RESEARCH ARTICLE

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2018, 9, 2091Synthesis and potential antineoplastic activity of
dehydroabietylamine imidazole derivatives†Fengyi Zhao,^{ab} Wen Lu,^b Fan Su,^c Li Xu,^{id} *^{bde} Dong Jiang,^b Xu Sun,^{cf} Jiuzhou Shi,^b
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To seek more efficient and lower toxicity anticancer compounds, several imidazole combining dehydroabietylamine derivatives including organic salts (L^1 – L^2) and amides (L^3 – L^5) were synthesized. Their antineoplastic activity against HeLa (cervix), MCF-7 (breast), A549 (lung) and HepG2 (liver) cells and HUVECs (umbilical vein, normal cells) *in vitro* were evaluated by MTT assay. The results unequivocally showed that nearly all compounds had better antineoplastic activity and lower toxicity than dehydroabietylamine (L^0). For MCF-7 cells, L^2 (0.75 μ M) and L^5 (2.17 μ M) had higher anti-MCF-7 activity than L^0 and DOX. For A549 cells, L^1 (1.85 μ M) and L^2 (4.37 μ M) had higher anti-A549 activity than L^0 ; in particular, the IC_{50} value of L^1 was much lower than that of DOX. Among these investigated compounds, L^2 and L^5 had lower IC_{50} values (0.75 μ M and 2.17 μ M) against MCF-7 cells and lower toxicity, which suggested that they may be potential future anticancer drugs. In addition, L^1 and L^2 could suppress cancer cell proliferation by inducing apoptosis. L^1 – L^5 could bind with DNA through intercalation.

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1. Introduction

Cancer has attracted enormous attention from all over the world, and extensive research (radiation and chemotherapy) has been devoted to the development of effective anticancer modalities.^{1,2} According to the statistics of the World Health Organization published in 2012, the number of annual cancer cases will rise from 14 million to 22 million within the next two decades.³

To the best of our knowledge, many chemotherapy drugs have been developed for the treatment of various cancers, but there are large side effects as well. Among the various cancer cures, the antineoplastic approach is one of the significant ways to control this deadly disease.⁴ Currently, numerous heterocyclic molecules, particularly imidazoles, have

shown great antineoplastic potential. As we know, imidazole-containing compounds have been found to be dynamically versatile as anticancer, antimicrobial, antibacterial, antifungal, antioxidant, anti-hypotensive and anti-inflammatory agents and they represent important therapeutic compounds.⁵ Some research papers have revealed that imidazoles possess the potential to overcome unsolved drawbacks of the presently available clinical drugs and could be used to develop new anticancer agents. Chen *et al.* reported seventeen imidazole and imidazoline analogues as antineoplastic agents for melanoma.⁶ Their IC_{50} values ranged from 16.1 to >100 μ M. Negi *et al.* reported the microwave synthesis of ten imidazoles conjugated with imines/amides and then screened them for anticancer activity against seven cancer cell lines.⁷ Among them, two imidazole Schiff-base derivatives and one imidazole amide derivative showed promising *in vitro* anticancer activity with low IC_{50} values (7.5–17 μ M) against A459, HepG2 and H460 cancer cell lines.

Recently, a number of bioactive natural products with tricyclic diterpene systems have drawn attention,^{8–14} such as dehydroabietylamine (L^0), which is a common diterpene from some resins.^{15–17} Possessing both a chiral scaffold and broad-spectrum biological properties,^{18,19} dehydroabietylamine and its derivatives have been widely explored in the fields of anticancer, antibacterial and pharmaceutical products, *etc.*^{20,21} For example, Muhammad and co-workers found a dehydroabietylamine derivative that demonstrated noticeable growth attenuation of the MCF-7 breast cancer cell line (IC_{50} = 4.8 μ M).²² Rao's group²³ screened a series of

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imines, amides and ureas with a dehydroabietyl skeleton for anticancer activity against SMMC7721 (liver), A549, C6 (glioma) and MCF-7 cancer cell lines with the smallest IC_{50} values of 6.65, 0.75, 0.81 and 10.65 μM . Robertson²⁴ concluded that dehydroabietylamine was 4.5 times more effective at inhibiting the survival of cultured melanoma cells than normal cells, with average IC_{50} values of 2.0 and 9.3 μM , respectively. Recently, our group has found that several Schiff-base compounds display highly potent inhibitory activities against HepG2 cells with IC_{50} values of 0.14 (L^7) and 0.24 μM (L^6).²⁵

When developing anticancer drugs, toxicity is a serious issue. Moreover, DNA is one of the primary target molecules for most anticancer and antiviral therapies.²⁶ It is well-known that one of the most active anticancer drugs, cisplatin, covalently binds to DNA; unfortunately, its usage is limited due to side-effects and acquired cellular resistance.²⁷ Thus, it is an urgent requirement to find new molecules with more effective, less toxic, and target-specific DNA binding activities for the treatment of cancer. In view of the fact that the aforementioned imidazole and dehydroabietylamine derivatives all have considerable anticancer activities, our group hopes to combine them to synthesize and screen target-specific DNA-binding, high anticancer activity and low toxicity dehydroabietylamine derivatives, including organic salts (L^1 – L^2) and amides (L^3 – L^5). Among them, several were designed and synthesized as potent anticancer agents. The antineoplastic activities of these new compounds were investigated against HeLa (cervix), MCF-7 (breast), A549 (lung), and HepG2 (liver) human cancer cell lines *in vitro* by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. It turned out that several compounds showed high antineoplastic activities against these four cancer cell lines and most of these compounds displayed more potent antineoplastic activities compared with L^0 . The apoptosis effect of L^1 or L^2 induced in A549 or MCF-7 cells was also investigated, which showed that L^1 and L^2 could trigger cell apoptosis, whilst our previous work confirmed the apoptosis-inducing ability of the compounds L^6 and L^7 .²⁵ In this paper, we wish to positively report this convenient synthesis procedure, which may be helpful in the development of future generations of anticancer drugs.

2. Results and discussion

2.1 Chemistry

(+)-Dehydroabietylamine ($[a]_D^{20} = +55.1$ (c 2.4 pyridine)), commercially available, was used as a starting material to synthesize organic salts L^1 – L^2 and amides L^3 – L^5 by a facile method (Scheme 1). For the purpose of studying the effect of different compound structures on biological activity, three imidazole carboxylic acids with different amounts of carboxylic acid and substituent groups were designed to react with dehydroabietylamine to prepare organic salts L^1 – L^2 and amides L^3 – L^5 . These reactions occurred smoothly under different condi-

Scheme 1 Synthesis and physicochemical data of L^1 – L^5 .

tions. L^3 – L^5 were in neutral to slightly alkaline conditions (pH 7.0–7.4) during the experimental process, and they are always stable due to large space steric hindrance from the aromatic diterpene structure with three rings. We have investigated the stability of compounds L^3 – L^5 by HPLC. After 24 hours, there were hardly any changes of the peak areas for L^3 – L^5 , which suggested that they had good stability (Table S1†).

Organic salts L^1 and L^2 exhibited novel structures, and both of them were colorless block-shaped crystals with monoclinic crystal systems. L^1 and L^2 crystallized in chiral space group $P2_1$ with a Flack parameter of $-0.6(13)$ and $0.0(6)$, respectively.²⁸ L^1 had two aromatic rings and two aliphatic rings with one molecular dehydroabietylamine, while L^2 had a $-CH_3$ electron-donating group, three aromatic rings and four aliphatic rings with two molecular dehydroabietylamines. For L^1 , the imidazole ring with C4, O1, O2, C5, O3, O4 and H3 was coplanar (Fig. 1a) and an intramolecular hydrogen bond is formed by O2, H3 and O3. The length of the new NH single bond N3–H3A/B/C (0.8900 Å) and the angles of H3A–N3–H3B/C and H3B–N3–H3C (109.50°) are consistent with the results of tetrahedral NH_3 . The molecules are continuously interleaved by two molecular imidazole carboxylic acids and two molecular dehydroabietylamines with classic $N-H\cdots O$, $N-H\cdots N$ and $O-H\cdots O$ hydrogen bonds from $-COOH$, $-NH_3$ and H_2O interacting strongly to assemble an infinite two-dimensional network structure along the a axis (Fig. 1b). For L^2 , the imidazole ring with C43, O1, O2, C44, O3 and O4 was coplanar and an intramolecular hydrogen bond is formed by O1, H67 and O4 (Fig. 2a). Similarly, the lengths of the new NH single bond and angles are in accordance with that of L^1 . One molecular imidazole carboxylic acid and one molecular

Fig. 1 a. Molecular structure of L^1 (hydrogen atoms connected with carbon atoms omitted for clarity); b. the two-dimensional network structure formed by hydrogen bond interactions during photodynamic therapy.

Fig. 2 a. Molecular structure of L^2 (hydrogen atoms connected with carbon atoms are omitted for clarity); b. the two-dimensional network structure formed by hydrogen bond interactions.

dehydroabietylamine are connected with classic $N-H\cdots O$ and $N-H\cdots N$ hydrogen bonds from $-COO^-$ and $-NH^{3+}$, and the other single molecular dehydroabietylamine is connected to $-NH^{3+}$ with a classic $N2-H2A\cdots N1$ bond; the strong interactions lead to the assembly of an infinite two-dimensional network structure along the b axis (Fig. 2b).

The stability of the novel molecular structure and crystal packing may contribute to better antineoplastic activity, which is caused by intermolecular hydrogen bonds. The

hydrogen bond parameters of L^1 and L^2 are shown in Table 1. Selected bond lengths and angles are presented in Table 2. The crystallographic data are shown in Table 4.

2.2 Biological evaluation

2.2.1 Antineoplastic activities. The antineoplastic activities of these compounds (L^1 – L^5) against human cancer cell lines HeLa, HepG2, MCF-7 and A549 and the normal cell line

Table 1 Hydrogen bonds of L^1 and L^2

Compd.	D–H $\cdots$ A	$d(D-H)$ (Å)	$d(H\cdots A)$ (Å)	$d(D\cdots A)$ (Å)	$\angle DHA$ (°)
L^1	$O3-H3\cdots O2^i$	0.8209(18)	1.6675(21)	2.4880(27)	178.047(136)
	$O1W-H1WA\cdots O1^{ii}$	0.8489(21)	1.9901(21)	2.8370(29)	175.326(151)
	$O1W-H1WB\cdots O2^{iii}$	0.8497(19)	2.2017(19)	2.8517(26)	165.275(35)
	$N1-H1A\cdots O3^{iv}$	0.8602(21)	2.1392(14)	2.9692(26)	161.998(145)
	$N3-H3A\cdots O1^{ii}$	0.8906(17)	1.9938(18)	2.8018(26)	150.195(123)
	$N3-H3B\cdots O4^v$	0.8898(21)	2.1850(21)	3.0156(31)	155.120(149)
	$N3-H3B\cdots N2^{iv}$	0.8898(21)	2.3294(21)	2.8890(31)	120.894(147)
	$N3-H3C\cdots O1W^{vi}$	0.8895(27)	2.0655(21)	2.8316(35)	143.672(184)
L^2	$N2-H2A\cdots N1^{vii}$	0.9098(28)	1.8959(34)	2.7932(44)	168.450(194)
	$N2-H2C\cdots N3^{vii}$	0.9101(23)	2.0022(24)	2.8685(33)	158.534(167)
	$N2-H2B\cdots O2^i$	0.9102(26)	2.0800(25)	2.8998(36)	149.242(174)
	$N2-H2C\cdots O3^{vii}$	0.9101(23)	2.5140(22)	3.0009(35)	113.943(181)
	$O4-H67\cdots O1^i$	0.8656(23)	1.5937(24)	2.4532(33)	171.508(176)

Symmetry code: i $x, 1 + y, z$; ii $1 + x, -1 + y, z$; iii $1 - x, 0.5 + y, 1 - z$; iv $1 - x, -0.5 + y, 1 - z$; v $-1 + x, 1 + y, z$; vi $1 - x, 0.5 + y, 1 - z$; vii $-x, 0.5 + y, 1 - z$.

Table 2 Selected bond lengths (Å) and angles (deg) for **L**¹ and **L**²

L ¹				L ²			
N1–C2	1.337(3)	O3–H3	0.8200	N1–C1	1.468(4)	N1–H1/8	0.8000/0.9000
N3–C19	1.483(3)	N3–H3A/B/C	0.8900	N2–C24	1.483(3)	N2–H2A/B/C	0.9100
C4–O1	1.244(3)	C5–O3–H3	109.50	O1–C43	1.278(4)	O1–C43–O2	125.0(3)
C4–O2	1.272(3)	O1–C4–O2	125.4(2)	O2–C43	1.237(4)	O3–C44–O4	121.6(3)
C5–O4	1.210(3)	O4–C5–O3	121.2(2)	O3–C44	1.223(4)	H1–N1–H8	107.0(3)
C5–O3	1.315(3)	H3A/B–N3–H3B/C	109.50	O4–C44	1.310(3)	H2A/B–N2–H2B/C	109.50

Table 3 Cytotoxicity of DOX, DDP, 5-FU and **L**⁰–**L**⁵ against axenic cancer cells and normal cells (IC₅₀ ± SE^a/μM)

Compd.	HeLa	HepG2	MCF-7	A549	HUVEC
L ⁰	2.02 ± 0.02	2.56 ± 0.04	19.45 ± 0.39	5.02 ± 0.19	1.27 ± 0.03
L ¹	18.74 ± 0.88*** ^b	4.62 ± 0.03 ^{nsd}	6.47 ± 0.25*** ^b	1.85 ± 0.53 ^{nsd}	1.21 ± 0.08 ^{nsd}
L ²	2.88 ± 0.15 ^{nsd}	2.91 ± 0.11 ^{nsd}	0.75 ± 0.07*** ^b	4.37 ± 0.07 ^{nsd}	3.98 ± 0.18 ^{nsd}
L ³	16.75 ± 0.65*** ^b	6.52 ± 0.18 ^{nsd}	12.08 ± 0.60*** ^b	12.34 ± 0.05*** ^b	32.99 ± 1.26*** ^b
L ⁴	48.47 ± 2.33*** ^b	50.49 ± 5.84*** ^b	9.85 ± 0.13*** ^b	17.02 ± 1.46*** ^b	44.92 ± 5.71*** ^b
L ⁵	20.95 ± 0.50*** ^b	12.02 ± 0.85*** ^c	2.17 ± 0.10*** ^b	53.23 ± 2.98*** ^b	57.78 ± 2.07*** ^b
L ⁶ (ref. 25)	3.22 ± 0.02 ^{nsd}	0.24 ± 0.01 ^{nsd}	4.28 ± 0.70*** ^b	3.17 ± 0.05 ^{nsd}	7.67 ± 0.43 ^{nsd}
L ⁷ (ref. 25)	7.86 ± 0.01*** ^b	0.14 ± 0.001 ^{nsd}	2.58 ± 0.15*** ^b	7 ± 1 ^{nsd}	26 ± 2*** ^b
DOX	3.55 ± 0.17 ^{nsd}	1.20 ± 0.04 ^{nsd}	14 ± 1.03*** ^b	3.35 ± 0.69 ^{nsd}	4.40 ± 0.55*** ^b

^a Average IC₅₀ values from at least three independent experiments. ^b ****p* < 0.001. ^c ***p* < 0.01. ^d ns = not significant, compounds compared with **L**⁰, respectively.

HUVEC were evaluated *in vitro* by MTT assay. DOX (doxorubicin) was used as a positive control, and the results are summarized as IC₅₀ values in Table 3. Actually, DOX is a widely used chemotherapy medication to treat cancer, which has the ability to penetrate tissues highly effectively.²⁹

Table 4 Crystallographic data for **L**¹ and **L**²

	L ¹	L ²
Empirical formula	C ₂₅ H ₃₇ N ₃ O ₅	C ₄₆ H ₆₈ N ₄ O ₄
Formula weight	459.58	741.03
Crystal system	Monoclinic	Monoclinic
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁
<i>a</i> (Å)	10.3659(9)	6.9872(5)
<i>b</i> (Å)	7.0531(6)	15.5151(10)
<i>c</i> (Å)	16.9815(15)	19.4250(13)
α (°)	90	90
β (°)	92.543(2)	98.467(2)
γ (°)	90	90
<i>V</i> (Å ³)	1240.32(19)	2082.9(2)
<i>Z</i>	2	2
<i>D</i> _{calcd} (g cm ^{−3})	1.231	1.182
μ (mm ^{−1})	0.086	0.075
Flack parameter	−0.6(13)	0.0(6)
<i>F</i> (000)	496.0	808.0
<i>T</i> (K)	294(2)	136(2)
Crystal size (mm ³)	0.30 × 0.26 × 0.15	0.20 × 0.12 × 0.18
θ range (°)	2.35–27.57	2.49–25.07
Reflections collected	7879	21 119
Independent reflections	4394	7187
Reflections observed	3446 (<i>R</i> _{int} = 0.0185)	5999 (<i>R</i> _{int} = 0.0452)
[<i>I</i> > 2σ(<i>I</i>)]		
Data/restraints/parameters	4394/1/298	7187/1/506
Goodness-of-fit on <i>F</i> ²	1.048	1.037
Final <i>R</i> indices [<i>I</i> > 2σ(<i>I</i>)]	0.0443/0.1109	0.0419/0.0795
<i>R</i> indices (all data)	0.0542/0.1165	0.0607/0.0863
Largest difference peak and hole (e Å ^{−3})	0.317, −0.155	0.180, −0.185

On the basis of the IC₅₀ values (Table 3), we could draw a histogram (Fig. 3) to compare the antineoplastic activity and cytotoxicity of **L**⁰, its organic salt (**L**¹–**L**²) and amide (**L**³–**L**⁵) derivatives and DOX against axenic cancer cells more clearly.

As shown in Fig. 3, **L**³, **L**⁴, **L**⁵ and **L**⁷ had lower toxicity than DOX, DDP (27.28 ± 0.01 μM),³⁰ and 5-FU (11.65 ± 1.28 μM) for HUVEC cells,³¹ and **L**⁶ had lower toxicity than DOX but higher toxicity than DDP and 5-FU. We used dehydroabietylamine (**L**⁰) as a negative control to compare the antineoplastic activities among its modified products, and DOX incubated with these five cells was used as a positive control. In general, despite the antineoplastic activity of **L**⁰ being slightly lower than that of DOX (except against HeLa cells), **L**⁰ had better potential antineoplastic activity than a large number of pharmaceutical products. Furthermore, probability values of *p* > 0.05 were considered and showed that the

Fig. 3 A comparison of the antineoplastic effects and cytotoxicity of DOX and **L**⁰–**L**⁵.

difference in antineoplastic activity between L^0 and DOX was statistically insignificant, except against MCF-7 cells. For HepG2 cells, the IC_{50} values of L^6 (0.24 μM) and L^7 (0.14 μM) were much lower than that of L^0 ; in particular, as shown in our previous report, L^7 was 180 times more effective at inhibiting cultured HepG2 cell survival than normal cells, with average IC_{50} values of 0.14 and 25.56 μM .²⁵ Unfortunately, although L^6 and L^7 had higher anti-HepG2 ability, their anti-MCF-7 (L^6 , 4.28 μM ; L^7 , 2.58 μM) and anti-A549 (L^6 , 3.17 μM ; L^7 , 7 μM) abilities were quite a bit lower than those of L^2 and L^1 . In fact, different compounds have diverse antineoplastic activity against a variety of cell lines. For MCF-7 cells, the IC_{50} values of all compounds were lower than that of L^0 , especially L^2 (0.75 μM) and L^5 (2.17 μM), which meant that they had higher anti-MCF-7 activity than L^0 and DOX. For A549 cells, the IC_{50} values of compounds L^1 (1.85 μM) and L^2 (4.37 μM) were lower than that of L^0 , which indicated that these three compounds have higher anti-A549 activity than L^0 ; in particular, the IC_{50} value of L^1 was much lower than that of DOX. When DOX was used as a control, the difference in cytotoxicity between L^1 and L^2 was statistically insignificant, which meant that L^1 and L^2 had similar cytotoxicities to DOX against normal cells (HUVECs). Among these tested compounds, L^2 and L^5 had lower IC_{50} values (0.75 μM and 2.17 μM) against MCF-7 cells and lower toxicity, which suggested that they may be a potential anticancer drug.

In comparison with the structure and cytotoxicity *in vitro* against the various cancer cell lines of L^1 and L^2 , we could see that the antineoplastic activity of the organic salt with two molecular dehydroabietyl amines (L^2) was higher than that with one molecular dehydroabietylamine (L^1). The reason for this may be that the increased amount of L^0 with high anticancer activity contributes more and the solubility of the organic salts is better. In imidazole rings, the "N" with a lone pair of electrons can easily form hydrogen bonds with

carboxyl and amino groups in the cancer cell lines. In addition, when the compounds possess a $-CH_3$ electron-donating group (L^3 , L^4 and L^5), the antineoplastic activities were lower in comparison to compounds without an electron-donating group (L^1 , L^6 and L^7). It has been reported that introducing a polar group containing a hydrogen bond donor could improve certain anticancer activity.³²

2.2.2 Induction of apoptosis. In recent years, many reports have indicated that various anticancer drugs or cancer chemopreventive agents, like DOX, can prevent tumor promotion and progression through inducing apoptosis. Apoptosis, a physiological mode of cell death, is related to the regulation of development and homeostasis, plays a central role in cancer and can provide important information to study modes of action.³³ To further evaluate the potential mechanism of the antineoplastic activity of L^1 and L^2 , an apoptosis assay was carried out using Annexin V-FITC/PI and flow cytometry in A549 and MCF-7 cells with DMSO as a negative control, and the results of the flow cytometric analysis are shown in Fig. 4.

According to Fig. 4, both L^1 and L^2 obviously exhibited apoptosis induction in a dosage-dependent manner. After incubation with A549 or MCF-7 cells, the number of live cells decreased and the number of apoptotic cells increased with an increase in the concentration of L^1 and L^2 from 0.1 to 5 $\mu g mL^{-1}$. When the concentration of L^1 was 1, 2 and 5 $\mu g mL^{-1}$, the apoptotic ratio was 12.47%, 30.64% and 76.86%, respectively. Similarly, when the concentration of L^2 was 0.1, 1 and 5 $\mu g mL^{-1}$, the apoptotic ratio was 25.40%, 39.49% and 54.21%, respectively. As discussed in our aforementioned report, L^6 and L^7 possess the ability to induce apoptosis in HepG2 cells;²⁵ nonetheless, their antineoplastic activity against A549 and MCF-7 cells was worse than that of equivalent L^1 and L^2 . To further evaluate the potential mechanism of cellular uptake, HeLa cells were treated with L^0 -FITC

Fig. 4 The apoptosis induced by L^1 and L^2 as determined by flow cytometric analysis. (1) A-C shows A549 cells treated with DMSO (negative control), and the remainder on the top row are L^1 at 1, 2 and 5 $\mu g mL^{-1}$, respectively. (2) M-C shows MCF-7 cells treated with DMSO (negative control), and the remainder on the bottom row are L^2 at 0.1, 1 and 5 $\mu g mL^{-1}$, respectively.

(equal L^0 and FITC were stirred with DIPEA as a catalyst at room temperature for 12 h) at 4 °C in the dark for 2 hours. There was hardly any fluorescence signal inside the cells at a lower temperature (4 °C), suggesting the inhibited internalization of the L^0 complex. This result indicated L^1 and L^2 could suppress cell proliferation by penetrating into the cytoplasm *via* endocytosis to induce apoptosis, which is greatly similar to the mechanism of DOX (shown in Fig. S1†).

For further research, the $\log P$ of all compounds was calculated using Hyperchem 8.0 (shown in Scheme 1). Among all compounds, the $\log P$ values of L^1 and L^2 were the smallest with the strongest hydrophilicity; moreover, compared with DOX (1.503), the $\log P$ values of L^1 (−0.51) and L^2 (0.05) were smaller, which suggested that they had higher solubility, and this may contribute to their remarkable cytotoxic activities. Liu and co-authors reported that good water solubility normally leads to a high drug potency and pleasant pharmacokinetic profiles.³⁴ Converting an organic compound into a salt is one of the crucial methods to enhance its physicochemical properties and can promote dissolution by increasing ionic hydration. For instance, DOX, the aforementioned positive control drug, is doxorubicin hydrochloride. The $\log P$ of a classic quinolone antibacterial drug, ciprofloxacin, is −1.20,³⁵ which signifies that L^1 and L^2 may be potential antiproliferation drugs. The $\log P$ of L^4 (5.27) was larger than that of the other compounds, which led to worse effects on cancer cells due to its poor solubility. L^3 and L^5 had stronger lipophilicity than DOX. Besides, the pK_b of all compounds was detected to be in the range of 7.10–15.30, which suggests that it has no regular effect on cytotoxic activity. Therefore, these compounds are ideal as potential antiproliferation drugs.

2.2.3 DNA binding modes. As mentioned above, DOX has the ability to remain inside nucleated cells, due to its lipophilic characteristics and DNA intercalating/binding properties. For further investigation, we studied the DNA binding properties of L^1 – L^5 . The DNA (Salmon Sperm DNA) binding modes were evaluated by ethidium bromide (EB) fluorescence displacement experiments. EB doesn't exhibit any appreciable emission in buffer solution, but upon addition of DNA, the fluorescence intensity is highly enhanced on account of its strong intercalation with DNA base pairs.^{36–38} If the DNA–EB

Fig. 5 Emission spectra of DNA–EB in the absence and the presence of increasing amounts of L^2 at room temperature ([EB] = 2×10^{-5} M, [DNA] = 1×10^{-4} M, [L^2] = 1.5×10^{-5} M).

Fig. 6 Absorption spectra of L^1 and L^2 (5×10^{-5} M) in the absence and presence of increasing amounts of DNA at room temperature in Tris–NaCl–HCl buffer (pH = 7.3). The arrow shows the absorbance change when increasing the DNA concentration.

emission can be decreased or quenched by the addition of a compound, the intercalation of the compound with the base pairs of DNA can be confirmed.³⁹ Subsequently, upon adding L^2 to DNA pretreated with EB, the emission intensity obviously reduced (shown in Fig. 5), which indicates that L^2 can bind to DNA at the sites occupied by EB, and its interaction with DNA occurs through intercalation.

The DNA binding modes of L^1 and L^2 were also investigated by the application of absorption spectral analysis (shown in Fig. 6). Through intercalation, a compound binding to DNA usually leads to hypochromism and bathochromism. On increasing the amount of DNA added to a solution of L^2 , the absorption spectra exhibit a hypochromic shift, which suggests an intercalative binding mode. This result is in accordance with that of the fluorescence studies. Both the absorption and fluorescence spectra proved that L^2 could bind with DNA through intercalation, similar to DOX. The DNA binding modes of L^3 , L^4 and L^5 were also intercalation (shown in Fig. S2†).

These findings were meaningful to understand the cytotoxicity and the DNA binding modes of these dehydroabietylamine analogues, and in the meantime, lay a foundation for the reasonable design of new and potential DNA targeting compounds.

3. Conclusions

In summary, we synthesized organic salts L^1 – L^2 and amides L^3 – L^5 , and revealed their high antineoplastic activity, relatively low toxicity and DNA binding modes. Compounds L^1 (1.85 μ M) and L^2 (4.37 μ M) had higher anti-A549 activity. Compounds L^2 (0.75 μ M) and L^5 (2.17 μ M) had higher anti-MCF-7 activity. Additionally, L^1 – L^5 could bind with DNA through intercalation. Besides, we verified that L^1 and L^2 suppressed cell proliferation by inducing apoptosis.

So far, anticancer drugs with rapid action, selective nature, efficient bioavailability, low toxicity and reduced or nearly no side effects are in demand. Owing to the high antineoplastic activity and relatively low toxicity of these compounds, the results reported here may be helpful in the exploration of future generations of selective anticancer drugs and building a foundation for the rational design of new and potential cellular targeting compounds. Further research will be required to define the mechanism of action of these compounds.

4. Experimental

4.1 General experimental procedures

All reagents and solvents were analytical reagent (AR) grade and used as received unless otherwise indicated. The IR spectra were recorded on a Bruker Vectex 80 FT-IR spectrometer with KBr discs in the 4000–500 cm^{-1} range. The ^1H and ^{13}C $\{^1\text{H}\}$ NMR spectra were measured on a Bruker Avance III NMR spectrometer (Billerica, MA, USA). ^1H and ^{13}C $\{^1\text{H}\}$ NMR spectra were recorded in CDCl_3 as a solvent unless otherwise stated. Chemical shifts (δ) are given as parts per million (ppm) relative to the NMR solvent signals (CDCl_3 7.26 and 77.00 ppm for ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR, respectively). J values were given in Hz. HRMS were measured to determine the purity of all tested compounds using an LTQ Orbitrap XL mass spectrometer (Thermo Electron, USA). Reactions were monitored by TLC using silica gel 60 F-254 in 0.25 mm-thick plates. Compounds on the TLC plates were detected under UV light at 254 nm. Purifications were performed by flash chromatography on silica gel (300–400 mesh). The DNA binding modes were investigated using a Lambda 950 (PerkinElmer) and LS55 Fluorescence Spectrophotometer (PerkinElmer). Cell imaging was carried out on a confocal fluorescence microscope (Nikon, Eclipse TE2000-E).

4.2 Synthesis of compounds L^1 – L^5

Organic salts L^1 – L^2 and amides L^3 – L^5 were synthesized by the following methods.

Synthesis of *N*-1*H*-imidazole-5-carboxyl-4-amino-carboxyl salt-dehydroabietylamine (L^1). A mixture of 4,5-dicarboxy-1*H*-imidazole (0.15 g, 1.0 mmol) and dehydroabietylamine (0.28 g, 1.0 mmol) in ethanol was cooled to room temperature, and after several days of slow evaporation, lots of colorless block single-shaped crystals of L^1 suitable for X-ray analysis were obtained. (0.142 g, 31%), IR (neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 3428, 2949, 1707, 1535, 1461, 1378, 1235, 1187, 1064, 996, 873, 626; ^1H NMR (CDCl_3 , 600 MHz) δ 0.96 (3H, s), 1.15–1.18 (9H, t, J = 2.4 Hz), 1.22–1.32 (2H, m), 1.34–1.43 (2H, m), 1.62–1.73 (4H, m), 2.28 (1H, d, J = 13.2 Hz), 2.70–2.89 (5H, m), 6.86 (1H, s), 6.96 (1H, dd, J = 1.8 Hz), 7.15 (1H, d, J = 8.4 Hz), 7.56–7.70 (3H, s, $-\text{NH}^{3+}$); $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 151 MHz) δ 17.77, 18.45, 19.25, 23.99, 25.46, 29.75, 32.02, 33.44, 36.54, 37.66, 42.81, 45.84, 46.63, 63.06, 123.90, 124.25, 126.85, 129.76, 134.46, 145.30, 146.22, 147.63, 161.61, 173.28, 206.31; L^1 in cation mode: M^+ m/z 286.2533 (calcd for $\text{C}_{20}\text{H}_{32}\text{N}^+$ 286.2529); L^1 in anion mode: M^- m/z 155.0108 (calcd for $\text{C}_5\text{H}_3\text{N}_2\text{O}_4^-$ 155.0098). Anal. calcd for $\text{C}_{25}\text{H}_{37}\text{N}_3\text{O}_5$: C, 65.34; H, 8.11; N, 9.14. Found: C, 65.29; H, 8.19; N, 9.05.

Synthesis of *N*-1-methyl-1*H*-imidazole-4,5-diamino-carboxy salt-dehydroabietylamine (L^2). A mixture of 4,5-dicarboxy-1-methyl-1*H*-imidazole (0.17 g, 1.0 mmol) and dehydroabietylamine (0.28 g, 1.0 mmol) in ethanol was cooled to room temperature, and several days later, colorless block-shaped single-crystals of L^2 appeared following slow evaporation of the solvent. (0.148 g, 20%), IR (neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 3381, 3287, 2959, 2866, 1707, 1552, 1515, 1465, 1378, 1254, 1161,

1056, 1012, 938, 876, 826, 783, 640; ^1H NMR (CDCl_3 , 600 MHz) δ 0.88 (3H, s), 1.13–1.16 (9H, t, J = 7.2 Hz), 1.20–1.28 (4H, m), 1.29–1.40 (2H, m), 1.45–1.47 (2H, m), 1.58–1.64 (4H, m), 1.66–1.74 (4H, m), 2.47 (2H, d, J = 12.6 Hz), 2.63–2.65 (2H, m), 2.76–2.88 (6H, m), 3.84 (3H, s), 4.63 (5H, s, $-\text{NH}_2$, $-\text{NH}^{3+}$), 6.84 (1H, s), 6.94 (1H, dd, J = 1.8 Hz), 7.14 (1H, d, J = 8.4 Hz); $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 151 MHz) δ 18.09, 18.30, 23.85, 23.91, 24.93, 29.19, 32.82, 34.49, 34.57, 36.14, 36.84, 37.82, 44.00, 51.30, 123.46, 123.91, 126.28, 128.92, 134.37, 137.43, 139.86, 144.89, 146.97, 161.88, 163.88; L^2 in cation mode: M^+ m/z 286.2526 (calcd for $\text{C}_{20}\text{H}_{32}\text{N}^+$ 286.2529); L^2 in anion mode: M^{2-} m/z 84.0133 (calcd for $\text{C}_6\text{H}_4\text{N}_2\text{O}_4^{2-}$ 84.0133). Anal. calcd for $\text{C}_{46}\text{H}_{68}\text{N}_4\text{O}_4$: C, 74.56; H, 9.25; N, 7.56. Found: C, 74.39; H, 9.29; N, 7.45.

Synthesis of *N*-1-methyl-1*H*-imidazole-4-acyl-5-carboxyl-dehydroabietylamine (L^3) and *N*-1-methyl-1*H*-imidazole-4,5-di-acyl-dehydroabietylamine (L^4). 4,5-Dicarboxy-1-methyl-1*H*-imidazole (0.34 g, 2.0 mmol) and HOBT (0.27 g, 2.0 mmol) were dissolved in ethyl acetate (40 mL), and stirred for 0.5 h at 0 °C. Then, DCC (0.41 g, 2.0 mmol) was slowly added, and the mixture was stirred for 2.5 h at 0 °C. An ethyl acetate solution (10 mL) of dehydroabietylamine (0.57 g, 2.0 mmol) was added to the reaction system slowly, and the mixture was stirred at room temperature for 8 h. After filtration to remove DCU, the filtrate was diluted to 200 mL and washed with 5% NaHCO_3 (3 $\times$ 20 mL), 10% citric acid (2 $\times$ 20 mL) and saturated salt water. The ethyl acetate layer was dried with anhydrous Na_2SO_4 for 2 h. The crude product was purified with SiO_2 column chromatography (hexane–ethyl acetate, 2 : 1). Compounds L^3 (0.49 g, 56%) and L^4 (0.45 g, 32%) were both obtained as white powders. L^3 : mp 130.4–132.2 °C; IR (neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 3392, 2931, 2863, 1717, 1650, 1583, 1379, 1230, 1043, 819, 633; ^1H NMR (CDCl_3 , 600 MHz) δ 1.01 (3H, s), 1.21–1.27 (9H, t, J = 6.6 Hz), 1.32–1.50 (3H, m), 1.71–1.82 (4H, m), 1.89–1.97 (1H, m), 2.29 (1H, d, J = 12.6 Hz), 3.16–3.58 (2H, dd, J = 7.2 Hz), 3.99 (3H, s), 6.89 (1H, s), 6.98 (1H, dd, J = 1.8 Hz), 7.15 (1H, d, J = 8.4 Hz), 7.45 (1H, s), 7.82 (1H, s, $-\text{NH}$), 16.52 (1H, s, $-\text{COOH}$); $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 151 MHz) δ 18.51, 18.83, 19.12, 23.87, 25.45, 25.57, 30.29, 33.34, 33.79, 35.42, 36.19, 37.52, 37.89, 38.11, 45.20, 49.93, 123.88, 124.13, 125.98, 126.94, 134.66, 136.21, 140.36, 145.64, 146.80, 159.44, 164.29; MS $[\text{M} + \text{H}]^+$ m/z 438.2549, $[\text{M} + \text{Na}]^+$ m/z 460.3341 (calcd for $\text{C}_{26}\text{H}_{35}\text{N}_3\text{O}_3$ 437.2678). Anal. calcd for $\text{C}_{26}\text{H}_{35}\text{N}_3\text{O}_3$: C, 71.37; H, 8.06; N, 9.60. Found: C, 71.28; H, 8.01; N, 9.72.

L^4 : mp 112.9–115.1 °C; IR (neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 3394, 2929, 2854, 1656, 1596, 1461, 1371, 1235, 1064, 962, 825, 626; ^1H -NMR (CDCl_3 , 600 MHz) δ 0.99 (3H, s), 1.01 (3H, s), 1.19–1.27 (18H, m), 1.33–1.48 (6H, m), 1.52–1.79 (8H, m), 1.96–2.08 (2H, m), 2.25 (2H, d, J = 6.0 Hz), 2.78–2.90 (6H, m), 3.08–3.20 (2H, dd, J = 6.0 Hz), 3.44, 4.12 (2H, dd, J = 7.2 Hz), 3.99 (3H, s), 6.85 (2H, s), 6.92 (2H, dd, J = 8.4 Hz), 7.14 (1H, d, J = 7.8 Hz), 7.30 (1H, s), 7.79 (1H, s, $-\text{NH}$), 11.38 (1H, s, $-\text{NH}$); $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 151 MHz) δ 18.93, 19.14, 19.17, 23.85, 23.90, 23.97, 24.05, 25.53, 25.55, 30.35, 30.45, 33.39, 36.08, 36.14, 36.16, 37.54, 37.79, 37.84, 38.19, 38.35, 45.06, 45.20,

49.58, 49.79, 60.34, 123.63, 123.78, 124.24, 126.79, 126.90, 127.87, 134.76, 134.89, 135.07, 138.82, 145.26, 145.49, 147.03, 147.31, 159.61, 163.54; MS $[M + H]^+$ m/z 705.5102 (calcd for $C_{46}H_{64}N_4O_2$ 704.5029). Anal. calcd for $C_{46}H_{64}N_4O_2$: C, 78.37; H, 9.15; N, 7.95. Found: C, 78.47; H, 9.21; N, 7.89.

Synthesis of N-1-methyl-1H-imidazole-5-acyl-dehydroabietylamine (L^5). 1-Methyl-1H-imidazole-5-carboxylic acid (0.13 g, 1.0 mmol), HOBT (1.0 mmol), DCC (1.0 mmol) and dehydroabietylamine (1.0 mmol) were used in the condensation reaction. The solution was dried with anhydrous Na_2SO_4 and evaporated to get a pale-yellow powder. (0.33 g, 83%), mp 150.8–152.3 °C; IR (neat) ν_{max}/cm^{-1} 3414, 2922, 2854, 1633, 1561, 1453, 1378, 1235, 1085, 819, 660; 1H -NMR ($CDCl_3$, 600 MHz) δ 0.99 (3H, s), 1.21–1.23 (9H, t, J = 3.6 Hz), 1.34–1.38 (2H, m), 1.45–1.48 (1H, m), 1.58–1.71 (2H, m), 1.77–1.82 (1H, m), 1.89–1.94 (2H, m), 2.30 (1H, d, J = 7.8 Hz), 2.78–2.83 (2H, m), 2.90–2.95 (1H, m), 3.24–3.38 (2H, dd, J = 4.2 Hz), 6.02 (1H, s, –NH), 6.88 (1H, s), 6.99 (1H, dd, J = 1.8 Hz), 7.16 (1H, d, J = 8.4 Hz), 7.12 (1H, d, J = 8.4 Hz), 7.40, 7.48 (2H, ds); $^{13}C\{^1H\}$ NMR ($CDCl_3$, 151 MHz) δ 20.06, 24.86, 25.85, 26.29, 26.55, 31.28, 34.86, 37.38, 38.44, 38.67, 39.26, 46.67, 50.00, 50.58, 124.83, 125.11, 127.81, 131.95, 135.52, 142.69, 146.58, 157.84, 161.46; MS $[M + H]^+$ m/z 394.2871 (calcd for $C_{25}H_{35}N_3O$ 393.2780). Anal. calcd for $C_{25}H_{35}N_3O$: C, 76.29; H, 8.96; N, 10.68. Found: C, 76.17; H, 8.87; N, 10.72.

4.3 X-ray crystal structure determination

Diffraction data were collected on a Bruker D8 VENTURE PHOTON 100 diffractometer using graphite-monochromated $MoK\alpha$ radiation (0.71073 Å) at 293 K in the ω – 2θ scan mode. In all cases, an empirical absorption correction by SADABS was applied to the intensity data. The structures were solved by direct methods and refined on F^2 by full-matrix least-squares methods using the SHELXTL crystallographic software package. All non-hydrogen atoms were refined anisotropically with hydrogen atoms included in calculated positions (riding model). Crystallographic data for compounds L^1 and L^2 are given in Table 4. CCDC 1816224 contains the supplementary crystallographic data for compound L^1 . CCDC 1478434 contains the supplementary crystallographic data for compound L^2 .

4.4 Biological assays

4.4.1 Cell culture, antineoplastic activities and cytotoxicity assay. Dulbecco's Modified Eagle's Medium (DMEM) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS) and penicillin/streptomycin were all purchased from Nanjing Keygen Biotech. Co. LTD.

HeLa, HepG2, MCF-7, A549 and HUVEC cells were used in the antineoplastic activity assay from Cell Bank, Shanghai Institutes for Biological Sciences, CAS. Cancer cells were seeded in 96-well plates with a density of 10^4 cells per well, and after 12 hours of incubation at 5% CO_2 and 37 °C, the culture medium was removed and the cells were incubated with L^1 – L^7 dissolved in DMEM at different concentrations (each concen-

tration repeated 3 times) for 36 h at 5% CO_2 and 37 °C. Subsequently, the culture medium was removed and fresh culture medium containing MTT (1 mg mL^{-1}) was added, followed by incubation for 4 h to allow the formation of formazan dye.^{40–42} After removing the medium, 200 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 595 nm in a microplate photometer. Cell viability values were determined (at least three times) according to the following formula: cell viability (%) = the absorbance of the experimental group/the absorbance of the blank control group $\times$ 100%.

4.4.2 Induction of apoptosis by flow cytometric analysis.

The induction of apoptosis assay was performed using a Becton Dickinson Ultra-high speed separation flow cytometry instrument and Annexin V-FITC/PI was purchased from Nanjing Keygen Biotech. Co. LTD.

We further investigated whether L^1 and L^2 could induce apoptosis, and DMSO was used as a negative control. A549 and MCF-7 cells (1×10^6) were cultured in 35 mm dishes and incubated at 37 °C for 24 h. After incubation with DMSO at 5 $\mu g mL^{-1}$, L^1 at 1, 2 or 5 $\mu g mL^{-1}$ and L^2 at 0.1, 1 or 5 $\mu g mL^{-1}$ for 24 h (each concentration repeated 3 times, the incubation time is optimum), the treated cells were washed, trypsinized (non-EDTA), and centrifuged (2000 rpm min^{-1}). Then the cells were collected and resuspended in 500 μL of buffer solutions loaded with Annexin V-FITC apoptosis detection reagent (with 5 μL Annexin V-FITC and 5 μL PI). The Annexin V-FITC-stained cells were incubated for 5–15 min in the dark, and approximately 10 000 cells were collected for flow cytometry analysis with a single 488 nm argon laser.

4.4.3 Confocal fluorescence images. In this work, HeLa cells were seeded on 35 mm glass dishes for 24 h, then incubated with L^0 -FITC (100 nM) in the dark for 2 h or 24 h at 5% CO_2 and 37 °C before fluorescence imaging. The control cells were treated with L^0 -FITC (100 nM) at 4 °C in the dark for 2 h. After incubation, the culture medium was removed and the cells were washed with PBS (pH = 7.4), and then incubated with fresh medium. Confocal fluorescence images were detected with excitation at 488 nm and emission at 519 nm.

Conflicts of interest

There are no conflicts of interest to declare.

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