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Cytotoxic activity of proflavine diureas: Synthesis, antitumor, evaluation and DNA binding properties of 1′,1″-(acridin-3,6-diyl)-3′,3″-dialkyldiureas

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Abstract—The synthesis of novel 1′,1″-(acridin-3,6-diyl)-3′,3″-dialkyldiureas was reported. Their biological activity to inhibit cell proliferation was assessed by a MTT assay on two cell lines, HeLa and HCT-116, at micromolar concentration. 1′,1″-(Acridin-3,6-diyl)-3′,3″-dihexyldiurea hydrochloride was active on a HCT-116 cell line with an IC_{50} value of 3.1 μM. The interaction of these compounds with calf thymus DNA was investigated by a variety of spectroscopic techniques including UV–vis, fluorescence and CD spectroscopy. From spectrofluorimetric titrations, binding constants for the DNA–drug complexes were determined ($K = 0.9$–$4.2 \times 10^5\ M^{-1}$). Antiproliferative activity of synthesized derivatives might be related to their intercalation into DNA.

1. Introduction

Oncological processes are characterized by uncontrolled increase in cell proliferation; hence the DNA is the biological target of many antitumor drugs and potential antineoplastic agents. Over the last four decades, intense research has been focused on the effect of small organic compounds which non-covalently bind to nucleic acids.[1] These interactions have been shown to disrupt the replication and/or transcription culminating in a cellular death.[2,3] Among different types of DNA interacting agents, intercalating[4–6] and groove-binding molecules[7,8] are important in molecular biology. Intercalators, by definition, bind into DNA by intercalating the flat aromatic ring between the base pairs of the DNA duplex. Typical examples of intercalators are acridinium salts, including proflavine chloride, and amino and glycoconjugates of acridine.[8]

Proflavine (3,6-diaminoacridine) causes a frameshift mutation in viruses, bacteriophages, and bacteria, but unfortunately may also show a DNA-breaking activity in mammalian cells. This and other chromosomal mutations seem to be related to the ability of proflavine to stabilize DNA-topoisomerase II intermediates.[9,10] The physicochemical properties of the proflavine moiety permit the use of sensitive spectroscopic techniques to examine the nature of interactions of appropriate derivatives with DNA.[11]

In our previous work,[12] we determined the binding affinity, binding constants and cytotoxic activity of proflavine-dithiazolidinones, promising novel DNA-targeted anticancer agents synthesized from thiourea precursors, with plasmid and calf thymus DNA. The activity observed inspired us to explore binding properties of relative but sulfur free 1′,1″-(acridin-3,6-diyl)-3′,3″-dialkyldiureas to exploit considerable intercalation

Keywords: Cytotoxic activity; Acridine derivatives; Intercalation; DNA.
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capability of fluorescent proflavine chromophore derivatized by more natural urea functionality. Attention was especially paid to the influence of structural changes on the intercalation properties, in particular, with reference to elongation of the urea alkyl chain in positions 3′, 3″ of proflavine.

UV–vis spectrophotometry, fluorescence titration, and CD spectroscopy all were employed to study the interaction of prepared compounds with calf thymus DNA (ctDNA). Anticancer properties of prepared derivatives were tested against two tumor cell lines, a human cervical epitheloid carcinoma HeLa and human colorectal cancer cells HCT-116.

2. Results and discussion

2.1. Chemistry

As a starting compound for the preparation of newly designed intercalators, 3,6-diisothiocyanatoacridine (**2**) obtained from a commercially available proflavine hemisulfate (**1**) was used. To incorporate aliphatic chains into resulting structures, intermediate dithioureas **3a**–**e** have been synthesized from **2** and aliphatic amines according to our procedure.[12] Conversion of dithioureas **3a**–**e** into corresponding diureas **4a**–**e** was carried out by mesitylnitrile oxide in methanol (Scheme 1). To improve the solubility and activity of products **4a**–**e**, their hydrochlorides **5a**–**e** were prepared by the treatment with methanolic HCl.

2.2. Biological studies

A MTT analysis employing the concentration range from 1 to 100 μM revealed an inverse relationship between the length of the carbon chain and IC_{50} of particular derivative (apart from **5c**) for both HeLa and HCT-116 cells (Table 1).

However, diverse reactions in two cell lines have been observed under further investigation. Analysis of floating cells percentage and total proliferation of HeLa cells (Table 2) revealed significant, though relatively weak reactions (24 h after the drug administration) which vanished later (48 h after administration of **5a**–**e**). HCT-116 cells proved to be more susceptible and their numbers dropped intensively as the percentage of floating cells increased (Table 2). The most intense effects within HCT-116 cells were observed after treatment with derivatives **5c**, **5d**, and **5e**. Derivatives **5a** and **5b** were ineffective in modulating a cell cycle progression (Table 3), however, they suppressed the cell proliferation significantly with no effect to percentage of floating cells or apoptosis incidence (Table 2). Derivatives **5c**, **5d** and **5e** caused an enormous increase in percentage of floating cells and depleted total cell population of HCT-116 down to almost 20% of untreated control. These changes were linked also with alterations in the cell cycle progression.

Analysis of a cell cycle distribution of HeLa cells revealed no significant changes when treated with **5a**–**d** and only a slight increase in the *S*-phase on account of G_0/G_1 when treated with **5e** (48 h) (Table 3). Incubation of HCT-116 cells with derivatives **5c**–**e** induced an intensive arrest in the G_2/M-phase 24 h (**5d** only) and 48 h (**5c**–**e**) accompanied by a decrease of cells in the *S*-phase (**5d** and **5e**, 24 h) or in the G_0/G_1-phase of cell cycle (**5e**, 48 h).

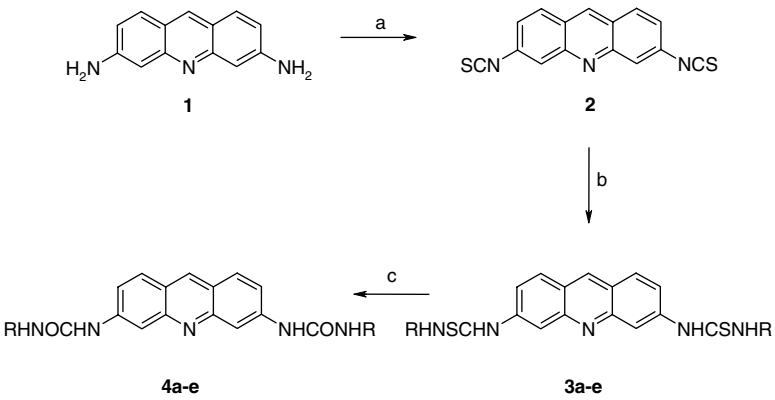
Apoptosis incidence with HeLa cells did not reveal any significant results. On the contrary, HCT-116 cells behaved differently and **5c**–**e** derivatives stimulated their apoptotic response already 24 h after the drug administration which deepened further after 48 h incubation with **5d** and **5e** (Table 2).

Qualitative evaluation of a DNA fragmentation by a flow cytometric analysis of cell cycle in logarithmic scale (evaluated as subG_0/G_1–subdiploid particles) by propidium iodide staining (PI) proved to be significant, highly intensive and correlated with the morphological analysis of apoptosis and cytokinetical analysis of total and floating cells percentage (Fig. 1). Whereas insignificant changes had been observed with HeLa (**5a**–**e**) and HCT-116 cells (**5a, 5b**), incubation of HCT-116 cells with **5c**–**e** induced intensive DNA fragmentation observable 24 h and 48 h after the drug administration (48 h analysis is only presented). These results support surmise that derivatives **5c**–**e** induced cell death in HCT-116 whereas **5a** and **5b** effected over cell proliferation that might be considered as inhibitory.

Comparison of HeLa and HCT-116 cells revealed a transitive inhibition of the cell metabolism in more slowly proliferating HeLa cells,[13] however, a strong inhibitory/cytotoxic impact on the proliferation, survival, and cell death of intensively growing colon carcinoma HCT-116 cells.[14] These differences are notable particularly with **5c**, **5d**, and **5e** derivatives. In addition, our results documented an extensive spectrum of cell responses to various derivatives at their IC_{50} doses evaluated by the MTT test. Complete analysis of all available data showed a strong inhibition of the oxidative metabolism with a relatively weak effect on the cell proliferation and the cell cycle progression within HeLa cells compared to a cell population depletion and an intensive cell death incidence in HCT-116. The IC_{50} determined 72 h after the **5e** drug treatment of HeLa cells was 3.695 ± 1.499 μM and it is in good agreement with a IC_{50} value for clinically established chemotherapeutic drugs: cisplatin (2.9 ± 0.6 μM) and etoposide (2.9 ± 1.0 μM).[15] Compound **5e** was cytotoxic against the HCT-116 cell lines, producing IC_{50} values of 3.094 ± 1.408 μM. This drug displayed a more potent cytotoxicity than that of miltirone (12.39 ± 0.75 μM).[16]

2.3. Spectral and DNA binding properties

The binding of compounds **5a**–**e** with ctDNA was measured using both absorption and fluorescence spectrophotometric titrations. The application of two methods was enabled by the low polarizability and strong absorption of diureas **5a**–**e** in the near UV region as well as their fluorescence due to a long-living triplet exited state of the aromatic system.[17]



Scheme 1. Synthesis of diureas **4a**–**e**. Reagents: (a) $CSCl_2$, $CHCl_3$, H_2O, Na_2CO_3; (b) R-NH_2, MeOH, R = ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl; (c) mesitylnitrile oxide, MeOH.

Table 1. Cytotoxic activity of **5a**–**e**[a]

	IC_{50} HeLa (μM)		IC_{50} HCT-116 (μM)	
	48 h	72 h	48 h	72 h
5a	63.940 ± 7.520	60.047 ± 3.727	27.624 ± 2.310	34.714 ± 3.842
5b	23.866 ± 4.441	29.256 ± 3.386	10.344 ± 1.654	61.510 ± 7.473
5c	5.411 ± 1.651	11.506 ± 4.846	76.968 ± 6.746	43.854 ± 5.006
5d	7.777 ± 0.886	13.349 ± 3.671	5.120 ± 1.440	3.476 ± 0.803
5e	3.491 ± 0.979	3.695 ± 1.499	4.765 ± 0.838	3.094 ± 1.408

[a] IC_{50} values were evaluated by MTT cytotoxicity test 48 or 72 h after the drug administration. Concentration ranges 1–100 μM (**5a**–**d**) or 1–25 μM (**5e**). Results are expressed as a mean ± SD ($n = 3$).

Table 2. Percentage of floating cells in total population, total cell population and cells with apoptotic morphology of nucleus

Cell populations				Floating (%)	Total (%)	Apoptic (%)
Cells	Time	Group	*c* (μM)			
HeLa	24 h	Control	0.00	3.41 ± 0.92	100.00 ± 4.60*	1.11 ± 0.57
		5a	64.00	3.58 ± 1.06	95.15 ± 3.30***	1.33 ± 0.54
		5b	24.00	3.84 ± 1.30*	91.36 ± 3.66*	0.89 ± 0.57
		5c	5.50	4.03 ± 1.14	92.75 ± 7.09***	1.22 ± 0.57
		5d	7.80	3.86 ± 1.16	87.47 ± 6.12***	1.33 ± 0.54
		5e	3.50	4.21 ± 1.19	87.42 ± 6.82***	0.75 ± 0.36
	48 h	Control	0.00	4.07 ± 0.45	100.00 ± 2.41	0.33 ± 0.27
		5a	64.00	4.63 ± 1.13	93.61 ± 14.54	0.89 ± 0.42
		5b	24.00	4.56 ± 1.01	89.95 ± 10.19**	1.00 ± 0.27
		5c	5.50	3.93 ± 0.33	96.5 ± 12.77	0.44 ± 0.16
		5d	7.80	3.62 ± 0.36**	101.34 ± 8.67	0.89 ± 0.42
		5e	3.50	4.41 ± 0.63	91.16 ± 9.21**	1.00 ± 0.82
HCT-116	24 h	Control	0.00	4.19 ± 1.10	100.00 ± 5.57	1.67 ± 0.27
		5a	27.00	6.20 ± 1.88***	79.40 ± 5.83***	3.22 ± 1.23
		5b	10.00	5.48 ± 1.85**	86.16 ± 5.98***	2.22 ± 1.10
		5c	77.00	51.08 ± 0.40***	47.40 ± 3.16***	9.67 ± 2.42*
		5d	5.10	78.81 ± 12.05***	45.85 ± 5.68***	10.67 ± 3.00*
		5e	4.80	77.94 ± 11.96***	48.10 ± 4.92***	5.67 ± 0.98*
	48 h	Control	0.00	4.00 ± 1.54	100.00 ± 3.36	1.78 ± 0.42
		5a	27.00	5.68 ± 3.54	75.37 ± 17.47***	3.44 ± 2.04
		5b	10.00	4.34 ± 1.25	71.30 ± 5.29***	2.89 ± 0.96
		5c	77.00	33.41 ± 16.40***	27.58 ± 3.32***	3.89 ± 1.81
		5d	5.10	88.58 ± 5.19***	21.22 ± 7.84***	11.33 ± 0.00***
		5e	4.80	88.55 ± 7.42***	19.84 ± 6.87***	12.67 ± 0.67***

The results are presented as a mean ± SD ($n = 3$), statistical significance: $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) for particular experimental group compared to untreated control.

The electronic absorption spectra of proflavine derivatives **5a**–**e** exhibited broad absorption bands in the region 325–475 nm typical for transitions between the π-electronic energy levels of the proflavine skeleton. The absorption spectra of **5a**–**e** (5.5 μM) were consequently recorded as a series against increasing

Table 3. Cell cycle distribution

Cell cycle phase				G_0/G_1 (%)	S (%)	G_2/M (%)
Cells	Time	Group	c (μM)			
HeLa	24 h	Control	0.00	62.01 ± 3.16	23.84 ± 2.55	14.15 ± 0.62
		5a	64.00	63.51 ± 0.92	23.73 ± 1.41	12.75 ± 1.54
		5b	24.00	62.48 ± 3.97	24.12 ± 3.15	13.40 ± 1.39
		5c	5.50	61.17 ± 3.78	24.95 ± 2.75	13.88 ± 1.46
		5d	7.80	61.00 ± 2.76	25.46 ± 2.69	13.54 ± 0.73
		5e	3.50	59.69 ± 1.77	27.08 ± 0.45	13.24 ± 1.34
	48 h	Control	0.00	74.71 ± 3.93	15.95 ± 2.19	9.35 ± 1.77
		5a	64.00	71.26 ± 2.11	19.50 ± 1.84	9.23 ± 0.30
		5b	24.00	72.81 ± 3.34	17.12 ± 2.71	10.06 ± 0.84
		5c	5.50	74.91 ± 2.95	15.50 ± 2.06	9.60 ± 0.89
		5d	7.80	72.24 ± 3.00	17.38 ± 2.54	10.39 ± 0.46
		5e	3.50	66.57 ± 0.50*	21.55 ± 0.41*	11.88 ± 0.31
HCT-116	24 h	Control	0.00	42.68 ± 3.81	38.42 ± 3.14	18.89 ± 0.92
		5a	27.00	47.44 ± 3.38	34.06 ± 2.38	18.50 ± 4.30
		5b	10.00	45.07 ± 2.89	35.80 ± 2.74	19.14 ± 1.83
		5c	77.00	39.97 ± 2.03	22.52 ± 9.09	37.51 ± 9.71
		5d	5.10	38.62 ± 6.81	17.34 ± 7.35*	44.05 ± 11.80*
		5e	4.80	33.52 ± 6.82	21.38 ± 7.93*	45.10 ± 14.59
	48 h	Control	0.00	56.06 ± 7.30	29.25 ± 4.14	14.70 ± 3.90
		5a	27.00	53.70 ± 1.31	31.45 ± 0.69	14.85 ± 1.25
		5b	10.00	57.74 ± 2.44	27.16 ± 1.94	15.11 ± 0.86
		5c	77.00	43.91 ± 2.81	27.24 ± 5.93	28.85 ± 4.72*
		5d	5.10	40.80 ± 7.31	22.16 ± 3.49	37.04 ± 6.32*
		5e	4.80	35.98 ± 5.56*	22.97 ± 0.61	41.06 ± 6.16**

The results are presented as a mean ± SD ($n = 3$), statistical significance: $p < 0.05$ (*) or $p < 0.01$ (**) for particular experimental group compared to untreated control.

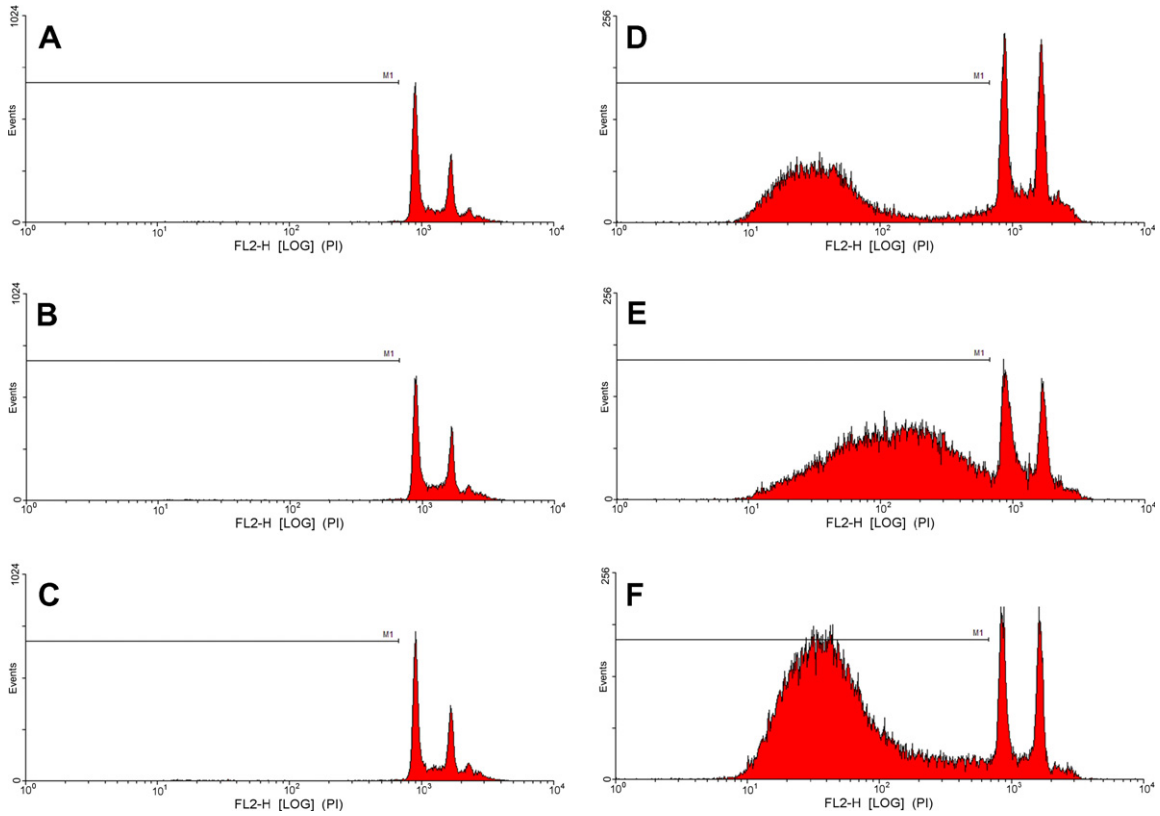


Figure 1. Qualitative evaluation of DNA fragmentation of HCT-116 cells 48 h after the drug administration. Debris had been avoided by gating on a similar cell population in FSC × SSC plot. Control (A), derivatives **5a** (B) and **5b** (C), **5c** (D), **5d** (E) and **5e** (F). Data are a representative result of the three independent experiments.

concentration of ctDNA (0–35 μM bp, Fig. 2), with corrections being made for the small volume changes that occurred during titration. The data revealed a decrease of peak intensities of **5a** with the absorbance reduction to 37% of its initial value. The hypochromism, due to a strong interaction between the electronic states of the intercalating chromophore and those of the DNA bases, was noted to be similar to other reports,[17] suggesting a close proximity of the proflavine chromophore and DNA. The presence of isosbestic points at 351 and 497 nm indicated spectroscopically distinct chromophores, namely, free and bound species. Such spectral behavior is generally associated with the intercalation as a dominant binding mode.[18] It is conceivable that side alkyl chains may also suit into the DNA grooves.

The fluorescence spectra of proflavine derivatives **5a**–**e**, exhibiting a broad emission band in the range 394–423 nm, were monitored at a fixed concentration of 4 μM. The titration with an increasing concentration of ctDNA continued until no further changes in the spectra of the drug–DNA complexes were recorded (Fig. 3A). Binding of the proflavine probes into the DNA helix was found to reduce the fluorescence of the complex thus independently proving the interaction of our compounds with DNA. A representative spectrofluorimetric titration of the proflavine hemisulfate is presented in Figure 3B.

CD experiment was performed to define the orientation of the compounds with respect to the DNA helix. The compounds have no circular dichroism (CD) spectrum when free in the solution but have an induced CD spectrum when in the complex with DNA. The B-form conformation of DNA shows two conservative CD bands in the UV region, a positive band at 278 nm due to the base stacking and a negative band at 246 nm due to the polynucleotide helicity.[19–21] When our compounds were incubated with ctDNA, the CD spectra displayed changes of both positive and negative bands (Fig. 4). The decrease of the negative bands with no significant red shift was observed for all the compounds. The helical band at 246 nm corresponding to the DNA unwound extent exhibited decrease for all the compounds in the same order as that

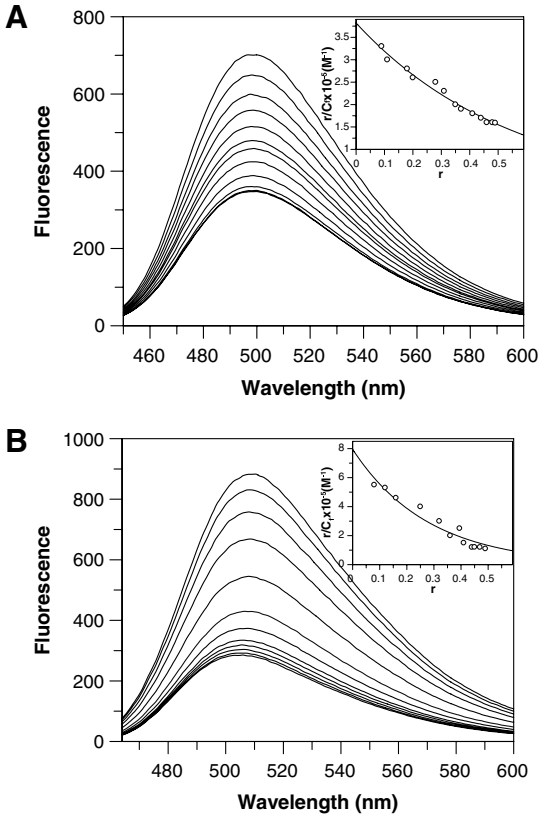


Figure 3. (A) Changes in the emission fluorescence spectra of **5b** (4 μM) in Tris buffer (0.01 M, pH 7.4, 25 °C) in the absence and presence of ctDNA (0–55 μM bp), λ_{ex} = 424 nm. (B) Changes in the emission fluorescence spectra of proflavine hemisulfate (4 μM) in Tris buffer (0.01 M, pH 7.4, 25 °C) in the absence and presence of ctDNA (0–55 μM bp), λ_{ex} = 444 nm.

of the binding affinity, **5a** > **5b** > **5c** > **5d** > **5e**. The positive band at 278 nm showed an increase of molar ellipticity with a mild red shift of the band maxima along with an increase of intensity upon addition of the proflavine complexes with DNA. This phenomenon could be due to the stabilization of the right-handed B-form of DNA by intercalation.

Fluorescence titration data have been used to determine the binding constants of probes with DNA by McGhee

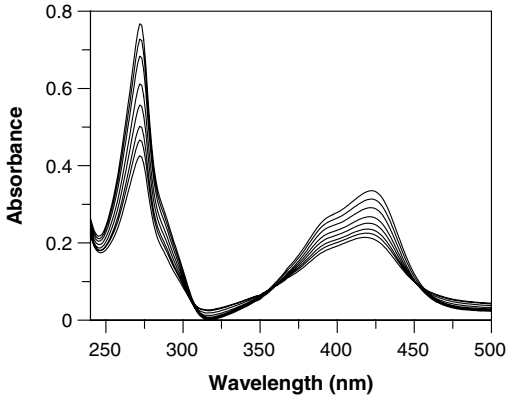


Figure 2. Absorption titration of compound **5b** (5.5 μM) in 0.01 M Tris buffer (pH 7.4, 25 °C) with an increasing concentration of ctDNA (from top to bottom: 0, 5, 10, 15, 20, 25, 30, 35 μM bp).

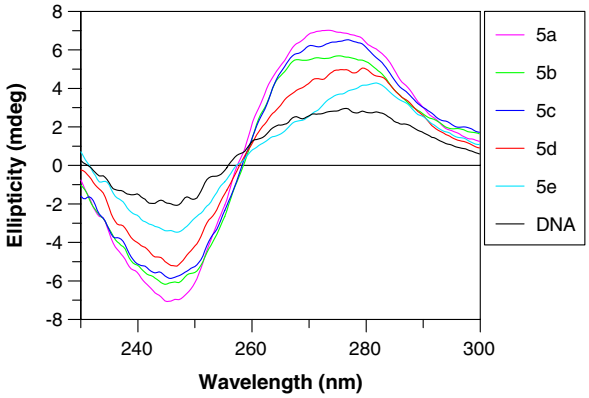


Figure 4. Circular dichroism spectra of the calf thymus DNA (200 μM bp) in the absence and presence of **5a**–**e** (50 μM) in 0.01 M Tris buffer (pH 7.4).

Table 4. Photophysical and binding parameters for **5a–e** from spectrofluorimetric analysis

Compound[a]	$K \times 10^5$ (M^{-1})	n	λ_{em} (nm)	Φ_f[b]	ΔG[c] (kJ mol^{-1})
5a	4.2	2.0	500	1.00	−19.27
5b	3.8	2.1	497	0.70	−19.52
5c	2.4	2.6	496	0.26	−20.66
5d	1.7	2.5	498	0.65	−24.51
5e	0.9	2.8	498	0.58	−23.09

[a] Proflavine sulfate was used as a standard ($K = 7.95 \times 10^5$ M^{-1}, $n = 1.2$).
[b] Fluorescence quantum yields were calculated using 1′,1″-(acridin-3,6-diyl)-3′,3″-diethyldiureas (**5a**) as standard ($\Phi_f = 1$).
[c] The average standard Gibb's free-energy change ($\Delta G^0 = -RT\ln K$) for derivatives **5a–e** is approximately −21.4 kJ mol^{-1} at 25 °C thereby indicating the spontaneous binding of the compounds into DNA.

and Hippel plots.[22,23] The constants were derived from nonlinear curve fitting which was described previously.[24] Calculated binding constants, K, and neighbor exclusion parameters, n, clearly indicated a direct correlation between intercalation capability and resulting structural changes. Estimations for n lay in the range 2.0–2.8 bp thus confirming the multiple site binding affected interaction at neighboring sites. The values of binding constants observed for **5a–e** (Table 4) are indicative of the high affinity of proflavine chromophore to the DNA-base pairs. The values of binding constants determined by spectrofluorimetry ranged from 4.2×10^5 M^{-1} to 0.9×10^5 M^{-1}. The binding affinity values for our derivatives with calf thymus DNA are in a good agreement with the K values of standard proflavine hemisulfate ($K = 7.95 \times 10^5$ M^{-1}, $n = 1.2$). Recently we reported the binding parameters K for proflavine-dithiazolidinone derivatives[12] which were about 10 times lower compared to our new diureas.

The relative binding constants K decreased in the order **5a > 5b > 5c > 5d > 5e.** Interestingly, a linear inverse relationship was found between the drug binding affinities and the chain length (Å) of the alkyl substituents ($k = -1.360$, $r = 0.993$; $S = 0.092$), that is, the binding decreased with an increasing substituent length, similar to our previous observation.[12]

3. Conclusion

We have synthesized a new series of bis alkylurea substituted proflavine derivatives. Compounds **5c**, **5d**, and **5e** exhibited potent cytotoxic properties and might be applicable against rapidly progressing tumors cells. The cells showed different sensitivity to drugs tested with HCT-116 cells which generally appeared more sensitive than HeLa ones. Interestingly, the compound **5e** with the low binding affinity showed much lower IC_{50} value than other compounds. It seems that the geometry of the intercalator–DNA complex contributes to antitumor properties more than the binding affinity. The efficient synthetic routes reported here provide a novel opportunity for designing DNA-targeted antitumor proflavine agents.

4. Experimental

4.1. Synthesis

All chemicals were purchased from Sigma–Aldrich, solvents were of laboratory grade. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were measured on a Varian Mercury Plus NMR spectrometer at room temperature in $CDCl_3$ using TMS as an internal standard (0 ppm for both nuclei). Melting points were determined using a Boetius hot-stage apparatus and are uncorrected. Elemental analyses were performed on a Perkin-Elmer analyser CHN 2400. Reactions were monitored by a thin-layer chromatography (TLC) using Silufol plates with detection at 254 nm. Preparative column chromatography was conducted using Kiesegel Merck 60 column, type 9385, grain size 250 nm.

4.1.1. General procedure for the preparation of 1′,1″-(acridin-3,6-diyl)-3′,3″-dialkyldiureas 4a–d. To a methanol suspension (3 mL) of 1′,1″-(acridin-3,6-diyl)-3′,3″-dialkyldithioureas **3a–e** (0.17 mmol), a three-molar excess of mesitylnitrile oxide (82.2 mg, 0.51 mmol) was added. The reaction mixture was stirred for approximately two days whilst the progress of the reaction was monitored by TLC (ethyl acetate/methanol, 8:1) until completion. The solid product was filtered off and dried in vacuo. The product was dissolved in acetone (0.5 mL) and an equimolar solution of 35% HCl (1 mL) in methanol (9 mL) was added. The mixture was stirred for 1 h followed by the addition of diethyl ether. The resulting precipitant was filtered off and dried in vacuo.

4.1.2. 1′,1″-(Acridin-3,6-diyl)-3′,3″-diethyldiurea (4a). Yield: 63%, mp: >360 °C. ^1H NMR (DMSO-d_6): δ 8.90 (br s, 2H, 2 × NH), 8.70 (s, 1H, 9-H), 8.15 (m, 2H, 4,5-H), 7.92 (d, 2H, 1,8-H, $J = 9.00$ Hz), 7.46 (dd, 2H, 2,7-H, $J_1 = 2.00$ Hz, $J_2 = 9.00$ Hz), 6.32 (t, 2H, 2 × NH, $J = 5.60$ Hz), 3.21–3.14 (m, 4H, 2 × NCH_2), 1.10 (t, 6H, 2 × CH_3, $J = 7.20$ Hz). ^{13}C NMR (DMSO-d_6): δ 154.82 (CO), 149.98 (C4a, C10a), 142.00 (C3, C6), 134.73 (C9), 128.84 (C1, C8), 121.11 (C8a, C9a), 119.33 (C2, C7), 110.75 (C4, C5), 33.95 (NCH_2), 15.30 (CH_3). Anal. Calcd for $C_{19}H_{21}N_5O_2$ (351.41): C, 64.94; H, 6.02; N, 19.93. Found: C, 64.82; H, 6.08; N, 19.77.

4.1.3. 1′,1″-(Acridin-3,6-diyl)-3′,3″-dipropyldiurea (4b). Yield: 65%, mp: >360 °C. ^1H NMR (DMSO-d_6): δ 8.90 (br s, 2H, 2 × NH), 8.70 (s, 1H, 9-H), 8.16 (m, 2H, 4,5-H), 7.92 (d, 2H, 1,8-H, $J = 8.40$ Hz), 7.46 (d, 2H, 2,7-H, $J = 8.40$ Hz), 6.37 (br s, 2H, 2 × NH), 3.16–3.08 (m, 4H, 2 × NCH_2), 1.55–1.44 (m, 4H, 2 × CH_2), 0.91 (t, 6H, 2 × CH_3, $J = 6.00$ Hz). ^{13}C NMR (DMSO-d_6): δ 154.92 (CO), 149.99 (C4a, C10a), 142.00 (C3, C6), 134.75 (C9), 128.87 (C1, C8), 121.11 (C8a, C9a), 119.30 (C2, C7), 110.71 (C4, C5), 40.82 (NCH_2), 22.84

(CH_2), 11.25 (CH_3). Anal. Calcd for $C_{21}H_{25}N_5O_2$ (379.47): C, 66.47; H, 6.64; N, 18.46. Found: C, 66.33; H, 6.76; N, 18.29.

4.1.4. 1′,1″-(Acridin-3,6-diyl)-3′,3″-dibutyldiurea (4c). Yield: 55%, mp: >360 °C. ^{1}H NMR (DMSO-d_6): δ 8.87 (br s, 2H, 2 × NH), 8.69 (s, 1H, 9-H), 8.13 (m, 2H, 4,5-H), 7.91 (d, 2H, 1,8-H, J = 9.00 Hz), 7.44 (dd, 2H, 2,7-H, J_1 = 2.00 Hz, J_2 = 9.00 Hz), 6.34 (t, 2H, 2 × NH, J = 6.00 Hz), 3.18–3.11 (m, 4H, 2 × NCH_2), 1.50–1.42 (m, 4H, 2 × CH_2), 1.39–1.29 (m, 4H, 2 × CH_2), 0.91 (t, 6H, 2 × CH_3, J = 7.60 Hz). ^{13}C NMR (DMSO-d_6): δ 154.89 (CO), 149.99 (C4a, C10a), 141.97 (C3, C6), 134.68 (C9), 128.82 (C1, C8), 121.09 (C8a, C9a), 119.29 (C2, C7), 110.73 (C4, C5), 38.66 (NCH_2), 31.68 (CH_2), 19.42 (CH_2), 13.58 (CH_3). Anal. Calcd for $C_{23}H_{29}N_5O_2$ (407.52): C, 67.79; H, 7.17; N, 17.19. Found: C, 67.83; H, 7.05; N, 17.03.

4.1.5. 1′,1″-(Acridin-3,6-diyl)-3′,3″-dipentyldiurea (4d). Yield: 67%, mp: >360 °C. ^{1}H NMR (DMSO-d_6): δ 8.86 (br s, 2H, 2 × NH), 8.70 (s, 1H, 9-H), 8.13 (m, 2H, 4,5-H), 7.92 (d, 2H, 1,8-H, J = 9.20 Hz), 7.45 (dd, 2H, 2,7-H, J_1 = 2.00 Hz, J_2 = 9.20 Hz), 6.34 (t, 2H, 2 × NH, J = 5.60 Hz), 3.17–3.11 (m, 4H, 2 × NCH_2), 1.52–1.44 (m, 4H, 2 × CH_2), 1.36–1.28 (m, 8H, 4 × CH_2), 0.90 (t, 6H, 2 × CH_3, J = 6.80 Hz). ^{13}C NMR (DMSO-d_6): δ 154.88 (CO), 150.00 (C4a, C10a), 141.97 (C3, C6), 134.71 (C9), 128.85 (C1, C8), 121.09 (C8a, C9a), 119.29 (C2, C7), 110.71 (C4, C5), 40.06 (NCH_2), 29.26 (CH_2), 28.51 (CH_2), 21.80 (CH_2), 13.87 (CH_3). Anal. Calcd for $C_{25}H_{33}N_5O_2$ (435.57): C, 68.94; H, 7.64; N, 16.08. Found: C, 68.85; H, 7.73; N, 15.94.

4.1.6. 1′,1″-(Acridin-3,6-diyl)-3′,3″-dihexyldiurea (4e). Yield: 62%, mp: >360 °C. ^{1}H NMR (DMSO-d_6): δ 8.88 (br s, 2H, 2 × NH), 8.71 (s, 1H, 9-H), 8.14 (m, 2H, 4,5-H), 7.92 (d, 2H, 1,8-H, J = 9.20 Hz), 7.45 (d, 2H, 2,7-H, J = 9.20 Hz), 6.34 (t, 2H, 2 × NH, J = 5.60 Hz), 3.17–3.10 (m, 4H, 2 × NCH_2), 1.51–1.42 (m, 4H, 2 × CH_2), 1.37–1.25 (m, 12H, 6 × CH_2), 0.88 (t, 6H, 2 × CH_3, J = 6.80 Hz). ^{13}C NMR (DMSO-d_6): δ 154.88 (CO), 149.92 (C4a, C10a), 142.09 (C3, C6), 134.81 (C9), 128.91 (C1, C8), 121.07 (C8a, C9a), 119.32 (C2, C7), 110.63 (C4, C5), 39.01 (NCH_2), 30.94 (CH_2), 29.54 (CH_2), 25.98 (CH_2), 22.02 (CH_2), 13.86 (CH_3). Anal. Calcd for $C_{27}H_{37}N_5O_2$ (463.63): C, 69.95; H, 8.04; N, 15.11. Found: C, 69.86; H, 8.12; N, 15.22.

4.1.7. 1′,1″-(Acridin-3,6-diyl)-3′,3″-diethyldiurea hydrochloride (5a). Mp 330–331 °C. ^{1}H NMR (DMSO-d_6): δ 10.19 (br s, 2H, 2 × NH), 9.34 (s, 1H, 9-H), 8.50 (m, 2H, 4,5-H), 8.19 (d, 2H, 1,8-H, J = 9.20 Hz), 7.55 (dd, 2H, 2,7-H, J_1 = 2.00 Hz, J_2 = 9.20 Hz), 6.90 (t, 2H, 2 × NH, J = 5.60 Hz), 3.24–3.16 (m, 4H, 2 × NCH_2), 1.11 (t, 6H, 2 × CH_3, J = 7.20 Hz). ^{13}C NMR (DMSO-d_6): δ 154.24, 148.06, 144.87, 141.62, 131.08, 120.00, 119.86, 100.22, 34.01, 15.05. Anal. Calcd for $C_{19}H_{21}N_5O_2$·HCl (387.87): C, 58.84; H, 5.72; N, 18.06. Found: C, 58.64; H, 5.65; N, 17.97.

4.1.8. 1′,1″-(Acridin-3,6-diyl)-3′,3″-dipropyldiurea hydrochloride (5b). Mp: 275–276 °C. ^{1}H NMR (DMSO-d_6): δ 10.06 (br s, 2H, 2 × NH), 9.35 (s, 1H, 9-H), 8.50 (m, 2H, 4,5-H), 8.20 (d, 2H, 1,8-H, J = 9.20 Hz), 7.53 (d, 2H, 2,7-H, J = 9.20 Hz), 6.88 (t, 2H, 2 × NH, J = 5.20 Hz), 3.18–3.11 (m, 4H, 2 × NCH_2), 1.56–1.45 (m, 4H, 2 × CH_2), 0.91 (t, 6H, 2 × CH_3, J = 7.60 Hz). ^{13}C NMR (DMSO-d_6): δ 154.35, 147.97, 144.88, 141.64, 131.11, 120.03, 119.89, 100.27, 40.84, 22.59, 11.21. Anal. Calcd for $C_{21}H_{25}N_5O_2$·HCl (415.93): C, 60.64; H, 6.30; N, 16.84. Found: C, 60.45; H, 6.20; N, 16.71.

4.1.9. 1′,1″-(Acridin-3,6-diyl)-3′,3″-dibutyldiurea hydrochloride (5c). Mp: 255–257 °C. ^{1}H NMR (DMSO-d_6): δ 10.28 (br s, 2H, 2 × NH), 9.33 (s, 1H, 9-H), 8.47 (m, 2H, 4,5-H), 8.18 (d, 2H, 1,8-H, J = 9.00 Hz), 7.54 (dd, 2H, 2,7-H, J_1 = 1.60 Hz, J_2 = 9.00 Hz), 6.97 (t, 2H, 2 × NH, J = 4.80 Hz), 3.21–3.14 (m, 4H, 2 × NCH_2), 1.51–1.43 (m, 4H, 2 × CH_2), 1.41–1.30 (m, 4H, 2 × CH_2), 0.91 (t, 6H, 2 × CH_3, J = 7.20 Hz). ^{13}C NMR (DMSO-d_6): δ 154.35, 148.00, 144.79, 141.62, 131.04, 119.92, 119.83, 100.17, 38.66, 31.47, 19.41, 13.57. Anal. Calcd for $C_{23}H_{29}N_5O_2$·HCl (443.98): C, 62.22; H, 6.81; N, 15.77. Found: C, 62.13; H, 6.92; N, 15.74.

4.1.10. 1′,1″-(Acridin-3,6-diyl)-3′,3″-dipentyldiurea hydrochloride (5d). Mp: 247–248 °C. ^{1}H NMR (DMSO-d_6): δ 10.02 (br s, 2H, 2 × NH), 9.34 (s, 1H, 9-H), 8.50 (m, 2H, 4,5-H), 8.20 (d, 2H, 1,8-H, J = 9.20 Hz), 7.52 (dd, 2H, 2,7-H, J_1 = 2.00 Hz, J_2 = 9.20 Hz), 6.84 (t, 2H, 2 × NH, J = 5.60 Hz), 3.20–3.14 (m, 4H, 2 × NCH_2), 1.56–1.45 (m, 4H, 2 × CH_2), 1.39–1.28 (m, 8H, 4 × CH_2), 0.89 (t, 6H 2 × CH_3, J = 6.40 Hz). ^{13}C NMR (DMSO-d_6): δ 154.32, 147.96, 144.26, 141.75, 131.09, 120.03, 119.90, 100.31, 39.02, 28.99, 28.41, 21.74, 13.84. Anal. Calcd for $C_{25}H_{33}N_5O_2$·HCl (472.04): C, 63.61; H, 7.26; N, 14.84. Found: C, 63.41; H, 7.31; N, 14.73.

4.1.11. 1′,1″-(Acridin-3,6-diyl)-3′,3″-dihexyldiurea hydrochloride (5e). Mp: 243–245 °C. ^{1}H NMR (DMSO-d_6): δ 9.95 (br s, 2H, 2 × NH), 9.33 (s, 1H, 9-H), 8.50 (m, 2H, 4,5-H), 8.19 (d, 2H, 1,8-H, J = 9.20 Hz), 7.51 (dd, 2H, 2,7-H, J_1 = 2.00 Hz, J_2 = 9.20 Hz), 6.81 (t, 2H, 2 × NH, J = 5.60 Hz), 3.20–3.13 (m, 4H, 2 × NCH_2), 1.53–1.44 (m, 4H, 2 × CH_2), 1.38–1.21 (m, 12H, 6 × CH_2), 0.88 (t, 6H, 2 × CH_3, J = 6.80 Hz). ^{13}C NMR (DMSO-d_6): δ 154.32, 147.79, 144.70, 141.84, 131.05, 120.04, 119.95, 100.39, 38.31, 30.87, 29.28, 25.88, 21.96, 13.83. Anal. Calcd for $C_{27}H_{37}N_5O_2$·HCl (500.08): C, 64.85; H, 7.66; N, 14.00. Found: C, 64.73; H, 7.53; N, 13.89.

4.2. Biological studies

4.2.1. Cell culture. HCT-116 (human colorectal cancer cells) and HeLa (human cervical epitheloid carcinoma, both ATCC, Rockville, MD, USA) were grown as the monolayer in a McCoy's medium (PAN Biotech, Aidenbach, Germany) supplemented with a 10% fetal calf serum (FCS, Gibco Invitrogen Corp., Carlsbad, CA, USA) and antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL, and amphotericin 25 μg/mL) (Gibco). The cultures were maintained at 37 °C in humidified atmosphere of 5% CO_2/95% air. Briefly, $1 \times 10^4/8 \times 10^5$

exponentially growing cells were seeded in 96-well microculture plates/∅ 60 mm Petri dish (both TPP, Switzerland) in a volume of 100 μL/5 mL. Prior to the drug administration cells were allowed to settle down for 24 h.

4.2.2. Quantification of total cell number and percentage of floating cells. Absolute numbers of adherent and floating cells within individual groups were evaluated by counting with Coulter Counter (Model ZF, Coulter Electronics Ltd, Luton, Bedfordshire, UK) and totalized to gather a total cell number. The total cell numbers were expressed as a percentage of the untreated control, and those of floating cells as a percentage of the total cell number.

4.2.3. Cytotoxicity evaluation. The cytotoxicity/survival of cells in the presence or absence of the experimental agent was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) as described previously. Cells harvested in the log phase of growth were counted and seeded in triplicates (10^4 cells/100 μL per well) in 96-well microculture plates. After 24 h incubation at 37 °C and 5% CO_2, cultures were treated with 1–100 μM concentration of **5a**–**d** or 1–25 μM concentration of **5e**. After 48 and 72 h exposure to the compound, MTT (0.5 mg/mL) was added to each well. After 4 h at 37 °C, the reaction was stopped and violet-blue crystals of formazan were dissolved by addition of sodium dodecylsulfate (SDS) to a final concentration 3.3%. Cells were incubated overnight (37 °C) and measured at 584 nm, using BMG FLUOstar Optima (BMG Labtechnologies GmbH, Offenburg, Germany). Blank value was subtracted and results were evaluated as a percentage of absorbance of the untreated control. The mean ± SD of three independent experiments for each compound was presented.

4.2.4. Cell cycle analysis and qualitative evaluation of DNA fragmentation. Adherent and floating cells (5×10^5) were harvested together 24 or 48 h after the drug administration, fixed in cold 70% ethanol and kept at −20 °C overnight. The ethanol was removed, and the pellet was washed in PBS. RNA was degraded with ribonuclease A (100 μg/mL) and nuclear DNA was stained with propidium iodide (Sigma, 20 μg/mL) in a Vindelov's solution at RT for 30 min. The DNA content was analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm argon-ion excitation laser. PI was exited at 488 nm and fluorescence analyzed at 585 ± 21 nm in a channel Fl-2. The ModFit 3.0 (Verity Software House, Topsham, ME, USA) software was used to generate DNA content frequency histograms and to quantify the number of cells in the different phases of the cell cycle. DNA fragmentation was evaluated by WinMDI (ver. 2.8) software on a logarithmic scale (evaluated as subG_0/G_1–subdiploid particles marked as M1).

4.2.5. Apoptosis frequency analysis by fluorescent microscopy. Analysis started 24 or 48 h after the drug administration when total cell population was harvested (by trypsinisation of adherent cells and collection of the medium with floating ones) by centrifugation, washed once in PBS and 1×10^6 cells were fixed in cold 70% ethanol and kept at −20 °C overnight. Prior to analysis, cell were stained in 70% ethanol with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (10 μg/mL) for 30 min in the dark at room temperature. After washing in PBS, cells were resuspended in 20 μL of PBS, mounted in Mowiol, kept at 4 °C till analysis, and evaluated with a fluorescent microscope (Nikon Eclipse 400, Nikon Instech Co., Ltd, Kawasaki, Japan). In each sample, 300 cells were evaluated and percentage of apoptotic cells defined by condensated or fragmented nucleus was determined.

4.3. DNA binding measurements

Proflavine hemisulfate was a product of Sigma–Aldrich (USA). A solution of the calf thymus DNA (Sigma Chemical Co.) in TE (Tris–EDTA) buffer was sonicated for 5 min and the DNA concentration determined from its absorbance at 260 nm. The purity of the DNA was determined by monitoring the value A_{260}/A_{280}. The concentration of DNA at 260 nm is expressed in the base pairs. UV–vis spectra were measured on a Varian Cary 100 UV–vis spectrophotometer in 0.01 M Tris buffer. Fluorescence measurements were made using a Varian Cary Eclipse spectrofluorimeter with a slit width 10 nm for the excitation and emission beams. Fluorescence intensity is expressed in arbitrary units. In fluorimetric titrations, excitation wavelengths 424 nm (**4a**, **4b**, **4d**, and **4e**) and 400 nm (**4c**) were used. CD spectra were recorded on a Jasco J-810 spectropolarimeter in 1 cm quartz cuvette and presented as a mean of three scans from which the buffer background had been electronically subtracted. The binding affinities were calculated using absorbance spectra according to the method of McGhee and von Hippel using data points from a Scatchard plot.[22,23] The binding data were fitted using GNU Octave 2.1.73 software.[25]

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