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Synthesis of 9,9-Dialkyl-4,5-diazafluorene Derivatives and Their Structure–Activity Relationships Toward Human Carcinoma Cell Lines

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A homologous set of 9,9-dialkyl-4,5-diazafluorene compounds were prepared by alkylation of 4,5-diazafluorene with the appropriate alkyl bromide and under basic conditions. The structures of these simple organic compounds were confirmed by spectroscopic techniques (FTIR, NMR, and FABMS). Their biological effects toward a panel of human carcinoma cells, including Hep3B hepatocellular carcinoma, MDAMB-231 breast carcinoma, and SKHep-1 hepatoma cells, were investigated; a structure–activity correlation was established with respect to the length of the alkyl chain and the fluorene ring structure. The relationship between the mean potency [$\log(1/IC_{50})$] and alkyl chain length was systematically studied. The results show that compounds with butyl, hexyl, and octyl chains exhibit good growth inhibitory effects toward these three human carcinoma cell lines, and the 9,9-dihexyl-4,5-diazafluorene further exhibits antitumor activity in athymic nude mice Hep3B xenograft models. For the structurally related dialkylfluorenes that lack the diaza functionality, in vitro cytotoxicity was not observed at clinically relevant concentrations.

Introduction

With respect to the classical fluorene-derived segments (9*H*-fluorene **I** and fluoren-9-one **II**), the bidentate ligands 4,5-diazafluorene (9*H*-cyclopenta[2,1-*b*:3,4-*b*′]bipyridine) and 4,5-diazafluoren-9-one (**III** and **IV**, respectively), in which the CH groups of the former are replaced by electron-donating nitrogen atoms, have significant structural differences. In analogy with the fluorene structure, the angle interior to the five-membered ring of **III**, centered at C2, is compressed to ~108°, and the angle exterior to the two fused rings is expanded to ~131°.[1] This type of in-plane distortion in the diazafluorene moiety increases the distance between the pyridyl nitrogen lone-pair electrons and thus influences their cooperativity.[2] Both **III** and **IV** show unique and distinctive properties relative to their structurally related 2,2′-bipyridine (bipy) and 1,10-phenanthroline (phen) analogues due to the fluorene moieties (Figure 1).[3] The fluorene skeletons can affect the metal chelation properties of these compounds, and there are clear differences in the coordinating and bonding properties between **III** and bipy (or phen).[4] Scaffold **III** remains essentially coplanar, and the methylene bridge in such 3,3′-annelated 2,2′-bipyridines distorts the bipyridine portion of the molecule; the resulting increase in the bite angle decreases the metal–nitrogen overlap.[3a, 5] Hence, **III** is a weaker σ-bonding ligand than bipy, and is lower than bipy in the spectrochemical series which causes a lower-

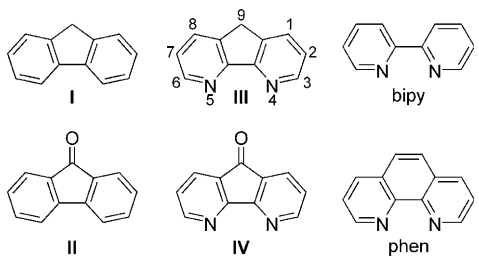

Figure 1. Structures of various 3,3′-annealed 2,2′-bipyridine ligands and related compounds.

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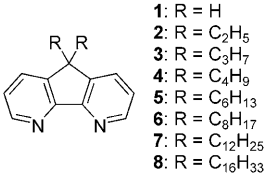
ing of any ligand-field states. This could promote the dissociation of one of the donor nitrogen atoms, and provides a unique pathway for the formation of labile binuclear complexes that could be catalytically active.[5] For instance, the two coordinating nitrogen atoms in **IV** have a larger bite distance (2.99 Å) relative to phen (2.65 Å), which would result in unusual coordination modes and would give rise to different redox and biological properties.[6] Specifically, the diazafluorene-type ligand was preferred over the bipyridyl analogue in some cases for its planar configuration.

While derivatives of bipy exhibit a rich coordination chemistry, their functional properties remain to be explored. Indeed, their bridged diquaternary derivatives have been studied extensively, with the potent herbicide diquat as an illustrative example.[7] Rebek et al. have also bridged bipy at the 3- and 3′-positions with ethylenedioxy units to create macrocycles capable of eliciting an allosteric effect by site-specific metal binding.[8] In another context, 2,7-bis[2-(*N*,*N*-diethylamino)ethoxy]-fluoren-9-one (tilorone) is known to be a low-molecular-weight interferon inducer,[9] and in view of its potential clinical applications, various biological activities of tilorone and its analogues have been widely studied.[10] It is believed that the in vitro interaction of tilorone with double-stranded DNA could be responsible for its various biological activities, including interferon induction and independent antiviral activity.[11] Novel classes of 1,8-diazafluorene and 4,7-phenanthroline derivatives were also investigated as DNA-complexing agents, and their cellular activities as well as interactions with nucleic acids in vitro were examined.[12] Recently, the metallated analogues of diazafluorene ligands[4] have also gathered attention in biochemical and biomedical applications, and Zaleski and co-workers saliently reported the use of bis(9-diazo-4,5-diazafluorene)copper(II) nitrate as an effective DNA photocleaving agent under anaerobic conditions using visible light. This complex can serve as a potential model for the action of kinamycin antitumor antibiotics such as kinamycin C.[13] The study of 4,5-diazafluorene derivatives is thus expected to continue to attract considerable research interest.

Herein we describe the preparation, chemical characterization and biological evaluation of the antitumor activity of compounds **1**–**8** (Figure 2) comprising a set of 9,9-dialkyl-4,5-diazafluorenes and their parent compound 4,5-diazafluorene. The growth inhibition potential of these compounds was screened with respect to alkyl chain length toward three human carcinoma cell lines: Hep3B hepatocellular carcinoma (HB-8064), MDAMB-231 breast carcinoma (HTB-26), and SKHep-1 hepatoma (HTB-52). Remarkably, the in vitro cytotoxicity of these compounds depends on the length of the alkyl substituents.



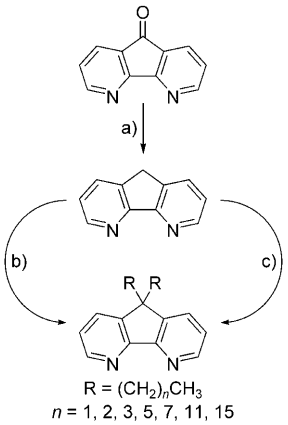
1: R = H
2: R = C_2H_5
3: R = C_3H_7
4: R = C_4H_9
5: R = C_6H_{13}
6: R = C_8H_{17}
7: R = $C_{12}H_{25}$
8: R = $C_{16}H_{33}$

Figure 2. Structures of the diazafluorene derivatives **1**–**8**.

Results and Discussion

Synthesis and characterization

The structures and synthesis protocols of the studied set of novel diazafluorene-based compounds are shown in Scheme 1. 4,5-Diazafluoren-9-one was prepared in one pot by oxidative ring contraction of phen with aqueous potassium permanganate in the presence of potassium hydroxide in water.[3a,14] In this step, it is important to increase the solution basicity and to add a dilute solution of potassium permanganate more slowly in order to favor ring contraction. This enables a more rapid benzyl–benzylic acid ring contraction of 1,10-phenanthroline-5,6-quinone to optimize the synthetic yield of 4,5-diazafluoren-9-one. 4,5-Diazafluorene **1** was obtained by reduction of 4,5-diazafluoren-9-one with neat hydrazine hydrate at 130 °C for 24 h.[1,3a,15] Two different methods were attempted to afford **2**–**8** in various overall yields via the alkylation of **1** with a suitable alkyl bromide using sodium hydride or sodium hydroxide as the base.[16] We initially used sodium hydride for several compounds, but the yields were quite unsatisfactory; however, the amount obtained was still sufficient for our studies. The use of sodium hydroxide gave a better yield in the majority of cases. These air-stable compounds were isolated in high purity as sticky oils or solids (depending on alkyl chain length) by column chromatography on silica gel with the proper eluent. These compounds were fully characterized by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (both fast atom bombardment (FAB) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) modes). However, with the exception of **1**, all attempts to get good-quality single crystals for X-ray diffraction analysis from solid samples have met with little success so far. We tested their possible biological effects toward a panel of human carcinoma cells, including Hep3B hepatocellular carcinoma, MDAMB-231 breast carcinoma, and SKHep-1 hepatoma cell lines.



Scheme 1. Synthesis of the new 9,9-dialkylated diazafluorene derivatives. Reagents and conditions: a) $NH_2NH_2{\cdot}H_2O$, 130 °C, 24 h; b) $Br(CH_2)_nCH_3$, NaH, THF, reflux, 2 h; c) $Br(CH_2)_nCH_3$, NaOH, $(PhCH_2)Et_3NCl$, DMSO, 60 °C, 24 h.

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Antitumor activities and structure–activity relationships

As shown in Figure 3, while the screening test was carried out starting from an initial concentration of 50 μM, we noted an increase in cytotoxic activity of these compounds with increasing alkyl chain length from $n=1$ to $n=6$; there is an optimum at $n=6$, and cytotoxicity then decreases for homologues with alkyl chains of $n=8$ or greater. The reason behind this observed increase in potency with increasing alkyl chain length is not yet known, but it may be due to an increase in lipophilicity and hence greater intracellular accumulation of the compounds.[17] Further lengthening of the alkyl chains beyond $n=6$ may be detrimental to cellular drug uptake through steric and solubility factors (see below).

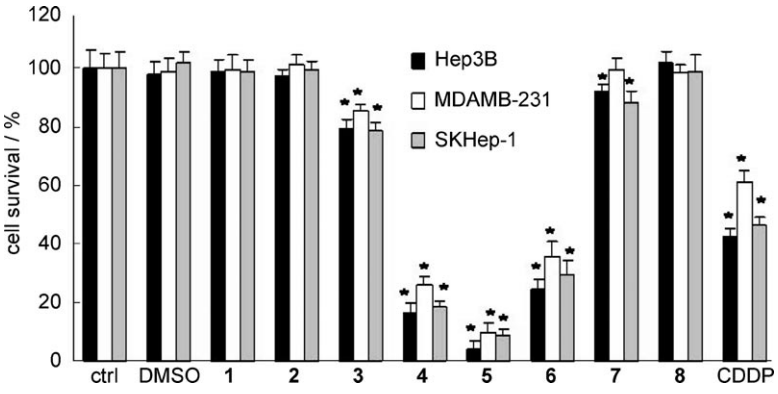

Figure 3. Survival of Hep3B, MDAMB-231, and SKHep-1 human carcinoma cells after treatment with compounds **1**–**8** (all at a final concentration of 50 μM) for 48 h shown as a percentage of survival of untreated control cells. Biological activity was determined by the sulforhodamine B protein staining assay. Cisplatin (CDDP) was used as a positive reference compound; DMSO was used at 0.05%. Each determination was made in triplicate; three independent experiments were performed, and similar results were obtained. Results represent the mean ±SD from one representative experiment; *$p<0.05$ relative to untreated control.

Because only compounds **4**, **5**, and **6** exhibit inhibition of cell growth to an extent >50% relative to untreated controls, we also determined their IC_{50} values.[18] Among them, compound **5** showed the highest cytotoxicity. Morphological investigation under inverted microscopy after staining with sulforhodamine B[19] revealed the formation of apoptotic bodies with both Hep3B and SKHep-1 cancer cells after treatment with compound **5** (Figure 4). However, under the same conditions, compound **5** showed a lower cytotoxic effect toward primary culture bone marrow cells obtained from two patients with a nonmalignant hematological disorder. This suggests that compound **5** may exhibit greater cytotoxicity toward malignant cells than nonmalignant bone marrow cells (Table 1).[20] We also tested the cytotoxicity of the non-nitrogen-containing model compounds of **4** and **5** (namely, 9,9-dibutylfluorene and 9,9-dihexylfluorene),[16b,21] and remarkably, both fluorene species are not cytotoxic, even at 100 μM, suggesting that the chelating nitrogen atoms play a key role in antitumor activity.

The caspase family plays an essential role in apoptosis,[20] among which, caspase-9 is an essential family member involved in the mitochondria-dependent programmed cell death pathway. Accordingly, we determined whether compound **5** is able to stimulate caspase-9 activation. As shown in Figure 5, incubation of cancer cells with compound **5** at 15 μM for 48 h significantly induced caspase-9 activity. Moreover, pre-incubation of the cancer cells with the pan-caspase inhibitor zVADfmk at 20 μM significantly reversed its activity; to determine whether caspase inhibition could reverse apoptosis induced by compound **5**, Hep3B, MDAMB-231, and SKHep-1 human cancer cells were pre-incubated with zVADfmk prior to the addition of compound **5** at 10 μM. After 48 h incubation, cell viability was again determined by the sulforhodamine B protein staining assay. As shown in Figure 6, pre-incubation of this pan-caspase inhibitor could only partially reverse the growth inhibitory effect of compound **5** on all the three cancer cell lines. We therefore hypothesize that the caspase-dependent pathway is only one of the possible ways to mediate the signal that is stimulated by treatment with compound **5**.

Athymic nude mice xenografted with human Hep3B hepatocellular carcinoma cells were employed to further demonstrate the possible antitumor activity of compound **5** in vivo.[22] Fourteen mice xenografted with Hep3B cancer cells exhibiting an average tumor volume of ~200 mm^3 received daily intraperitoneal injections of either buffer carrier (50 μL) or an equal volume of compound **5** at 10 mg kg^{-1} for a period of 9 days. As shown in Figure 7, on day 9, mean tumor size of the test group was roughly half that of the vehicle control group. Representative mice are also shown in Figure 8a.

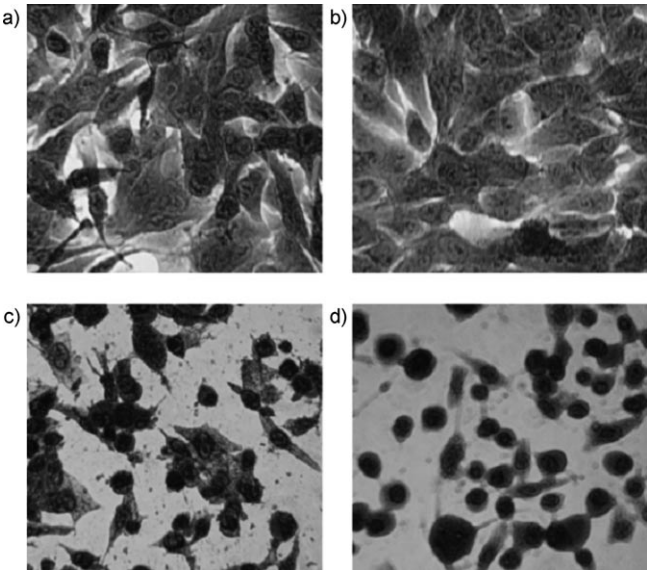

Figure 4. Morphological changes of Hep3B [a) and c)] and SKHep-1 [b) and d)] human carcinoma cells after treatment with compound **5**, staining with sulforhodamine B, and comparison with DMSO vehicle control [a) for Hep3B and b) for SKHep-1]. Compound **5** was added at 12.5 μM for a cell culture period of 48 h.

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Table 1. IC_{50} values of compounds **4**, **5**, **6**, cisplatin (CDDP), and doxorubicin (DOX) toward the three human carcinoma cell lines tested after 48 h incubation.[a]

Cell line	4	5	6	CDDP	DOX
Hep3B	13.3±3.2	6.6±1.5	21.8±4.3	38.2±2.9	3.1±1.2
MDAMB-231	18.6±2.4	10.3±0.8	26.4±2.1	79.3±5.3	9.7±0.3
SKHep-1	14.7±2.6	7.9±1.3	23.6±0.7	34.7±2.6	3.4±1.4
BM1	ND	14.7±0.6	ND	ND	ND
BM2	ND	13.2±2.2	ND	ND	ND

[a] Results are based on the sulforhodamine B assay. CDDP and DOX were used as positive reference compounds. Each determination was performed in triplicate. Three independent experiments were performed, and similar results were obtained. Data represent the mean ±SD from one representative experiment; ND = Not determined; BM = Nonmalignant hematological disorder bone marrow cells.

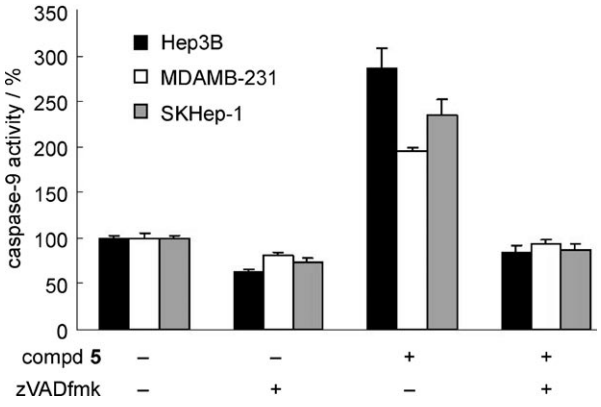

Figure 5. Caspase-9 activity assay with Hep3B, MDAMB-231, and SKHep-1 human cancer cells to study the apoptosis-inducing potential of compound **5** after 48 h incubation at a concentration of 15 μM; zVADfmk (20 μM) is an inhibitor of caspase-9 activation. Results are expressed as a percentage of the control and represent the mean ±SD from triplicate tests; the data shown are from a representative experiment taken from three independent experiments giving similar results.

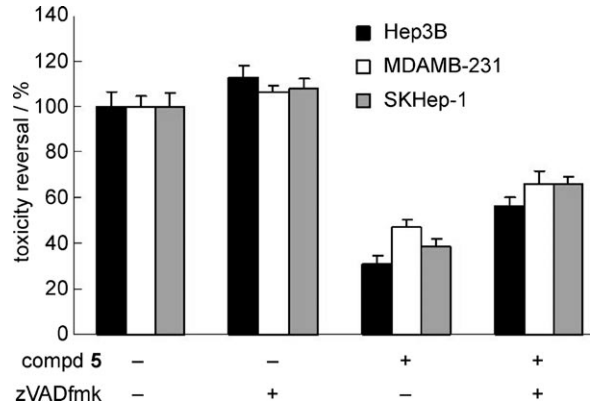

Figure 6. Analysis of the possible reversal effects of the pan-caspase inhibitor zVADfmk (20 μM) on the cytotoxicity of compound **5** after 48 h incubation at a concentration of 15 μM toward Hep3B, MDAMB-231, and SKHep-1 cancer cells. Results shown represent the mean ±SD from triplicate tests; the data shown are from a representative experiment taken from three independent experiments giving similar results.

To further determine whether compound **5** exerts any toxic effects in the kidney, hematoxylin and eosin (H and E) staining

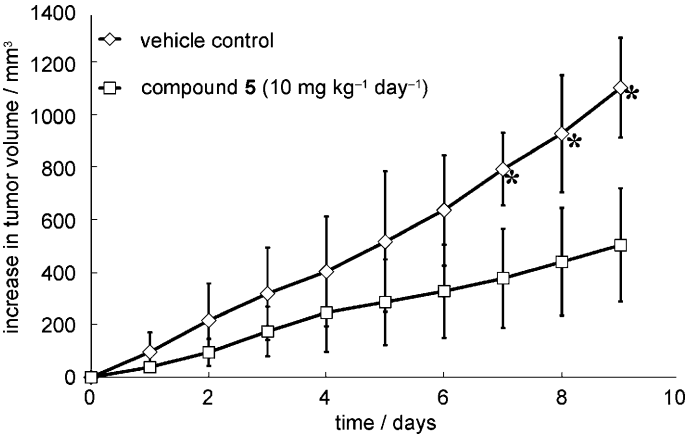

Figure 7. Representative results showing the changes of tumor volume (mm^3) as a function of time for mice treated with vehicle or compound **5**. Intraperitoneal injection started for both groups when the mean tumor volume of mice reached ~200 mm^3 on day 0. A total of 14 mice were randomly divided into two groups. On day 9, after measuring individual tumor volume, all mice were sacrificed for plasma collection. Results represent the mean ±SD from seven animals; $*p < 0.05$ relative to untreated controls.

of kidney sections were investigated. We found no observable necrotic features from autopsy samples of all the 14 mice analyzed (Figure 8b). Furthermore, analysis of plasma liver functional enzymes, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) showed normal liver functions in the mice treated with compound **5** (Table 2). H and E staining of tumor sections showed high cellular integrity, which suggests that **5** does not induce necrosis in the xenograft under the therapeutic dosage used (Figure 8c).

For comparison, the in vitro antitumor activities of all candidates in the homologous series against Hep3B, MDAMB-231, and SKHep-1 cell lines were expressed as the concentration of compounds that exhibit 50% inhibition (IC_{50}). The relationship between the mean potency [$\log(1/IC_{50})$] and n (excluding the ethyl and decyl analogues, which exhibit potencies much lower than 4.3) is illustrated in Figure 9. The hexyl analogue appears to be the best candidate for antitumor activity, with the highest cytotoxicity toward the selected cell lines. These results suggest a better permeability of this candidate across the cell membrane, which in turn explains its greater cytotoxicity, consistent with some published reports.[17b,c] However, with $n > 6$, the compounds may tend to aggregate in the culture medium, probably owing to stronger dipole–dipole interactions among the side chains.[23] Hence, the variations in solubility and aggregation tendencies in the culture medium within this compound series could explain the observed trend in cytotoxicity with $n = 6$ and greater. It appears that the hexyl compound shows the best balance between solubility and lipophilicity.

Conclusions

We have developed a novel class of 9,9-dialkylated derivatives of 4,5-diazafluorene that show a good therapeutic potential to be exploited in bioorganic and medicinal chemistry studies.

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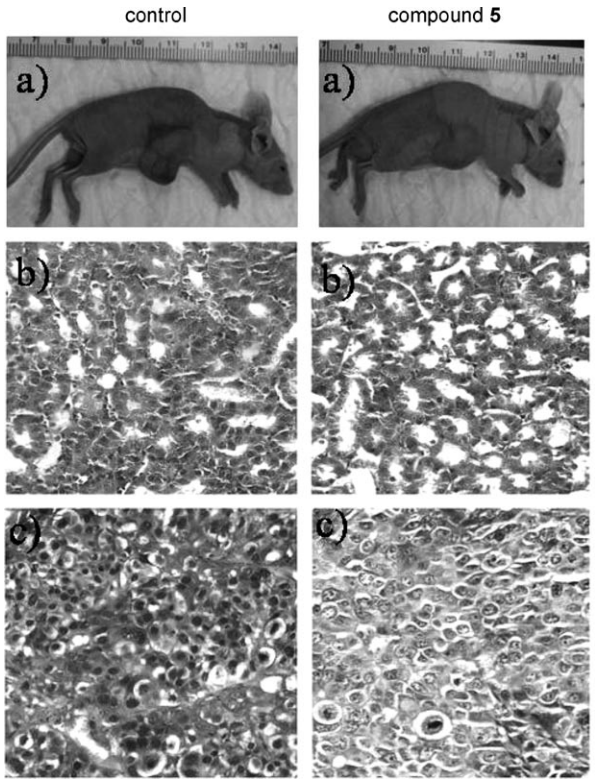

Figure 8. a) Representative Hep3B xenografted nude mice treated with vehicle (left column) or compound **5** (right column) at 10 mg kg^{-1} for nine consecutive days. The tumor size of the Hep3B xenograft treated with vehicle is larger than that of the animal treated with compound **5**. b) Histochemistry study of kidney sections from sacrificed mice to observe any possible kidney toxicity; neither necrotic nor damaged tissue was observed. c) Histochemistry study of Hep3B tumor sections from sacrificed mice to detect any possible xenograft tumor necrosis; no systemic necrosis was observed.

Table 2. Plasma liver enzyme assays for vehicle control and compound **5**-treated Hep3B xenografted athymic nude mice.[a]

Enzyme	Vehicle	**5**	Reference value
ALT	67.9 ± 12.8	57.1 ± 14.7	28-132
AST	197.0 ± 41.5	161.9 ± 45.3	59-246

[a] Number of mice = 7 for both vehicle control group and test group treated with compound **5**. Enzyme levels were determined by Vet biochemistry assay kits (IDEXX Laboratories instrument) and are expressed as units per liter. Results represent the mean ± SD.

We evaluated their possible utility as anticancer agents and also studied the structure–activity correlation between antitumor efficacy, alkyl chain length, and the nature of fluorene ring. There is a clear relationship between the mean potency [log(1/IC_{50})] and alkyl chain length. The results obtained on the in vitro antitumor activity of compounds **1**–**8** toward the three different human carcinoma cell lines demonstrate that the hexyl congener is the best candidate for the selected cell lines; these results are compatible with improved lipophilic entry of this compound across the cell membrane. As alkyl chain length increases beyond $n = 6$, the compounds show a decreased cellular uptake because of solubility/lipophilicity issues and/or a greater aggregation tendency. In the absence of the diaza functionality, cytotoxicity is abolished for the dialkylfluorenes at clinically relevant concentrations. The particular action of these compounds toward different cancer cell lines of human origin could be of great importance in the future search for novel antitumor drugs. Research is ongoing to investigate the factors that may modify the biological properties of these compounds. The influence of metal complexation with these diazafluorene-type ligands on their biological effects is worthy of future examination.

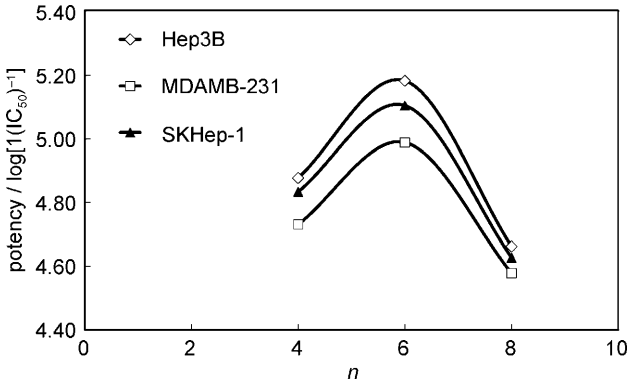

Figure 9. Cytotoxic potency of the homologous series plotted as a function of alkyl chain length (*n*).

Experimental Section

General

All reactions were performed under N_2 atmosphere. Solvents were carefully dried and distilled from appropriate drying agents prior to use. Commercially available reagents were used without further purification unless otherwise stated. 4,5-Diazafluoren-9-one was prepared according to published procedures.[3a, 14] All reactions were monitored by thin-layer chromatography (TLC) with Merck pre-coated glass plates. Flash-column chromatography was carried out with silica gel from Merck (230–400 mesh). FABMS data were recorded in *m*-nitrobenzyl alcohol matrices on a Finnigan MAT SSQ710 system, and high-resolution (HR) MALDI-TOF mass spectra were obtained on an Autoflex Bruker MALDI-TOF MS instrument. ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ on a Bruker 400 MHz FT-NMR spectrometer, and chemical shifts (δ) are reported in ppm relative to $(CH_3)_4Si$.

Synthesis of 1: This compound was prepared according to a slight modification of published procedures.[1, 3a, 15] A mixture of 4,5-diazafluoren-9-one (500 mg, 2.74 mmol) and $NH_2NH_2{\cdot}H_2O$ (5 mL) was heated at 130 °C for 24 h. After cooling, the mixture was extracted with CH_2Cl_2 (3 × 25 mL). The organic layers were combined and dried over anhydrous Na_2SO_4; solvents were removed in vacuo. The crude product was purified by column chromatography on silica gel, eluting with EtOAc to provide **1** (295 mg, 1.75 mmol) as a white solid in 64% yield. $R_f = 0.28$ (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.70$–8.75 (d, $J = 3.6$ Hz, 2H), 7.89–7.90 (d, $J = 7.2$ Hz, 2H), 7.29–7.32 (m, 2H), 3.88 ppm (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 159.01$, 149.73, 137.55, 133.07, 122.84, 32.45 ppm; FAB-

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MS: *m/z* 168 $[M]^+$; MALDI-TOF HRMS: *m/z* calcd for $C_{11}H_8N_2$: 169.0766, found: 169.0757.

General procedures for the synthesis of 9,9-dialkylated diazafluorene derivatives 2–8

Method A: 4,5-Diazafluorene (0.20 mmol) and NaH (60%, 0.60 mmol) were dissolved in THF (5 mL) in a round-bottomed flask. The appropriate alkyl bromide (0.60 mmol) was added in one portion. The mixture was held at reflux for 3 h, and then the reaction was quenched with H_2O. The solvent was evaporated, and the reaction mixture was extracted with Et_2O and dried over anhydrous Na_2SO_4. The target product was isolated by purification of the crude product using column chromatography on silica gel (first using hexane as eluent to remove excess alkyl bromide, and then CH_2Cl_2/EtOAc (3:1 *v/v*) to elute the product).

Method B: 4,5-Diazafluorene (0.20 mmol) was added to a mixture of DMSO (5 mL) and aqueous 50% NaOH (0.5 mL) in a round-bottomed flask. A catalytic amount of benzyltriethylammonium chloride was added to the reaction mixture followed by the addition of alkyl bromide (0.80 mmol). The mixture was stirred at 60 °C for 24 h. The resulting mixture was poured into EtOAc (30 mL), and the slurry was filtered to remove the solid bromide salt. The filtrate was washed with 1 M HCl and H_2O (2×10 mL), and then dried over anhydrous Na_2SO_4. The desired product was obtained by purification via column chromatography on silica gel (initially using hexane as eluent to remove the excess organic bromide, followed by CH_2Cl_2/EtOAc (3:1 *v/v*) to isolate the product). It was found that this method gave better synthetic yields than method A. We expect that the product yields for those compounds prepared below based on method A only could be improved greatly with the use of method B instead.

9,9-Diethyl-4,5-diazafluorene (2): Sticky dark solid (10% based on method A). R_f = 0.27 (CH_2Cl_2/EtOAc 3:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.71–8.69 (m, 2H), 7.72–7.70 (m, 2H), 7.31–7.28 (m, 2H), 2.10–2.04 (m, 4H), 0.40–0.36 ppm (t, J = 7.4 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.84, 149.57, 144.15, 130.67, 122.93, 52.27, 31.64, 8.60 ppm; FAB-MS: *m/z* 225 $[M]^+$; MALDI-TOF HRMS: *m/z* calcd for $C_{15}H_{16}N_2$: 225.1392, found: 225.1390.

9,9-Dipropyl-4,5-diazafluorene (3): Brown solid (14% based on method A). R_f = 0.37 (CH_2Cl_2/EtOAc 3:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.69–8.68 (m, 2H), 7.73–7.71 (m, 2H), 7.30–7.28 (m, 2H), 2.00–1.96 (m, 4H), 0.70–0.68 ppm (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.46, 149.48, 144.87, 130.58, 122.86, 51.44, 41.50, 17.35, 14.33 ppm; FAB-MS: *m/z* 252 $[M]^+$; MALDI-TOF HRMS: *m/z* calcd for $C_{17}H_{20}N_2$: 253.1705, found: 253.1700.

9,9-Dibutyl-4,5-diazafluorene (4): Dark oil (11% based on method A). R_f = 0.47 (CH_2Cl_2/EtOAc 3:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.70–8.68 (m, 2H), 7.73–7.70 (m, 2H), 7.31–7.28 (m, 2H), 2.02–1.98 (m, 4H), 1.11–1.06 (m, 4H), 0.70–0.62 ppm (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.52, 149.50, 144.89, 130.59, 122.93, 51.23, 38.98, 26.18, 22.94, 13.74 ppm; FAB-MS: *m/z* 281 $[M]^+$; MALDI-TOF HRMS: *m/z* calcd for $C_{19}H_{24}N_2$: 281.2018, found: 281.2026.

9,9-Dihexyl-4,5-diazafluorene (5): Brown oil (22% based on method A and 60% based on method B). R_f = 0.54 (CH_2Cl_2/EtOAc 3:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.70–8.68 (m, 2H), 7.72–7.70 (m, 2H), 7.30–7.27 (m, 2H), 2.01–1.97 (m, 4H), 1.12–1.01 (m, 12H), 0.78–0.74 (t, J = 7.2 Hz, 6H), 0.67–0.63 ppm (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.49, 149.47, 144.90, 130.58, 122.92, 51.31, 39.21, 31.39, 29.54, 23.96, 22.47, 13.95 ppm; FAB-MS: *m/z* 337 $[M]^+$; MALDI-TOF HRMS: *m/z* calcd for $C_{23}H_{32}N_2$: 337.2643, found: 337.2667.

9,9-Dioctyl-4,5-diazafluorene (6): Dark oil (25% based on method A and 63% based on method B). R_f = 0.54 (CH_2Cl_2/EtOAc 3:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.70–8.68 (m, 2H), 7.72–7.70 (m, 2H), 7.30–7.28 (m, 2H), 2.00–1.98 (m, 4H), 1.21–1.04 (m, 20H), 0.83–0.80 (t, J = 7.2 Hz, 6H), 0.64 ppm (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.73, 149.71, 145.15, 130.81, 123.14, 51.54, 39.43, 31.94, 30.10, 29.36, 29.32, 24.21, 22.78, 14.27 ppm; FAB-MS: *m/z* 393 $[M]^+$; MALDI-TOF HRMS: *m/z* calcd for $C_{27}H_{40}N_2$: 393.3270, found: 393.3276.

9,9-Didodecyl-4,5-diazafluorene (7): Pale-yellow oil (12% based on method A). R_f = 0.67 (CH_2Cl_2/EtOAc 3:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.70–8.68 (m, 2H), 7.72–7.70 (m, 2H), 7.30–7.27 (m, 2H), 2.01–1.98 (m, 4H), 1.28–1.04 (m, 36H), 0.90–0.85 (t, J = 7.1 Hz, 6H), 0.67–0.63 ppm (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.51, 149.49, 144.92, 130.58, 122.91, 51.31, 39.20, 31.89, 29.88, 29.58, 29.51, 29.45, 29.31, 29.20, 24.00, 22.68, 14.12 ppm; FAB-MS: *m/z* 505 $[M]^+$; MALDI-TOF HRMS: *m/z* calcd for $C_{35}H_{56}N_2$: 505.4522, found: 505.4515.

9,9-Dihexadecyl-4,5-diazafluorene (8): Pale-yellow solid (56% based on method B). R_f = 0.71 (CH_2Cl_2/EtOAc 3:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.70–8.68 (m, 2H), 7.72–7.69 (m, 2H), 7.78–7.30 (m, 2H), 2.00–1.96 (m, 4H), 1.29–1.04 (m, 52H), 0.89–0.86 (t, J = 7.0 Hz, 6H), 0.67–0.64 ppm (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.52, 149.49, 144.91, 130.57, 122.90, 51.31, 39.20, 32.81, 31.92, 29.87, 29.65, 29.57, 29.44, 29.35, 29.19, 25.73, 24.00, 22.69, 18.44, 14.12 ppm; FAB-MS: *m/z* 617 $[M]^+$; MALDI-TOF HRMS: *m/z* calcd for $C_{43}H_{72}N_2$: 617.5773, found: 617.5812.

Cancer cell culture

The human cancer cell lines Hep3B, MDAMB-231, and SKHep-1 were cultured in RPMI-1640 medium with 5% fetal bovine serum (complete cell culture medium) at 37 °C under an atmosphere containing 5% CO_2 in a humidified incubator.

Bone marrow aspirate

Bone marrow cells from patients with nonmalignant hematological disorder during presentation were obtained after informed consent with approval obtained from the ethical committee of the institute (Haematology Division, Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong). Mononuclear cells were further isolated after Ficoll–Paque separation, and cells were re-suspended in complete cell culture medium.

Sulforhodamine B assays for cell viability

Cancer cells were removed from sterile cell culture flasks with trypsin and neutralized with fetal bovine serum. After washing twice with phosphate buffered saline and centrifugation, cells were re-suspended in complete cell culture medium at a concentration of ~1×10^5 cells mL^{-1} and counted manually using a hemocytometer under an inverted microscope. Cancer cells seeded in 96-well microtiter plates for 24 h were prepared for the screening of our synthesized compounds. The compounds were dissolved in molecular-biology-grade DMSO. Cisplatin (CDDP) and doxorubicin (DOX) were used as the positive reference compounds, and were added at a starting concentration of 50 μM. Test compounds were added at a starting concentration of 50 μM followed by twofold serial di-

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lutions and incubated for a further period of 48 h. The maximum concentration of DMSO employed was 0.1% by volume. Untreated controls received either total complete cell culture medium or 0.1% DMSO. Afterward, evaluation of possible antiproliferative activity of the compounds was performed by sulforhodamine B protein staining methods. Briefly, cancer cells were fixed with trichloroacetic acid (TCA), washed, and stained with sulforhodamine B. Cells were then washed again with acetic acid, and stained cells were resuspended in un-buffered Tris base. Finally, optical absorptions were measured at λ 575 nm using a microplate reader (Victor V, PerkinElmer, Life Sciences). The IC_{50} values of these compounds and of CDDP were calculated from these experimental results.

Caspase inhibition assays

Cancer cells were seeded at a concentration of $\sim 1\times10^5$ cells mL^{-1} and counted manually using a hemocytometer under an inverted microscope. After 24 h, the culture medium was changed, and cells were pre-incubated with a pan-caspase inhibitor (zVADfmk) for 2 h before addition of test compounds. After a further incubation of 48 h, cellular viability was determined by the sulforhodamine B protein staining methods as described above.

In vivo athymic nude mice experiments

Eight-week-old athymic nude mice, weighing ~15–20 g, were purchased from the animal unit of The Chinese University of Hong Kong and maintained in a sterile facility, in accordance with the institutional guidelines on animal care, with the required consistent temperature and relative humidity. All the procedures were approved by the Animal Research Ethics Committee [accreditation number: (10-17)inDH/HA&P/8/2/4Pt.3]. Fourteen athymic nude mice were injected subcutaneously with human hepatocellular carcinoma Hep3B cells. They were housed under sterile conditions. Tumor size was measured daily by electronic calipers. When the tumor size reached a mean volume of ~200 mm^3 [where tumor volume is determined by: $(l\times w\times h)/2$], they were randomly divided into two groups. Compounds at a concentration of 10 mg kg^{-1} body weight were administered intraperitoneally each day for a continuous 9-day period starting from day 1. The control group received only buffer carrier [physiological saline for injection with 2% poly(ethylene glycol)]. Each group consisted of seven mice. On day 10, mice were sacrificed, and H and E staining of autopsy analysis including kidneys from all animals were investigated for any significant toxicology effects, including the occurrence of any necrotic tissue. Tumors were also collected for investigation. Whole blood was also collected, and plasma liver enzymes including ALT and AST were measured by Vet biochemistry assay kits (for the IDEXX Laboratories instrument) to detect any possible liver failure phenomena from both groups of mice by comparison with normal control ranges.

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