^+$ 646.3005, found 646.3005.

4.4.4.3. 2-(6-(2-(Dimethylamino)ethylamino)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3H)-yl)-4-methyl-*N*-(4-sulfamoylphenyl)pentanamide (7c). Yield: 60%. Orange red solid: mp 136.6–138.6 °C.^{1}H NMR (400 MHz, DMSO-d_6): δ 9.835 (s, 1H), 8.701 (d, J = 8.4 Hz, 1H), 8.468 (d, J = 7.6 Hz, 1H), 8.287 (d, J = 8.4 Hz, 1H), 7.786 (s, br, 1H), 7.751–7.690 (m, 5H), 7.202 (s, 2H), 6.879 (d, J = 8.8 Hz, 1H), 5.631 (dd, J_1 = 4.8 Hz, J_2 = 9.2 Hz, 1H), 3.659 (d, J = 4.8 Hz, 2H), 3.062 (s, br, 2H), 2.596 (s, br, 4H), 2.596 (s, 6H), 2.245–2.176 (m, 1H), 1.940–1.871 (m, 1H), 1.488–1.404 (m, 1H), 0.926 (d, J = 6.4 Hz, 3H), 0.840 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.43, 164.51, 163.81, 150.69, 142.54, 138.73, 134.79, 131.44, 130.18, 129.18, 126.73, 124.98, 122.79, 120.79, 120.20, 109.07, 104.58, 52.89, 44.19, 37.45, 25.35, 23.62, 22.60; FTIR (KBr, cm^{-1}): 3281, 3096, 2948, 2859, 1680, 1648, 1580, 1535, 1450, 1361, 1242, 1153, 830, 774; HRMS (ES+) calcd for $C_{28}H_{34}N_5O_5S$ ([M+H])$^+$ 552.2281, found 552.2244.

4.4.5. General procedure for the preparation of *N*-(substituted aryl)-2-(1,3-dioxo-6-(2-(phenylthio)ethylamino)-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-4-methylpentanamide (8a–c)

To a solution of compound **6** (50 mg, 0.108 mmol) in $CHCl_3$ (2 mL) the corresponding substituted arylamine (0.108 mmol), EDCI (42 mg, 0.216 mmol) and DMAP (27 mg, 0.216 mmol) were added. The solution was stirred at room temperature under nitrogen for 48 h and concentrated. The residue was purified on silica gel column chromatography by using CH_2Cl_2/CH_3OH 50:1 (v/v) as the eluent, affording **8a–c** as orange/khaki solid. Yield: 55–65%.

4.4.5.1. *N*-(4-(4-Aminophenylsulfonyl)phenyl)-2-(1,3-dioxo-6-(2-(phenylthio)ethylamino)-1*H*-benzo[de]isoquinolin-2(3H)-yl)-4-methylpentanamide (8a). Yield: 65%. Orange yellow solid. mp 135.5–137.5 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.840 (s, 1H), 8.640 (d, J = 8.0 Hz, 1H), 8.431 (d, J = 7.2 Hz, 1H), 8.211 (d, J = 8.4 Hz, 1H), 7.951 (t, J = 5.6 Hz, 1H), 7.713–7.674 (m, 5H), 7.470 (d, J = 8.8 Hz, 2H), 7.407 (d, J = 7.6 Hz, 2H), 7.321 (t, J = 8.0 Hz, 2H), 7.198 (t, J = 7.2 Hz, 1H), 6.737 (d, J = 8.8 Hz, 1H), 6.577 (d, J = 8.8 Hz, 2H), 6.093 (s, 2H), 5.591 (q, J_1 = 5.2 Hz, J_2 = 9.2 Hz, 1H), 3.631 (d, J = 6.0 Hz, 2H), 2.219–2.149 (m, 1H), 1.887–1.817 (m, 1H), 1.479–1.396 (m, 1H), 0.912 (d, J = 6.4 Hz, 3H), 0.816 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.56, 164.45, 163.43, 153.82, 150.66, 143.33, 139.48, 138.93, 134.83, 130.41, 130.22, 129.81, 129.10, 128.98, 127.80, 126.50, 124.93, 122.73, 120.64, 120.45, 113.39, 108.78, 104.38, 52.60, 42.64, 37.99, 31.37, 25.28, 23.52, 22.63; FTIR (KBr, cm^{-1}): 3362, 2940, 2918, 2837, 1680, 1639, 1576, 1535, 1517, 1383, 1361, 1287, 1242, 1138, 1101, 830, 771, 685, 548; HRMS (ES+) calcd for $C_{38}H_{36}N_4O_5S_2Na$ ([M+Na])$^+$ 715.2025, found 715.2034.

4.4.5.2. *N*-(4′-Amino-3,3′-dimethoxybiphenyl-4-yl)-2-(1,3-dioxo-6-(2-(phenylthio)ethylamino)-1*H*-benzo[de]isoquinolin-2(3H)-yl)-4-methylpentanamide (8b). Yield: 55%. Viscous khaki solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.461 (d, J = 8.4 Hz, 1H), 8.415 (s, 1H), 8.264–8.216 (m, 2H), 8.030 (d, J = 8.0 Hz, 1H), 7.416 (d,

J = 7.6 Hz, 2H), 7.325 (t, J = 8.0 Hz, 1H), 7.287–7.192 (m, 3H), 7.145 (d, J = 8.4 Hz, 1H), 7.042–7.007 (m, 3H), 6.781 (d, J = 8.0 Hz, 1H), 6.420–6.390 (m, 1H), 6.323 (s, 1H), 5.949 (q, J_1 = 5.6 Hz, J_2 = 8.8 Hz, 1H), 3.918 (s, 3H), 3.904 (s, 3H), 3.530 (d, J = 5.6 Hz, 2H), 3.244 (t, J = 6.4 Hz, 2H), 2.407–2.338 (m, 1H), 2.199–2.130 (m, 1H), 1.670–1.604 (m, 1H), 1.063 (d, J = 6.8 Hz, 3H), 0.991 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.77, 164.53, 163.89, 149.65, 148.37, 147.54, 137.59, 135.48, 134.75, 134.59, 131.83, 131.27, 130.44, 129.49, 129.17, 126.85, 126.10, 124.62, 122.01, 120.07, 119.98, 119.64, 119.20, 115.11, 109.46, 109.36, 108.56, 104.00, 56.00, 55.62, 53.90, 42.63, 38.06, 32.76, 25.83, 23.29, 22.33; FTIR (KBr, cm^{-1}): 3355, 2955, 2859, 1680, 1648, 1576, 1535, 1506, 1454, 1387, 1361, 1283, 1238, 1164, 1023, 771; HRMS (ES+) calcd for $C_{40}H_{40}N_4O_5SNa$ $([M+Na])^+$ 711.2617, found 711.2614.

4.4.5.3. 2-(1,3-Dioxo-6-(2-(phenylthio)ethylamino)-1*H*-benzo[*de*]isoquinolin-2(3H)-yl)-4-methyl-*N*-(4-sulfamoylphenyl)pentanamide (8c). Yield: 60%. Orange yellow solid. mp 120.9–122.9 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.822 (s, 1H), 8.659 (d, J = 8.4 Hz, 1H), 8.445 (d, J = 7.2 Hz, 1H), 8.225 (d, J = 8.4 Hz, 1H), 7.988 (t, J = 5.2 Hz, 1H), 7.939 (s, 1H), 7.723–7.685 (m, 5H), 7.409 (d, J = 7.6 Hz, 2H), 7.322 (t, J = 7.2 Hz, 2H), 7.216–7.179 (m, 3H), 6.748 (d, J = 8.8 Hz, 1H), 5.622 (dd, J_1 = 4.8 Hz, J_2 = 8.8 Hz, 1H), 3.636 (dd, J_1 = 6.4 Hz, J_2 = 12.8 Hz, 2H), 3.306 (s, 2H), 2.224–2.167 (m, 1H), 1.930–1.861 (m, 1H), 1.452–1.420 (m, 1H), 0.921 (d, J = 6.4 Hz, 3H), 0.836 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.47, 158.69, 158.38, 158.08, 157.78, 150.68, 142.54, 138.72, 135.94, 134.82, 131.39, 130.04, 129.61, 129.09, 126.71, 126.49, 124.93, 122.78, 120.66, 120.23, 119.19, 116.80, 108.82, 104.37, 52.84, 42.65, 37.96, 31.36, 25.86, 23.68, 22.59; FTIR (KBr, cm^{-1}): 3407, 3266, 2955, 2925, 1677, 1576, 1535, 1469, 1368, 1205, 1149, 849, 797, 722, 604, 518; HRMS (ES+) calcd for $C_{32}H_{32}N_4O_5S_2Na$ $([M+Na])^+$ 639.1712, found 639.1704.

4.5. Cytotoxic evaluation in vitro

The target compounds were submitted to the Chinese National Center for Drug Screening and School of Pharmacy in East China University of Science and Technology for in vitro antitumor activity assays. Growth inhibitory effects on the cell lines (HeLa, P388, HL-60, MCF-7, HCT-8 and A375) were measured by using MTT assay.[46] For A549 cell line, the growth inhibition effect was tested by sulforhodamine B (SRB) assay.[45]

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