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Synthesis and cytotoxicity of novel bis(9-aminoacridines)

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This paper is dedicated to the memory of Marianne Byrn Caple, X-ray practitioner extraordinaire

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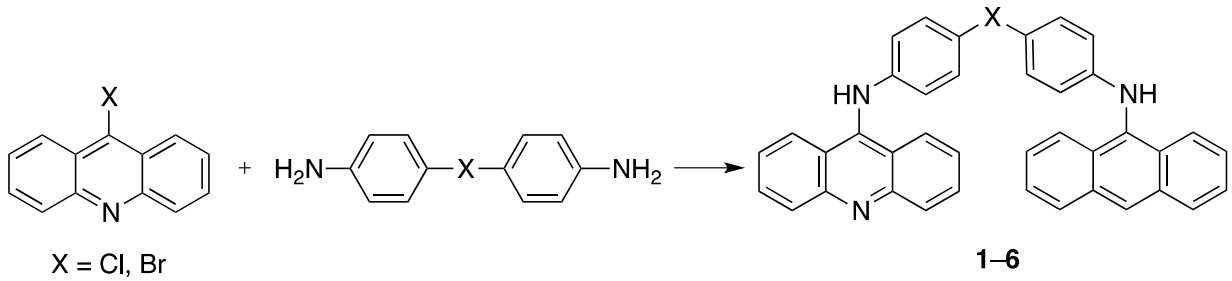
Abstract

We describe the synthesis and cytotoxicity towards L-1210 murine leukemia cells in culture of six novel bis(9-aminoacridines) **1**–**6** prepared from 9-chloroacridine or 9-bromoacridine and the appropriate diamine.



Keywords: bis(9-aminoacridines), DNA intercalation, L-1210 cytotoxicity

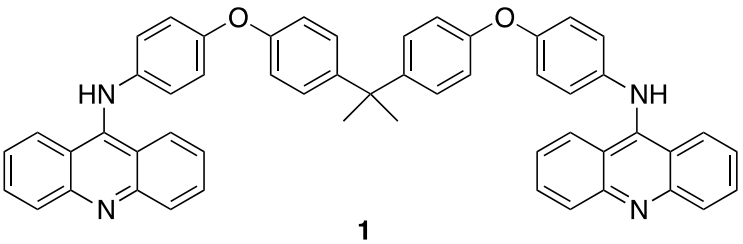
Introduction

In continuation of our studies on potential DNA-bis-intercalating agents[1–5] and related acridines,[6–8] we now describe the synthesis of six new bis(9-aminoacridines) **1**–**6** and their cytotoxicity against L-1210 murine leukemia cells *in vitro*. DNA-bis intercalators possessing semi-rigid tethers typically display improved antitumor activity over those compounds with flexible tethers.[9–12] For example, ditercalinium is in clinical trials for the treatment of cancer.[13] Moreover, because the planar acridine ring *per se* is an excellent DNA intercalator, it is no surprise that several studies have demonstrated acridines and bis-acridines possess anticancer properties,[14] one of which is the drug amsacrine.[15] DNA binding and other properties of bis-intercalators are also well established,[16–23] and novel functionalized acridines display unique physical and biophysical properties.[24–27]

Results and Discussion

We have previously found the incorporation of solvent molecules or water into the bis-acridines.[1] These solvent molecules could be seen spectroscopically (i.e. H-NMR) and, more significantly, were present as determined by elemental analysis. It has been shown[1,28] that solvent molecules commonly are found included into bis-acridinamines, and that the removal of these molecules is difficult at best. Drying *in vacuo* at elevated temperatures (60–100 °C) over long periods of time (3 days) does not remove the solvent molecules. We observed similar phenomena.

The included solvent molecules are proposed to be associated with the cleft of the bis-acridines, an indication that the bis-acridinamines might form “tweezers” around the solvent molecule. Another possible location of the solvent molecule in the crystal may be between two adjacent acridine units of different molecules. When the acridine moiety of the bis-acridinamine may be closer to an acridine in a neighboring molecule than to the acridine within the same compound, this is the expected location. Evidence that bis-acridines can exist with charge-transfer pi acceptor molecules (2,4,7-trinitro-9-fluorenone, TNF) bound in the cleft presented by the bis-acridine, can be found in the work of Zimmerman.[28,29] Although his bis-acridines do not possess the 9-amino functionality, they do describe the intercalation of TNF into the bis-acridine cleft. Further evidence of this “tweezer”-like orientation is found in CPK model studies[30] of each of the compounds. Those studies indicate that both acridine moieties can exist on the same side of the tether, and that they can be parallel or pseudo-parallel. Although some of these parallel conformations may be higher in energy, as in **4** [*N,N*-methylenedi-(5,2-*m*-xylyl)-bis-(9-acridinamine)], the energy returned by the inclusion of a solvent molecule may exceed the energy of the conformation.



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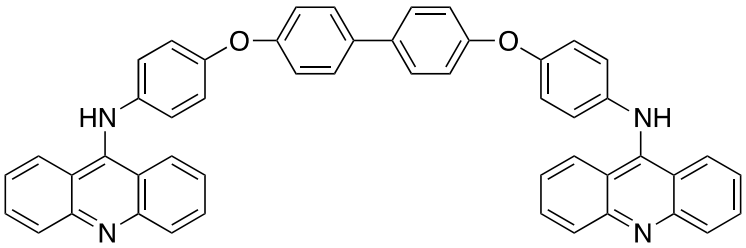

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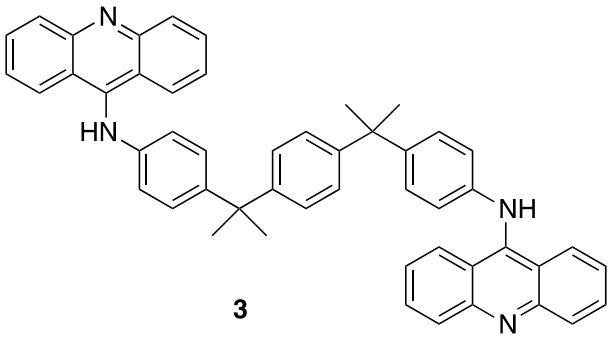

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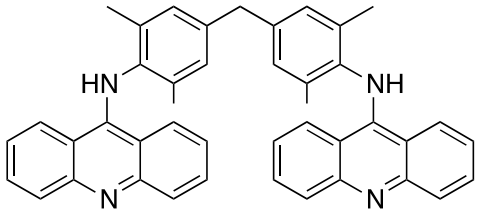

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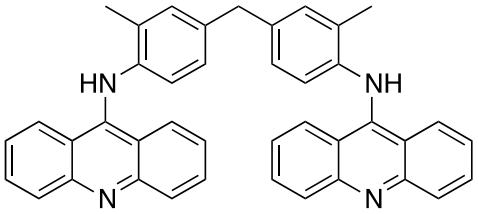

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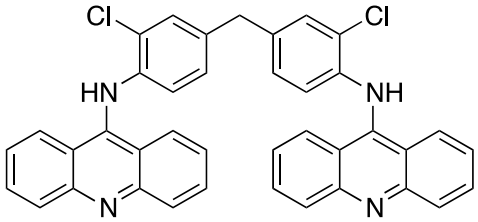

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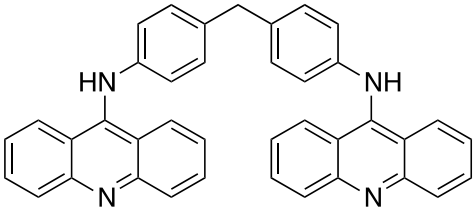

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Compounds **1**, **2**·2HCl, and **3**, were synthesized to explore the possibility of a larger base-pair bis-intercalator. Because the neighbor-exclusion rule of bis-intercalation requires a spacing of no less than 8–10 Å between the two acridine nuclei, a longer distance may still be suitable for this interaction. The existence of three base-pairs between the acridine nuclei is predicted to require a minimum distance of approximately 14 Å, which could produce a better fit of the bis-intercalator with the DNA macromolecule. In addition, the tethers in these three cases (**1**, **2**·2HCl, and **3**) are allowed, by our CPK-model studies, to be in a conformation that corresponds to the helical twist of DNA. Thus, an increase in their interaction with double-stranded DNA may be possible.

The synthesis of both **1** and **3** was quite straight-forward. The corresponding diamine tethers were condensed with an excess of 9-chloroacridine in warm phenol, extracted with chloroform or methylene chloride, washed with dilute hydroxide, dried and then purified by flash chromatography on silica gel. Compound **2**·2HCl, on the other hand, was less soluble in chloroform and methylene chloride than the other compounds. Initial attempts to wash the dissolved reaction mixture with aqueous base or water resulted in the isolation of the product as the dihydrochloride salt. Instead, **2**·2HCl was precipitated from the organic phase, and then recrystallized from ethanol/water to give a deep red powder. All attempts to isolate the free base resulted in extremely low yield (<3%), due to decomposition of the bis-adduct into its component parts (9-acridanone, mono-adduct, and the diamine tether). A possible reason for the insolubility of **2** in the organic phase may be the result of the incorporation of two and a half water molecules into the crystal structure of the compound. The water is present by elemental analysis and may be tightly hydrogen-bonded to the bis-adduct. The increase in the polarity of the compound would then give rise to the decrease in the solubility of **2** in the organic phase.

Because hydrolysis or thiolysis *in vivo* is the primary step in metabolism of these compounds that results in the rapid loss of cytotoxicity, the synthesis of **4**, **5**, and **6** was undertaken to explore hydrolysis of these bis-acridinamines under physiological conditions. For example, **7**, previously reported by Jaycox[1], exhibits an LD_{50}(L1210) of 0.031 μM. However, this compound has a short half-life under physiological conditions due to thiolysis. Compound **7** might have a much lower LD_{50} if hydrolysis and/or thiolysis were inhibited. Therefore, bis-adducts **4**, **5**, and **6** were designed with substituents that may sterically or electronically inhibit the addition-elimination mechanism of hydrolysis/thiolysis. Our CPK-model studies do support the ability of the methyl groups to block the face of the 9 position of the acridine on the internal surface of the molecular tweezer, yet they also cause extreme steric constraints to the rotation and conformation of the molecules, which could only serve to decrease any observable biological activity. Indeed, the ΔT_m decreases to one-tenth of that for ethidium bromide, indicating even poorer intercalation into DNA.

The inclusion of methyl groups on the tether ortho to the amino group (compounds **4** and **5**) seems to indicate, by CPK models, a reduced amount of rotational freedom for the acridine nuclei as well as flattening of the tether-amino nitrogen-acridine (C9) bond angle. CPK models also show a steric inhibition of the acridine nucleus to insert between the base-pairs of DNA. The data seem to indicate that although the compounds may be protected from hydrolysis or thiolysis, the acridine nuclei cannot occupy the same hemisphere of the molecule, nor can the acridines intercalate completely between adjacent base-pairs of DNA. This leads to a non-intercalative complex with DNA, and any observed binding must surely arise from association with the outer surfaces of the macromolecule (type II interaction) which is the weaker form of interaction. Compound **6** should also exhibit this steric inhibition. This compound does have an added bonus in that it may be able to participate in nucleophilic displacements at or near the intercalation site. An event such as this would increase the effectiveness of the compound as a drug by eliminating reversible binding to DNA (e.g., the compound would be permanently intercalated). However, this does not seem to be the case, due to the very small ΔT_m value seen.

Table 1. ΔT_m Values for some compounds in this study

Compound	ΔT_m (calf thymus DNA)
1	11.6 ± 0.4
3	10.9 ± 0.31
4	0.92 ± 0.15
5	10.7 ± 0.3
6	0.28 ± 0.02
7[1]	16.7 ± 0.5
ethidium bromide	11.9 ± 1.1

Table 2. L-1210 cytotoxicity data (LD_{50})

Compound	μM
1	5.4
3	>13.0
4	3.0
5	0.55
6	11.8
7[1]	0.031

The ΔT_m values (Table 1) of compounds **1**, **3**, and **5** indicate that there is a associative interaction with the DNA duplex. This interaction does result in a noticeable change in the melting point of calf thymus DNA, yet it is no greater than that of the well-known mono-intercalator ethidium bromide. The L-1210 cytotoxicity *in vitro* LD_{50} data from the University of Vermont are shown in Table 2. Compound **7** has a very low LD_{50} (0.031 μM),[1] compared to that of **4**, **5**, and **6**. The addition of 2 methyl groups (**5**) decreases the cytotoxicity by a factor of 10. The addition of another set of methyl groups (**4**) results in another decrease in cytotoxicity by a factor of 10. Addition of the halogen to the tether (**6**) reduces the cytotoxicity even further. This trend agrees with that shown by the ΔT_m values in Table 1. While the hydrolysis and/or thiolysis rate may be reduced under physiological conditions, the cytotoxicity suffers considerably. As for the longer tethers found in compounds **1**, **2**, and **3**, only **1** shows limited cytotoxicity. Compared to the measured ΔT_m values, these compounds are slightly less effective DNA intercalators than is 9-aminoacridine. This may prove to be the case *in vivo*, but lack of strong thermal denaturation ability is not an accurate predictor for cytotoxicity.

Conclusions

The compounds studied do not appear to be exceptional bis-intercalators of DNA using ΔT_m and LD_{50} (L-1210) metrics. Larger base-pair tweezers (**1**, **2**, and **3**) appear to cause a loss of expected biological activity. This may be due to the added entropy of the longer tether, and/or steric hindrances between the tether and the DNA macromolecule during the formation of bis-intercalative interactions. The inclusion of steric blocking groups against hydrolysis or thiolysis under physiological conditions reduces the bis-intercalative abilities from that of the parent compound. Thus, other methods of solving the decomposition of these compounds in the blood stream may prove to be of more importance than the steric inhibition in compounds **4**, **5**, and **6**.

Experimental Section

General. Melting points were determined in open capillary tubes with a Büchi 510 melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were recorded on a Varian XL-300 multinuclear Fourier transform instrument and are reported in parts per million from tetramethylsilane on the δ scale. The internal standard was trifluoroacetic acid, unless otherwise noted, which shows an absorption at 11.50 δ. Data are reported as follows: chemical shift [multiplicity (s = singlet, m = multiplet), integration]. Mass spectra were recorded either using fast atom bombardment (FAB) provided by the NIH Regional Facility located at the Massachusetts Institute of Technology (Cambridge, MA), or electron impact (20 eV) courtesy of Dr. M. Cory at Burroughs-Wellcome. Samples on which exact masses were measured exhibited no significant peaks at *m/e* greater than that of the parent. Combustion analyses were performed by Atlantic Microlabs (Norcross, Alabama).UV-visible spectra were recorded on a Hewlett-Packard 8451A Diode-Array Spectrophotometer from 300 nm to 820 nm. Extinction coefficients (ε) were not determined. The λ_{max} values reported indicate the largest absorption in the range studied. Absorptions between 300 nm and 190 nm are not reported. Flash chromatography was performed on silica gel (Kieselgel 60, 230–400 mesh ASTM) from EM Science. Analytical thin layer chromatography (TLC) was carried out on 200 µm Kieselgel 60 F_{254} coated plastic sheets (Merck). The spots were visualized, after use of a mobile phase (diethyl ether, unless otherwise noted), with 254 nm UV light or by spraying with concentrated H_2SO_4 and heating. All reactions were carried out under a nitrogen or argon atmosphere in oven-dried flasks unless otherwise stated.

9-Chloroacridine. To a flask containing freshly distilled phosphorus oxychloride (80 mL) was added acridanone (3.77 g, 19.3 mmol) with stirring. The resulting green slurry was heated to reflux for 1 h, by which time the mixture had become dark and homogeneous. The mixture was stirred at room temperature overnight before slow addition to cracked ice (500 g); (caution! induction period). The mixture was maintained near 0 °C during neutralization with concentrated ammonium hydroxide. The resulting tan precipitate was collected by filtration, washed with ice-cold water, recrystallized from ethanol/water, and dried in vacuo overnight to give 3.37 g (82%) as tan needles: mp 118–120 °C (lit.[31] mp 119–120 °C) (lachrymator and irritant); ^{1}H NMR ($CDCl_3$) δ 8.50–8.10 (m, 4H); 7.95–7.50 (m, 4H).

9-Bromoacridine. To a flask containing phosphorus oxybromide (4.97 g, 17.3 mmol) under argon was added acridanone (0.70 g, 3.58 mmol). The two solids were heated together on an oil bath slowly for 5 min before acetonitrile (11 mL) was added. The green slurry was stirred and heated to reflux, which caused the formation of a brownish-yellow color. After 10 min at reflux, the mixture was cooled and added cautiously to crushed ice (100 g), neutralized at 0–5 °C with concentrated ammonium hydroxide, and the green precipitate filtered. Flash chromatography (short path/hexane-ether, 8:1) gave 0.58 g (63%) as a yellow powder: mp 105–107 °C (lit.[31] mp 116 °C).

***N,N'*-2,2-Propylenebis-(4,1-phenylene-oxy-4,1-phenylene)-bis-(-acridinamine) (1).** 9-Chloroacridine (0.64 g, 2.99 mmol) was added to a flask containing phenol (10 g). This was warmed to 80 °C and then 2,2-bis(4-(4-aminophenoxy)phenyl)propane (BAPP) (0.42 g, 1.02 mmol) was added turning the yellow solution deep red. The temperature was maintained at 80–90 °C for 5 h, then cooled to room temperature overnight. The red solidified reaction mixture was then taken up in chloroform (100 mL), washed with 0.1 M NaOH (4 x 100 mL), distilled water (100 mL), and brine (100 mL), and dried ($MgSO_4$). The orange organic phase was then filtered of drying agent and adsorbed onto silica gel and chromatographed (silica gel/hexane-ether, gradient elution). The appropriate fractions were combined, and the solvent removed in vacuo. The orange powder was then recrystallized from THF/hexane to give 0.18 g (23%) of **1** as a bright orange powder. UV-vis ($CHCl_3$): λ_{max} 408

nm; ^{1}H NMR (TFA-d_1): 8.2–7.1 δ (m, 32H); 1.75 δ (s, 6H); MS EI: *m/e* (rel. intensity): 608 (9); 471 (253); 431 (100); 193 (4); 179 (6); 134 (12); MS FAB: *m/e* 765 (MH^+); High Res MS FAB: Calcd for $C_{53}H_{41}N_4O_2$ (MH^+): 765.3230, Found: 765.3228; *Anal.* Calcd for $C_{53}H_{40}N_4O_2 + H_2O$: C, 81.29%; H, 5.41%; N, 7.16%. Found: C, 81.35%; H, 5.52%; N, 6.81%.

***N,N'*-4,4'-Biphenylenedi-(oxy-4,1-phenylene)-bis-(9-acridinaminium) dihydrochloride (2**·2HCl**).** To phenol (15 g) was added, with stirring, 9-chloroacridine (1.082 g, 5.06 mmol). The mixture was heated to 80 °C and bis-(aminophenoxy)biphenyl (BAPB) (0.420 g, 1.14 mmol) was added. The orange reaction mixture turned a deep red color instantly upon addition of the diamine. The mixture was stirred for 5.5 h at 85 °C, then cooled to room temperature and triturated with ether (3 x 25 mL) to yield 1.04 g (87%) of a bright orange precipitate, presumed to be the dihydrochloride salt. This was filtered and recrystallized from ethanol/water to give 0.3 g of **2**·2HCl as a brick-red powder. UV-vis ($CHCl_3$) λ_{max} = 408 nm; *Anal.* Calcd for $C_{50}H_{34}N_4O_2 + 2HCl + 2.5\ H_2O$: C, 71.43%; H, 4.91%; N, 6.66%. Found: C, 71.51%; H, 4.70%; N, 6.50%.

***N,N'*-4.4'-Phenylenedi-(1-methylethylidene-4,1-phenylene)-bis-(9-acridinamine) (3).** To a stirred solution of 9-chloroacridine (0.94 g, 4.40 mmol) in phenol (15 g) at 80 °C was added 4,4'-(1,4-phenylenebis(1-methylethylidene))-bis-aniline (Bisaniline P) (0.35 g, 1.06 mmol). This was maintained at 80 °C for 7 h, then cooled to room temperature overnight. The red mixture was taken up in methylene chloride (100 mL), washed with 0.1 M NaOH (5 x 100 mL), distilled water (2 x 100 mL), brine (100 mL), and dried ($MgSO_4$). After adsorbtion onto silica gel the product was isolated by flash chromatography (silica gel, hexane/acetone, gradient elution) and after recrystallization (THF/hexane) dried in vacuo to give 0.55 g (74%) of **3** as an orange powder. UV-vis ($CHCl_3$) λ_{max} = 416 nm; ^{1}H NMR (TFA-d_1): 8.2–7.2 δ (m, 28H); 1.8 δ (s, 12H); MS EI: *m/e* (rel. intensity): 698 (23, M^+); 683 (18); 334 (16); 179 (6); 94 (100); 66 (37); MS FAB: *m/e* 699 (MH^+); High Res MS FAB: Calcd for $C_{50}H_{43}N_4$ + 1 THF (C_4H_8O): C, 84.12%; H, 6.53%; N, 7.27%. Found: C, 83.53%; H, 6.17%; N, 6.70%.

***N,N'*-Methylenedi-(5,2-xylyl)-bis-(9-acridinamine) (4).** To a molten mixture (80 °C) of 9-chloroacridine (1.00 g, 4.68 mmol) in phenol (15 g) was added methylene-bis-(5,2-xylidine) (MBXD) (0.32 g, 1.26 mmol) which turned the yellow solution deep red. This was stirred at 80 °C for 4.5 h, and cooled to room temperature overnight. The mixture was dissolved in methylene chloride (75 mL), washed with 0.1 M NaOH (4 x 100 mL), distilled water (2 x 100 mL), brine (100 mL), and dried ($MgSO_4$). The clear red-orange organic phase was then filtered and after adsorbtion onto silica gel, flash chromatographed (silica gel, hexane/ether, gradient elution) to give 0.13 g (17%) of **4** as an orange powder. UV-vis ($CHCl_3$) λ_{max} = 394 nm; ^{1}H NMR (TFA-d_1): 8.1–7.2 δ (m, 20H); 4.1 δ (s, 2H); 2.5–2.2 δ (m, 12H); MS EI: *m/e* (rel. intensity) 587 (2); 479 (12); 463 (4); 285 (34); 268 (32); 255 (14); 205 (29); 179 (9); 159 (11); 97 (12); 91 (11); 72 (44); 71 (61); 57 (33); 42 (100); *Anal.* Calcd for $C_{43}H_{36}N_4$ + H_2O: C, 82.39%; H, 6.11%; N, 8.94%. Found: C, 82.05%; H, 6.79%; N, 9.24%.

***N,N'*-Methylenedi-(5,2-tolyl)-bis-(9-acridinamine) (5).** To a yellow solution of warm (80 °C) phenol (15.1 g) and 9-bromoacridine (1.08 g, 4.18 mmol) was added with stirring 4,4'-methylene bis-di-*o*-toluidine (MBOT) (0.23 g, 1.02 mmol). The red solution was stirred at 75–80 °C for 10 h, then cooled to room temperature overnight. The solidified deep red reaction mixture was taken up in methylene chloride (100 mL) and washed with 0.1 M NaOH (4 x 100 mL), distilled water (2 x 100 mL), and brine (100 mL). After drying (Na_2SO_4) the orange organic phase was adsorbed onto silica gel. Flash chromatography (silica gel/hexane-ether, gradient elution) gave 0.46 g (78%) of **5** as an orange powder which showed one low R_f spot by TLC. UV-vis ($CHCl_3$) λ_{max} = 404 nm; ^{1}H NMR (TFA-d_1): 8.2–7.2 δ (M, 22H); 4.2 δ (S, 2H); 2.25 δ (S, 6H); MS EI: *m/e* (rel. intensity): 581 (27, M^++1); 580 (65, M^+); 403 (19); 297 (72); 290 (100); 282 (93); 268 (16); 193 (6); 180 (35); 179 (20); *Anal.* Calcd for $C_{41}H_{32}N_4 + 2H_2O$: C, 79.84%; H, 5.88%; N, 9.08%. Found: C, 79.90%; H, 5.86%; N, 8.94%.

***N,N'*-Methylenedi-(5,2-chlorophenyl)-bis-(9-acridinamine) (6).** A mixture of phenol (15.1 g) and 9-bromoacridine (1.22 g, 4.73 mmol) was heated to 80 °C with stirring. To this yellow solution was added 4,4'-methylene bis-(2-chloroaniline) (Bis-Amine A) (0.33 g, 1.24 mmol) and the reaction mixture stirred at 100 °C for 10 h. After cooling to room temperature overnight, the solidified red reaction mixture was taken up in methylene chloride (100 mL), washed with 0.1 M NaOH (4 x 100 mL), distilled water (100 mL), and brine (100 mL), and dried ($MgSO_4$). The organic phase was filtered and adsorbed onto silica. Flash chromatography (silica gel/hexane, ether, gradient elution) gave 0.32 g (42%) of **6** as an orange powder which showed a single spot (R_f = 0.2) by TLC. UV-vis ($CHCl_3$) λ_{max} = 406 nm; ^{1}H NMR (TFA-d_1): 8.2–7.3 δ (M, 22H); 4.2 δ (M, 2H); MS EI: *m/e* (rel. intensity) 622 (33, M^++2); 620 (49, M^+); 585 (9); 547 (4); 317 (90); 303 (16); 292 (100); 281 (86); 275 (97); 268 (43); 205 (22); 179 (31); 151 (24); 94 (13); MS FAB: *m/e* 621 (MH^+); High Res. MS FAB: Calcd for $C_{39}H_{26}N_4Cl_2$ + 2 THF (C_4H_8O): C, 73.72%; H, 5.53%; N, 7.32%; Cl, 9.26%. Found: C, 73.25%; H, 5.61%; N, 7.33%; Cl, 9.33%.

Procedure for determining L-1210 activity. The individual compounds were tested by Professor Miles P. Hacker, Department of Pharmacology, University of Vermont. L-1210 cells (10^5) maintained as suspension cultures in McCoy's 5A medium (containing 10% horse serum, glutamine, penicillin, and streptomycin) were exposed to known concentrations (0.01, 0.1, and 10 μg/ml in DMSO) of the compounds in an atmosphere of 10% carbon dioxide and 90% air at 37 °C. After 72 h of continuous exposure the population of each culture was determined by a Coulter Counter (Model ZBF, Hialeah, FL) and compared to the control. The ratio of exposed cell population to control cell population was used to calculate the percent cell growth relative to control.[1] This was used to calculate the LD_{50} value (the drug concentration required to inhibit cell growth by 50% of control).

Procedure for determination of ΔT_m values. The thermal denaturation studies were done by the method of Cory[16] on a Varian 2290 UV-visible spectrophotometer with a heating rate of 18 °C/h. The five cuvettes were on a 2 min cycle time with a 5 s dwell time. ΔT_m were calculated from the printout using the difference between the compound T_m and the DNA. The calf thymus DNA had a T_m of 56.8 °C.

Acknowledgements

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