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DNA threading bis(9-aminoacridine-4-carboxamides): Effects of piperidine sidechains on DNA binding, cytotoxicity and cell cycle arrest

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Abstract—We describe the synthesis of a series of DNA-threading bis(9-aminoacridine-4-carboxamides) comprising ethylpiperidino and *N*-methylpiperidin-4-yl sidechains, joined via neutral flexible alkyl chains, charged flexible polyamine chains and a semi-rigid charged piperazine linker. Their cytotoxicity towards human leukaemic cells gives IC_{50} values ranging from 99 to 1100 nM, with the ethylpiperidino series generally being more cytotoxic than the *N*-methylpiperidin-4-yl series. Measurements with supercoiled DNA indicate that they bisintercalate.

1. Introduction

Cytotoxic DNA intercalating agents inhibit cell growth by two well-established mechanisms: poisoning topoisomerases[1–3] and inhibiting transcription.[4] The majority of intercalating cancer drugs in clinical use are of the former type, with examples including adriamycin, mitoxantrone and amsacrine.[1–3] Surprisingly, little attention has been paid to the development of template transcription inhibitors, despite their impressive experimental antitumour activity, in part, because of the difficulty of endowing chemically accessible compounds with the capacity to form the bulky long-lived reversible complexes needed to inhibit the passage of RNA polymerase. Intercalating transcription inhibitors are exemplified by the antitumour antibiotics actinomycin D, the bisintercalator echinomycin and the threading agent nogalamycin.[5–7] In previous work, we combined the bisintercalation motif of echinomycin with the threading mode of nogalamycin, to synthesize a series of bis(9-aminoacridine-4-carboxamides) linked via the 9-position with various linkers, in which the carboxamide sidechains comprise *N*,*N*-dimethylaminoethyl and ethylmorpholino groups.[8] The compounds were designed to bisintercalate into DNA, sandwiching two basepairs and to thread their sidechains through the helix to form hydrogen bonding contacts with the O6/N7 atoms of guanine in the major groove: a hydrogen bonding scheme observed in DNA complexes of the 9-aminoacridine-4-carboxamide monomer 9-amino-DACA.[9]

Our initial findings showed that the targeted bis(9-aminoacridine-4-carboxamides) bisintercalate via a threading mode as anticipated,[8] and that their complexes dissociate many orders of magnitude more slowly than simple intercalators, some members forming complexes that have lifetimes comparable to those of the natural products.[8] The most active agents are cytotoxic towards human leukaemic CCRF-CEM cells with IC_{50} values of 35–50 nM in a range extending over 20-fold, with neither the dimethylaminoethyl nor the ethylmorpholino series being intrinsically more toxic.[8] Cell cycle progression studies indicated that the ethylmorpholino series generally has no effect on cycle distribution in CEM cell populations, whereas the *N*,*N*-dimethylaminoethyl series, with two exceptions, cause G2/M arrest in the manner of topoisomerase poisons, consistent with possible

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involvement of topoisomerases in their mode of action.[8] Subsequent studies of drug effects on global gene expression using DNA microarrays, showed that selected examples of the bis(9-aminoacridine-4-carboxamides) have profound effects on transcription, in a similar manner to that of actinomycin, echinomycin and nogalamycin, producing patterns of perturbation that depend both on linker and sidechain structure.[10]

Here we provide a further attempt to develop bisintercalative transcription template inhibitors utilizing the bis(9-aminoacridine-4-carboxamide) framework. Previous crystallographic studies of monomeric 9-aminoacridine-4-carboxamides with dimethylaminoethyl and ethylmorpholino sidechains[9,11] established that the compounds bind to DNA with their carboxamide sidechains lying in the major groove, forming hydrogen bonds with the O6/N7 atoms of guanine. In these structures, the ethylmorpholino sidechain, being more bulky, covers two basepairs, in contrast to the *N*,*N*-dimethylaminoethyl sidechain, which extends just to the bonding guanine.[9,11] Surprisingly, our previous work revealed that the kinetic stability of the complex of the C3NC3-linked ethylmorpholino compound is no greater than that of its *N*,*N*-dimethylaminoethyl analogue.[8] This was taken to suggest that either the hydrogen bonding capacity of the cyclic sidechain is weaker than that of *N*,*N*-dimethylaminoethyl, or that during dissociation the sidechain rotates into the intercalation plane, thereby minimizing the difference in 'structural drag'' between the two threading groups. In the present work, we have replaced the *N*,*N*-dimethylaminoethyl and ethylmorpholino groups with ethylpiperidino and *N*-methylpiperidin-4-yl sidechains in an attempt to ameliorate these effects. Our intention with the ethylpiperidino sidechain is to enhance the strength of the guanine hydrogen bond, since piperidine, with a pK of ≈11, is substantially more basic than morpholine, whose pK is about 8, and the *N*-methylpiperidin-4-yl group is designed to remove a number of degrees of freedom from the flexible *N*,*N*-dimethylaminoethyl sidechain, while maintaining the protonated amine in a suitable position for hydrogen bond formation.

In this report, we describe the synthesis of a series of bis(9-aminoacridine-4-carboxamides) with ethylpiperidino and *N*-methylpiperidin-4-yl sidechains, joined via neutral flexible alkyl chains, charged flexible polyamine chains and a semi-rigid charged piperazine linker (Fig. 1). Their cytotoxicity towards human leukaemic CCRF-CEM cells gives IC_{50} values ranging from 99 to 1100 nM, with the ethylpiperidino series generally being more cytotoxic than the *N*-methylpiperidin-4-yl series. However, comparison of the structure–activity relationships of the new agents with that of their *N*,*N*-dimethylaminoethyl- and ethylmorphorpholino homologues is not straightforward.[8] Analysis of their effects on cell cycle progression shows that the ethylpiperidino series fail to invoke G2/M arrest, whereas the *N*-methylpiperidin-4-yl series does, providing some evidence for topoisomerase poisoning in their mode of action. Helix unwinding angle measurements with supercoiled DNA indicate that the new agents intercalate, but suggest that the binding affinity of the *N*-methylpiperidin-4-yl series is lower than that of its congeners.

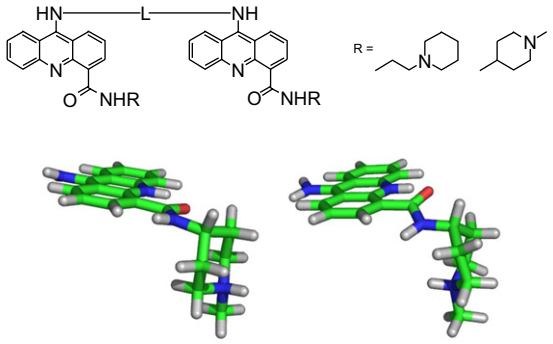
2. Results

2.1. Chemistry

The acridine compounds studied (Fig. 1) were synthesized as outlined in Scheme 1. The 9-oxoacridan-4-carboxylic acid was prepared as described by Denny and colleagues,[12] the 9-chloroacridine-4-carbonyl chloride being obtained by treatment with thionyl chloride in the presence of catalytic amounts of DMF. The 4-carboxamide sidechains were coupled using an excess of *N*-piperidinylethyleneamine or 1.1 equivalents of *N*-methyl-4-aminopiperidine in the presence of sodium carbonate in anhydrous dichloromethane. *N*-Methyl-4-aminopiperidine was prepared via a reductive amination by treatment of *N*-methyl-4-piperidone with ammonium formate and 10% palladium on carbon in methanol-water (Scheme 2).[13] The 9-aminoacridine-4-carboxamide monomers were prepared by reacting the 9-chloroacridine-4-carboxamides with ammonia gas in hot phenol solution. The bis(9-aminoacridine-4-carboxamides) were coupled in the manner previously described by us,[8] as shown in Scheme 1. The 9-chloroacridine-4-carboxamides were dissolved in hot phenol and treated with a half molecular equivalent of the α,ω-diamine linkers. After heating at 125 °C for 2 h, and suitable workup, the diacridine free bases were recovered from chloroform and purified by flash chromatography or recrystallization. Their corresponding hydrochloride salts were prepared by treating the free base in methanol with concentrated hydrochloric acid at pH 3. Most of the α,ω-diamine linkers used are commercially available. The tetraamine required for the C6N2 linker was purchased as the tetrahydrochloride salt, and treated as described previously by us before use.[8] 1,4-Bis(aminoethyl)piperazine, required for the C2pipC2 linker, was prepared as reported previously.[8,14]

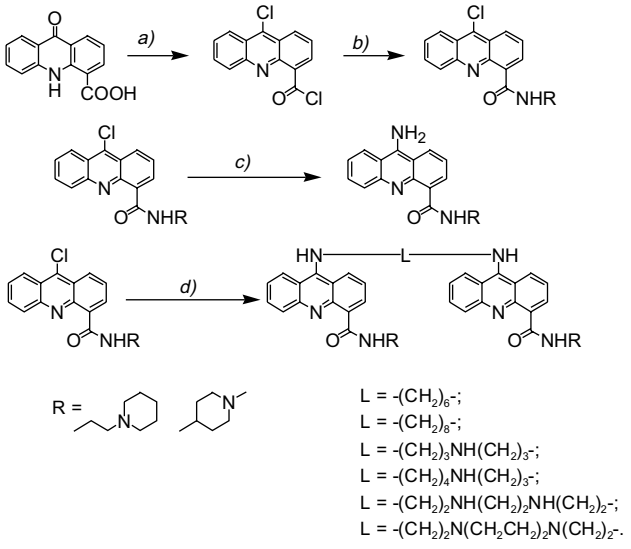
2.2. DNA binding

To establish whether the dimers bind to DNA by a bisintercalative threading mode, we investigated their capacity to remove and reverse the supercoiling of closed circular pBR322 DNA as assessed by electrophoretic mobility measurements on agarose gels.[8,15,16] Figure 2 shows typical titration curves for C3NC3 Pip, C2pipC2 Pip and C3NC4 NMP in 40 mM TEA buffer, and reveals equivalence binding ratios, where all the supercoiling have been removed and the closed and nicked circular species co-migrate, in the range 0.13–0.21 ligand molecules per base pair. Calibrating the titrations using the C8-linked DMAE threading dimer of known unwinding angle 43°,[8] and equivalence binding ratio of 0.075, enables simple calculation of helix unwinding angles, as recorded in Table 1. The DNA complexes of both the ethylpiperidino and *N*-methylpiperidin-4-yl monomers dissociate on the gels, so precluding a determination. The ethylpiperidino dimers behave uniformly, with unwinding angles of 20° to

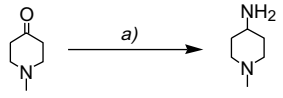


Linker (L)	Linker name	Flexibility	Linker charge	Inter-chromophore distance, Å[a]
$(CH_2)_6$	C6	Flexible	Neutral	8.8
$(CH_2)_8$	C8	Flexible	Neutral	11.3
$(CH_2)_3NH(CH_2)_3$	C3NC3	Flexible	+1	9.8
$(CH_2)_3NH(CH_2)_4$	C3NC4	Flexible	+1	11.4
$(CH_2)_2NH(CH_2)_2NH(CH_2)_2$	C6N2	Flexible	+2	11.3
$(CH_2)_2N(CH_2CH_2)_2N(CH_2)_2$	C2pipC2	Semi-rigid	+2	10.1

Figure 1. Structure of the threading diacridines studied. Compounds are described in the text by reference to their linker and sidechain structure, the abbreviations for the latter being Pip, ethylpiperidino and NMP, *N*-methylpiperidin-4-yl. Energy-minimised structures of the NMP monomer with the sidechain in chair (left) and twisted-boat (right) conformations are also shown. These structures were modeled using Discovery Studio 1.7 and illustrated using PyMOL. The chair conformer would not be able to make hydrogen bonding interactions to guanine, whereas the twisted-boat conformer would. In our previous report we described compounds with an *N*,*N*-dimethylaminoethyl sidechain, designated DMAE, and an ethylmorpholino sidechain, designated morpholino[8]. $N(CH_2CH_2)_2N$ denotes piperazine. [a]Ref. 15.



Scheme 1. Synthesis of 9-aminoacridine-4-carboxamides and dimers. Reagents and conditions: (a) $SOCl_2$; (b) RNH_2/dcm; (c) NH_3 (gas), phenol, 125 °C; (d) α, ω-diamine, phenol, 125 °C.



Scheme 2. Synthesis of *N*-methyl-4-aminopiperidine. Reagent: (a) 10% Pd/C, $HCOONH_4$, CH_3OH.

25°, their complexes giving no evidence of dissociation on the gel in 40 mM buffer. Whilst this range is low compared to the expected values of 30–35° for bisintercalation, given that the 9-aminoacridinecarboxamides have unwinding angles of 15–17°,[17] it is comparable to the values found for bisintercalating simple piperazine-linked diacridines and to the piperazine-linked DMAE and morpholino bis(9-aminoacridinecarboxamides).[15] In contrast, the *N*-methylpiperidin-4-yl dimers give titration responses which depend on the ionic strength of the buffer and the nature of the linker, indicating that their affinity for DNA is somewhat lower than that of their ethylpiperidino homologues. Accordingly, for the *N*-methylpiperidin-4-yl dimers, Table 1 records the findings in 10 mM TAE buffer, pH 7.5, where the cation concentration is about 8–9 mM. Under these conditions, the observed unwinding angles range from 13 to 30°. The values for the alkyl- and C3NC3-linked dimers should be viewed as lower limits since these agents show the greatest salt-dependent variations (Table S1). In contrast, the C3NC4- and C6N2-linked dimers give the same result in both 10 and 40 mM buffer, indicating that these are quantitatively bound in both circumstances, and have unwinding angles of 16 and 30° which span the traditional range of mono- to bifunctional reaction. In distinction to the other members of the *N*-methylpiperidin-4-yl series, the semi-rigidly linked C2pipC2 NMP dimer removes but fails to reverse DNA supercoiling in 10 mM TAE, and there is a clear evidence of complex dissociation. Thus, it would seem that its DNA affinity is anomalously low for a dimer with a doubly charged linker.

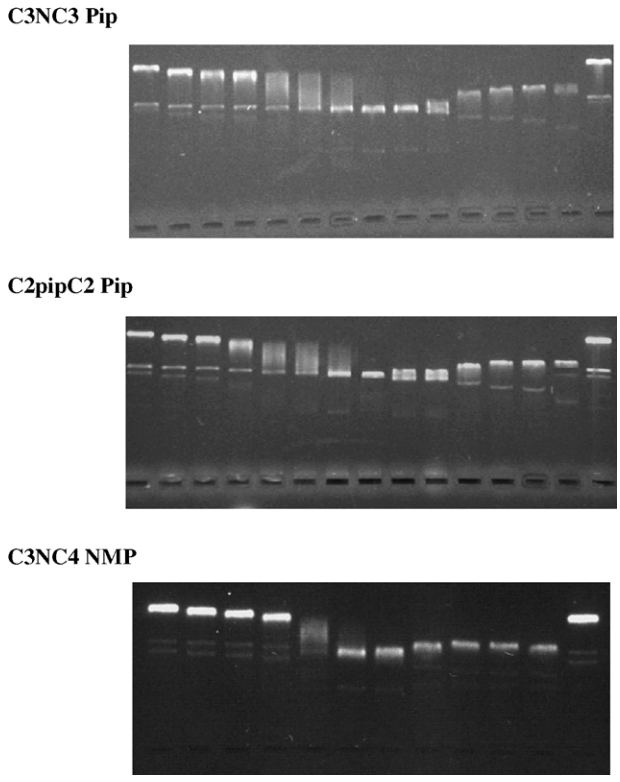


Figure 2. Effects of the C3NC3 Pip, C2pipC2 Pip, and C3NC4 NMP threading diacridines on the supercoiling of pBR 322 DNA. Measurements were made in 40 mM tris acetate buffer. Drug-to-DNA ratios in lanes 1–15 for C3NC3 Pip are 0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, 0.20, 0.25, 0.30, 0.35, and 0. Drug-to-DNA ratios in lanes 1–15 for C2pipC2 Pip are 0, 0.04, 0.05, 0.06, 0.07, 0.08, 0.10, 0.10, 0.12, 0.14, 0.16, 0.18, 0.20, 0.25, and 0. Drug-to-DNA ratios in lanes 1–12 for C3NC4 NMP are 0, 0.05, 0.075, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.50 and 0.

2.3. Cytotoxicity

The cytotoxicity of the target bis(9-aminoacridine-4-carboxamides) and related monomers against human leukaemia CEM cells is shown in Table 1. Data for the topoisomerase II poisons amsacrine and 9-amino-DACA, and the transcription inhibitor actinomycin D, are presented for the purposes of comparison.[8] The monofunctional 9-aminoacridinecarboxamides have IC_{50} values of 600 and 800 nM, the more potent being the NMP analogue, and are thus about 5- to 7-fold less toxic than 9-amino-DACA with the *N*,*N*-dimethylaminoethyl sidechain. In the ethylpiperidino dimer series, all compounds are more potent than the parental monomer, around 3- to 4-fold more so in the case of the C6-, the C3NC4-, the C3NC3- and C6N2-linked compounds, and about 7- to 8-fold more so in the case of the C8- and C2pipC2-linked dimers. Thus, it is apparent that for the ethylpiperidino series dimerization consistently enhances cytotoxicity, with the uncharged octamethylene linker promoting the greatest activity. In contrast, for the *N*-methylpiperidin-4-yl series, dimerization can enhance or weaken the biological potency with IC_{50} values varying up to ~10-fold, depending on the nature of the linker. Here, the C8-, the C6- and the C3NC4-linked NMP dimers are, respectively, 6-, 3.5- and 2-fold more cytotoxic than the NMP monomer, whereas the NMP dimers with doubly charged linkers, C6N2 and C2pipC2 are both less potent than the monomer. Once again, the octamethylene linker yields the most active compound.

2.4. G2/M arrest

The effects of the threading agents on cell cycle progression were investigated by using flow cytometry. CEM cells were fixed, labeled with propidium iodide and analyzed after incubation for 24 h in the presence of the ligands at concentrations ranging from the IC_{50} (based on 72 h incubation), upward to 2, 5 and 10 times that level. Once again, the topoisomerase poisons amsacrine and 9-amino-DACA, and the transcription inhibitor actinomycin D, were used as comparators. Figure 3a and b show typical profiles for amsacrine and 9-amino-DACA, where, as found previously,[8] the cells accumulate in G2/M in a dose-dependent manner as a consequence of causing topoisomerase II-induced DNA strand breaks.[2] The proportion of cells in G2/M not exposed to drugs was routinely found to be about 20%. By contrast, also as previously reported,[8] actinomycin has no effect on the cell cycle (data not shown), an outcome it shares with other transcription template inhibitors like echinomycin and nogalamycin.[8] With respect to the threading dimers, none of the ethylpiperidino series, including the monomer, caused perturbation of the cycle (data not shown), from which we infer that the mechanism of action of these agents does not involve topoisomerase poisoning. However, all members of the NMP series studied, including the monomer, arrest cells in G2/M as shown in Table 1, examples of the profiles for the monomer and C8 NMP being illustrated in Figure 3c and d. We take this finding to infer that compounds bearing an *N*-methylpiperdin-4-yl sidechain, do engage topoisomerase II in their mode of action, at least in part.

3. Discussion

The bis(9-aminoacridine-4-carboxamides) were designed to bisintercalate into DNA in a ''staple-like'' conformation, sandwiching 2 basepairs, with the carboxamide sidechains threading through the helix to form hydrogen bonding contacts with the O6/N7 atoms of guanine in the major groove.[8] Our previous work illustrated that bis(9-aminoacridine-4-carboxamides) with *N*,*N*-dimethylaminoethyl and ethylmorpholino sidechains do indeed bisintercalate by a threading mode, that their DNA complexes dissociate slowly, and that they are potently cytotoxic in a manner that depends both on linker and sidechain structure.[8] The intention was to develop inhibitors of transcription that mimic the RNA polymerase-blocking activity of antitumour antibiotics like actinomycin, echinomycin and nogalamycin,[5–7] and experiments with DNA microarrays illustrated that they perturb transcription profiles in a similar way to these natural products.[10] In the work presented here we sought to enhance the putative sidechain-guanine hydrogen bonding interaction in two ways, and to deter-

Table 1. Cytotoxicity, cell cycle effects and helix unwinding angles for the compounds studied

Compound	Cytotoxicity IC_{50} (nM)	Equivalence binding ratio	Helix unwinding angle (deg)	G2/M arrest
Controls				
Amsacrine	130 ± 39[a]	—	21[b]	Yes: 1 × IC_{50} (68%)[a]
9-Amino-DACA	120 ± 25[a]	—	17[c]	Yes: 10 × IC_{50} (61%)[a]
Actinomycin D	1.6 ± 1.0[a]	—	18[d]	No[a]
Pip series				
Monomer	800 ± 50	No effect on supercoiling	—	No
C6 Pip	200 ± 10	0.140 ± 0.005	23 ± 2	No
C8 Pip	99 ± 24	0.160 ± 0.010	20 ± 3	No
C3NC3 Pip	260 ± 100	0.140 ± 0.010	23 ± 2	No
C3NC4 Pip	230 ± 50	0.135 ± 0.010	24 ± 2	—
C6N2 Pip	270 ± 30	0.160 ± 0.015	20 ± 3	No
C2pipC2 Pip	130 ± 5	0.130 ± 0.005	25 ± 1	No
NMP series				
Monomer	600 ± 130	No effect on supercoiling	—	Yes: 5 × IC_{50} (60%)
C6 NMP	210 ± 20	0.250 ± 0.050	13 ± 2	—
C8 NMP	130 ± 25	0.175 ± 0.025	19 ± 3	Yes: 10 × IC_{50} (43%)
C3NC3 NMP	680 ± 90	0.240 ± 0.030	14 ± 2	Yes: 10 × IC_{50} (50%)
C3NC4 NMP	340 ± 5	0.210 ± 0.025	16 ± 2	—
C6N2 NMP	1100 ± 50	0.110 ± 0.020	30 ± 4	Yes: 10 × IC_{50} (40%)
C2pipC2 NMP	800 ± 150	No reversal	—	Yes: 5 × IC_{50} (52%)

Cytotoxicity was measured after 72 h incubation with CCRF-CEM human lukaemia cells. Cell cycle effects were studied on the basis of proportion (%) of CCRF-CEM cells arrested at G2/M phase determined by flow cytometry after treatment with the compounds at concentrations equal to the IC_{50} or at 2×, 5× or 10× that concentration, control preparations being left untreated. Helix unwinding angles were measured by agarose gel electrophoresis using pBR322 covalently closed circular DNA at pH 7.5. Pip dimers measured in 40 mM tris acetate buffer, and NMP dimers measured in 10 mM tris acetate buffer. Equivalence binding ratios are expressed in basepair units.

[a] Data taken from Ref. 8.

[b] Data taken from Ref. 18.

[c] Data taken from Ref. 17.

[d] Data taken from Ref. 19.

mine the effects of the modifications on cytotoxicity. Replacing the morpholino group, pK ≈8, with the more basic piperidine moiety, pK ≈11, was intended to strengthen the hydrogen bond energy directly, whereas the *N*-methylpiperidin-4-yl group was designed as a rigid analogue of the *N*,*N*-dimethylaminoethyl group: one that would restrict rotation of the sidechain about the carboxamide bond and reduce its degrees of freedom from 3 to 1, so making it more difficult to withdraw the bulky sidechain back through the helix.

The helix unwinding angle measurements (Table 1 and Fig. 3) show that the ethylpiperidino series form stable complexes with circular DNA on agarose gels, and that they intercalate with unwinding angles in the range 20–25°, in a manner independent of linker structure. Although these values are low for typical bisintercalating diacridines, they are within the range found for simple piperazine-linked diacridines and the piperazine-linked DMAE and morpholino bis(9-aminoacridinecarboxamides).[8,15] The findings suggest that the ethylpiperidino group binds as anticipated, that DNA affinity remains high, but that the helicoidal parameters of the complexes are distorted compared to those of the *N*,*N*-dimethylaminoethyl complexes. In contrast, the complexes of several members of the *N*-methylpiperidin-4-yl series show clear evidence of dissociation on agarose gels at the standard TEA concentration of 40 mM, and stable complexes are only observed at the lower buffer concentration of 10 mM (Tables 1 and S1, Fig. 3). This is most notable for the uncharged C6 and C8 alkyl-linked dimers, and for the C2PipC2-linked dimer. These results strongly suggest that the DNA affinity of the *N*-methylpiperidin-4-yl series is diminished, and implies that the rigid sidechain is unable to make the desired favourable interactions with the guanine O6/N7 atoms. The most likely explanation for this is that the cyclic *N*-methylpiperidin-4-yl moiety more-readily adopts a chair-like conformation in the complex, and directs the protonated nitrogen away from the guanine base (Fig. 1). Notwithstanding this, the unwinding angles of the C8-linked and all the linear polyamine-linked dimers have values approaching, or are typical of, bisintercalation (Table 1), implying that the *N*-methylpiperidin-4-yl moiety does not intrinsically inhibit bifunctional reaction in this structural framework. However, the failure of the C2PipC2 NMP dimer to reverse DNA supercoiling at any ionic strength implies that this compound is unable to bisintercalate, despite the positive changes on its linker and chromophore, suggesting a structural impediment to bifunctional reaction in this case.

The cytotoxicity of the ethylpiperidino series appears essentially independent of linker structure (Table 1), the IC_{50} values varying over a range of about 3-fold implying that all bisintercalated ethylpiperidino complexes are equally biologically active, caveats about cellular uptake notwithstanding. Taken as whole, this is a different response to the *N*,*N*-dimethylaminoethyl and

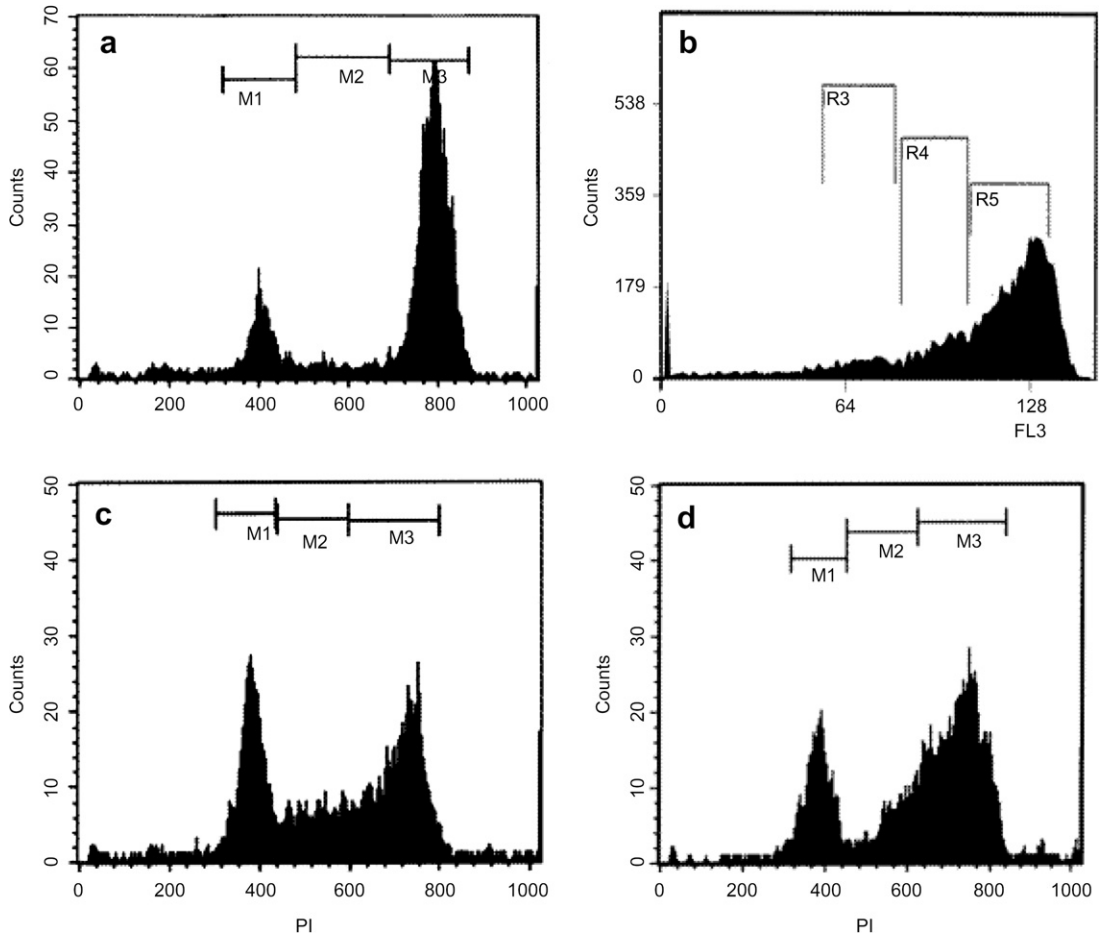


Figure 3. Flow cytometric profiles for selected agents, based on staining of CCRF-CEM cells with propidium iodide, illustrating accumulation of cells having G2+M DNA content >40% following exposure to amsacrine at 1× IC_{50} (a), 9-amino DACA at 10× IC_{50} (b), C8 NMP at 10 × IC_{50} (c) and the NMP monomer at 5 × IC_{50} (d) for 24 h.

the ethylmorpholino series, whose toxicities spread over a greater range and are more linker-specific.[8] Despite these different profiles, the C8- and C2pipC2-linked dimers are amongst the most potent in both the DMAE and ethylpiperidino series (IC_{50} in the range 35–130 nM), comparable activity being found for the C3NC3- and C3NC4-linked ethylmorpholino dimers.[8] The cytotoxicity of the *N*-methylpiperidin-4-yl series presents yet a different structure activity profile, the C8 NMP dimer, with an IC_{50} of 130 nM, being the only example that might be considered amongst the 'active' bisthreading agents (Table 1). Curiously, although the linear amine-linked NMP dimers have greater DNA affinity, they are less cytotoxic, a tendency being most pronounced with the clearly bisintercalating C6N2 dimer which is the least toxic of the series. More re-assuringly, the C2pipC2 NMP dimer, which appears not to be a bisthreader, is also amongst the least active (Table 1). Whilst DNA microarray studies confirm the threading bis(9-aminoacridine-4-carboxamides) as transcription inhibitors,[10] the possibility exists that these agents, being dimers of known topoisomerase II poisons, may also involve topoisomerase biology in their mode of action. However, the cell cycle measurements show that the ethylpiperidino series, like the transcription inhibitors actinomycin, nogalamycin and actinomycin,[10] fails to elicit the typical G2/M block (Table 1) associated with DNA strand breaks caused by trapping of the topoisomerase II cleavable complex, which mitigates against this possibility. In contrast, the *N*-methylpiperidin-4-yl series, as a whole, including the monomer (Table 1), causes clear evidence of G2/M arrest, implying that the rigid cyclic analogue of the *N*,*N*-dimethylaminoethyl sidechain may interact favourably with topoisomerase II.

4. Conclusion

In this work we have attempted to enhance the strength of the putative hydrogen bonding interaction between the carboxamide sidechain of bisintercalated threading bis(9-aminoacridine-4-carboxamides) and the O6/N7 atoms of guanine in their DNA complexes, and to assess the effects of these modifications on cytotoxicity. We find that an ethylpiperidino sidechain, employed because of its greater basicity, yields compounds with high DNA affinity and cytotoxic potency, whereas an *N*-methylpiperidin-4-yl moiety, designed as a rigid sidechain with reduced degrees of rotational freedom to

provide greater kinetic stability, destabilizes DNA binding and diminishes biological activity. We ascribe the latter effect to the *N*-methylpiperidin-4-yl moiety adopting an unfavourable chair-like conformation that directs hydrogen bonding away from the guanine base (Fig. 1). Evidently, the design of bulky threading side-chains remains a challenging task in the development of bisthreading agents with enhanced biological activity.

5. Experimental

Melting points were measured on a Reichert 'thermopan' microscope hot-stage apparatus up to 300 °C and are recorded uncorrected. Microanalyses were performed by either the Microanalytical Unit, Australian National University, Canberra, Australia, or the Campbell Laboratory, University of Otago, New Zealand. NMR spectra were recorded on a Bruker AM-300 spectrometer operating at 300.17 and 75.48 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts are reported as δ values in ppm relative to internal tetramethylsilane. The solvent was deuterated chloroform, unless otherwise specified. DEPT-135 was used to identify proton-bound carbon atoms in ^{13}C NMR spectra. MALDI-TOF mass spectra were obtained using a hydroxycinnamic acid matrix on a PerSeptive Biosystems Voyager mass spectrometer. All solvents were laboratory grade and used without further purification unless otherwise stated. Phenol was redistilled and kept under nitrogen. Dichloromethane was stored over anhydrous calcium chloride, refluxed for 4 h, and then distilled and stored over 4 Å molecular sieves. All chemical reagents were used as obtained from commercial suppliers without further purification unless otherwise indicated.

5.1. *N*-Methyl-4-aminopiperidine[13]

A solution of 4-methylpiperidone (4.9 g, 43 mmol) in MeOH (112 mL) was vigorously mixed with ammonium formate (25 g, 400 mmol) in water (12.5 mL). After complete dissolution, 10% Pd/C (5.1 g, 4.8 mmol) was added and the reaction mixture stirred overnight at room temperature. On completion of the reaction, the catalyst was filtered off on Celite and the solution concentrated under reduced pressure. *N*-methyl-4-aminopiperidine was distilled off under reduced pressure as a colourless oil (1.8 g, 37%), bp 120 °C (28 mm Hg). ^{1}H NMR: 1.22–1.35 (m, 2 H, CH_2), 1.73–1.78 (m, 2H, CH_2), 2.02–2.10 (m, 2H, CH_2), 2.16 (s, 3H, CH_3), 2.56–2.65 (m, 1H, CH), 2.74–2.78 (m, 2H, CH_2); ^{13}C NMR: 35.7 (CH_3), 46.0 (CH_2), 47.9 (CH_2), 54.49 (CH).

5.2. 9-Chloroacridine-4-carbonyl chloride

9-Oxoacridan-4-carboxylic acid was prepared as described by Rewcastle et al.[12] and the acid chloride, prepared according to Atwell et al.,[17] used in the next step without further purification.

5.3. *N*-[2-(*N*-Piperidinyl)ethyl]-9-chloroacridine-4-carboxamide

An ice-cold solution of 1-(2-aminoethyl)piperidine (0.96 g, 7.5 mmol) in dichloromethane (10 mL), containing sodium carbonate (1 g), was added over 5 min to a stirred suspension of the above carbonyl chloride (7.5 mmol) in dichloromethane (10 mL) cooled on ice. The mixture was stirred at room temperature for 4 h, the sodium carbonate removed by filtration, and the organic filtrate washed and condensed to give the product as a pale yellow solid (2.69 g). It was recrystallized from benzene and petroleum ether as a yellow powder (1.98 g, 71%), mp 116–117 °C. ^{1}H NMR: 1.48–1.54 (m, 2H, CH_2), 1.65–1.73 (*m,* 4H, $2CH_2$), 2.56–2.60 (m, 4H, $2CH_2$), 2.75 (*t,* 2H, J = 6.2 Hz, CH_2), 3.81 (q, 2 H, J = 6.2 Hz, CH_2), 7.65–7.76 (m, 2H, 2ArH), 7.83–7.88 (m, 1H, ArH), 8.35 (d, 1H, J = 8.6 Hz, ArH), 8.43 (d, 1H, J = 8.6 Hz, ArH), 8.59 (dd, 1H, J = 8.6, 1.5 Hz, ArH), 8.99 (dd, 1H, J = 7.2, 1.5 Hz, ArH), 11.74 (br s, 1H, CONH). ^{13}C NMR: 24.4 (CH_2), 25.9 (CH_2), 37.3 (CH_2), 54.6 (CH_2), 58.0 (CH_2), 123.8 (C), 124.4 (C), 124.6 (CH), 126.5 (CH), 127.4 (CH), 128.3 (CH), 129.0 (C), 129.6 (CH), 131.1 (CH), 135.4 (CH), 142.7 (C), 146.3 (C), 147.3 (C), 165.3 (C).

5.4. *N*-(*N*-Methylpiperidin-4-yl)-9-chloroacridine-4-carboxamide

This compound was prepared as above, using *N*-methyl-4-aminopiperidine (0.45 g, 4.0 mmol) and 9-chloroacridine-4-carbonyl chloride (2.0 mmol), to give *N*-(*N*-methyl-4-piperidinyl)-9-chloroacridine-4-carboxamide as a pale yellow solid (0.4 g, 57%). ^{1}H NMR: 1.99–2.06 (m, 4H, $2CH_2$), 2.25–2.30 (m, 2H, CH_2), 2.46 (*s*+m,5H, CH_3+$2CH_2$), 3.0–3.05 (m, 2H, CH_2), 4.20–4.30 (m, H, CH), 7.67–7.78 (m, 3H, 3ArH), 7.85–7.88 (m, 1H, ArH), 8.17 (d, 1H, J = 9.0 Hz, ArH), 8.43 (d, 1H, J = 9.0 Hz, ArH), 8.61 (dd, 1H, J = 9.0, 1.6 Hz, ArH), 8.99 (dd, 1H, J = 7.1, 1.6 Hz, ArH), 11.80 (br s, 1H, CONH). ^{13}C NMR: 34.0 (CH_2), 47.7 (CH_2), 48.0 (CH_2), 56.1 (CH_2), 125.6 (C), 126.3 (C), 126.5 (CH), 128.3 (CH), 129.2 (CH), 130.2 (CH), 130.5 (C), 130.8 (CH), 133.4 (CH), 137.4 (CH), 144.8 (C), 148.1 (C), 148.9 (C), 166.3 (C).

5.5. *N*-[2-(*N*-Piperidinyl)ethyl]-9-aminoacridine-4-carboxamide

N-[2-(*N*-Piperidinyl)ethyl]-9-chloroacridine-4-carboxamide (0.37 g, 1.0 mmol) was dissolved in anhydrous phenol (3 g) in a flask immersed in a 100 °C oil bath. Dry ammonia gas was bubbled through the mixture for 15 min, and the temperature raised to 115 °C for 30 min, whilst the addition of the ammonia continued. After cooling, the reaction mixture was stirred with 20% sodium hydroxide (20 mL), and the resulting suspension extracted with chloroform. The organic extract was washed with saturated sodium carbonate and dried over sodium sulfate. Evaporation of the solvent gave the title compound, which was recrystallized from benzene/petroleum ether as a yellow solid (0.21 g, 60%), mp 178.5–180 °C. ^{1}H NMR: 1.49–1.53 (m, 2H, CH_2),

1.65–1.72 (m, 4H, $2CH_2$), 2.56–2.59 (m, 4H, $2CH_2$), 2.77 (t, 2H, J = 6.2 Hz, CH_2), 3.81 (q, 2H, J = 6.2 Hz, CH_2), 6.01 (br s, 2H, NH_2), 7. 23 (t, 1H, J = 7.2 Hz, ArH), 7.36 (t, 1H, J = 7.5 Hz, ArH), 7.66 (t, 1H, J = 7.2 Hz, ArH), 7.86–7.99 (m, 3H, 3ArH), 8.73 (d, 1H, J = 6.8 Hz, ArH), 12.49 (br s, 1H, CONH). The di-hydrochloride salt of this compound was obtained as a yellow solid, mp 220.5–222 °C, by treating a solution of the free base in methanol with hydrogen chloride gas (Found: C, 54.89; H, 6.55; N, 12.05; $C_{21}H_{24}N_4O{\cdot}2HCl{\cdot}2H_2O$ requires: C, 55.14; H, 6.61; N, 12.25).

5.6. *N*-(*N*-Methylpiperidin-4-yl)-9-aminoacridine-4-carboxamide

This compound was prepared as above, using *N*-(*N*-Methyl-4-piperidinyl)-9-chloroacridine-4-carboxamide (0.2 g, 0.56 mmol), to give *N*-(*N*-Methyl-4-piperidinyl)-9-aminoacridine-4-carboxamide as a brown solid (~0.2 g) which was further recrystallized from acetonitrile as a yellow solid (0.13 g, 68%). ^{1}H NMR: 1.65–1.72 (m, 2H, CH_2), 1.78–1.90 (m, 2H, CH_2), 2.02–2.15 (m, 2H, CH_2), 2.27 (s, 3H, CH_3), 4.14 (br s, 1H, 1CH), 5.67 (*s,* 2H, NH_2), 7.38 (m, 2H, 2ArH), 7.64 (m, 1H, ArH), 7.83–7.97 (m, 3H, 3ArH), 8.81 (m, 1H, ArH), 12.51 (br s, 1H, CONH); MALDI-MS: 335.18 $(M+1)^+$. The di-hydrochloride salt of this compound was prepared by treating a methanolic solution with hydrogen chloride gas followed by precipitation with ethyl acetate. It was obtained as a yellow solid, mp 235–236 °C (Found: C, 51.72; H, 6.73; N, 11.67; $C_{20}H_{22}N_4O{\cdot}2HCl{\cdot}3H_2O$ requires: C, 52.06; H, 6.55; N, 12.14).

5.7. 1,8-Bis{4-[*N*-(*N*-methylpiperidin-4-yl)carbamoyl]acridin-9-yl}-diaminootane (C8 NMP), and general procedure for synthesis of bis(9-aminoacridine-4-carboxamides)

A mixture of *N*-(*N*-methylpiperidin-4-yl)-9-chloroacridine-4-carboxamide (0.177 g, 0.5 mmol), 1,8-diaminooctane (0.036 g, 0.25 mmol) and phenol (2.0 g) was heated at 125 °C with stirring for 2 h. On cooling, the reaction mixture was added to a mixture of diethyl ether/ethyl acetate (90 mL/45 mL) and the solution decanted. The solid residue was dissolved in methanol (3–5 mL), added to a 3% sodium hydroxide solution (100 mL) and extracted twice with chloroform (2× 50 mL). The combined organic extract was washed with 10% sodium carbonate, then with saturated brine and dried over sodium sulfate. Evaporation of the solvent gave the product as a brownish oil (0.13 g), which was recrystallized from hot acetonitrile as a yellow solid (0.09 g, 46%). ^{1}H NMR: 1.10–1.35 (m, 12H, $6CH_2$), 1.66–1.73 (m, 4H, $2CH_2$), 1.80–2.0 (m, 4H, $2CH_2$), 2.1–2.2 (m, 4H, $2CH_2$), 2.34 (*s,* 6H, $2CH_3$), 2.86–2.89 (m, 4H, $2CH_2$), 3.65–3.80 (m, 4H, $2CH_2$), 4.16 (br m, 2H, 2CH), 5.16 (br s, 2H, 2NH), 7.34–7.42 (m, 4H, 4ArH), 7.66 (t, 2H, J = 7.5 Hz, 2ArH), 7.93 (d, 2H,J = 8.3 Hz, 2ArH), 8.01 (d, 2H,J = 8.3 Hz, 2ArH), 8.15 (d, 2H,J = 7.9 Hz, 2ArH), 8.81 (d, 2H, J = 6.8 Hz, 2ArH), 12.49 (*s,* 2H, 2CONH). MALDI-MS: 776.48 (M^+). The tetrahydrochloride salt was obtained as a yellow solid, 255–258 °C (Found: C, 52.89; H, 7.57; N, 10.09; $C_{48}H_{58}N_8O_4{\cdot}4HCl{\cdot}9H_2O$ requires: C, 53.04; H, 7.42; N, 10.31).

5.8. 1,6-Bis{4-[*N*-(*N*-methylpiperidin-4-yl)carbamoyl]acridin-9-yl}-diaminohexane (C6 NMP)

This was prepared in the manner of the C8 NMP dimer, from *N*-(*N*-methylpiperidin-4-yl)-9-chloroacridine-4-carboxamide (0.22 g, 0.62 mmol) and hexamethylenediamine (0.036 g, 0.31 mmol), to give the product as a yellow solid (0.18 g, 77%). ^{1}H NMR: 1.30–1.50 (m, 4H, $2CH_2$), 1.65–1.70 (m, 8H, $4CH_2$), 1.80–1.90 (m, 4H, $2CH_2$), 2.10–2.20 (m, 4H, $2CH_2$), 2.31 (*s*, 6H, $2CH_3$), 2.81–2.84 (m, 4H, $2CH_2$), 3.72–3.8 (m, 4H, $2CH_2$), 4.14 (br s, 2H, 2CH), 5.11 (br s, 2H, 2NH), 7.29–7.39 (m, 4H, 4ArH), 7.60–7.65 (*m,* 2H, 2ArH), 7.90–7.98 (m, 4H, 4ArH), 8.09–8.12 (d, 2H, 2ArH), 8.81 (d, 2H, 2ArH), 12.45 (br s, 2H, 2CONH). MALDI-MS: 748.45 (M^+). The tetrahydrochloride salt was obtained as a yellow solid, mp 225–226.5 °C (Found: C, 50.80; H, 7.12; N, 10.11; $C_{48}H_{54}N_8O_2{\cdot}4HCl{\cdot}10H_2O$ requires: C, 51.30; H, 7.30; N, 10.40).

5.9. 1,7-Bis{4-[*N*-(*N*-methylpiperidin-4-yl)carbamoyl]acridin-9-yl}-1,4,7-triaza-heptane (C3NC3 NMP)

This was prepared as for the C8 NMP dimer, from *N*-(*N*-methylpiperidin-4-yl)-9-chloroacridine-4-carboxamide (0.212 g, 0.6 mmol) and *N*-(3-aminopropyl)-1,3-propane-diamine (0.0393 g, 0.3 mmol). The product so obtained was impure, purification being achieved by converting the free base to its pentahydrochloride salt followed by re-basification with 3% sodium hydroxide. The free base was then extracted with chloroform and recrystallized from chloroform/hexane as a yellow powder (0.09 g, 39%). ^{1}H NMR: 1.48–1.51 (m, 4H, $2CH_2$), 1.67–1.72 (m, 8H, $4CH_2$), 2.08–2.12 (*m,* 4H, $2CH_2$), 2.29 (s, 6H, $2CH_3$), 2.79–2.87 (m, 8H, $4CH_2$), 3.95–3.97 (m, 4H, $2CH_2$), 4.15 (br m, 2H, 2CH), 7.16–7.30 (m, 4H, 4ArH), 7.56 (d, 2H, 2ArH), 7.86–8.14 (m, 6H, 6ArH), 8.76 (d, 2H, 2ArH), 12.53 (br s, 2H, 2CONH). MALDI-MS: 763.46 (M^+). The pentahydrochloride salt was obtained as a yellow solid, mp 284.5–286 °C (Found: C, 55.40; H, 6.81; N, 12.40; $C_{48}H_{55}N_9O_2{\cdot}5HCl{\cdot}3H_2O$ requires: C, 55.12; H, 6.64; N, 12.58).

5.10. 1,10-Bis{4-[*N*-(*N*-methylpiperidin-4-yl)carbamoyl]acridin-9-yl}-1,5,10-triaza-decane (C3NC4 NMP)

This was prepared as for the C8 NMP dimer, using *N*-(*N*-methylpiperidin-4-yl)-9-chloroacridine-4-carboxamide (0.203 g, 0.57 mmol) and spermidine (0.041 g, 0.28 mmol), followed by flash chromatography on alumina (ethyl acetate/di(isopropyl)amine) to give a yellow solid (0.05 g, 22%) (Found: C, 72.13; H, 7.24; N, 16.46; $C_{46}H_{55}N_9O_2$ requires: C, 72.37; H, 7.37; N, 16.16). ^{1}H NMR: 1.55–1.95 (m, 12H, $6CH_2$), 2.23 (m, 2H, CH_2), 2.36 (s, 6H, $2CH_3$), 2.67 (m, 2H, CH_2), 2.84 (m, 4H, $2CH_2$), 3.36 (m, 2H, CH_2), 3.79 (m, 2H, CH_2), 3.95 (m, 2H, CH_2), 3.98 (t, 2H, CH_2), 4.14 (br, 2H, 2CH), 7.25–7.31 (m, 4H, 4ArH), 7.60 (m, 2H, 2ArH), 7.89–8.17 (*br m,* 6H, 6ArH), 8.77 (br m, 2H, 2ArH), 12.45

(br s, 2H, 2CONH). MALDI-MS: 777.47 (M^+). The pentahydrochloride salt was obtained as a yellow solid, mp 235–236 °C.

5.11. 1,10-Bis{4-[*N*-(*N*-methylpiperidin-4-yl)carbamoyl]acridin-9-yl}-1,4,7,10-tetra-azadecane (C6N2 NMP)

This was prepared as for the C8 NMP dimer, using *N*-(*N*-methylpiperidin-4-yl)-9-chloroacridine-4-carboxamide (0.253 g, 0.7 mmol) and triethylenetetramine (0.052 g, 0.35 mmol). The product was dissolved in hot acetonitrile and cooled at 4 °C to give an orange semi-solid which was solidified by tritriation with cold acetonitrile (0.09 g, 32%). ^{1}H NMR: 1.60–1.90 (m, 8H, $4CH_2$), 2.22 (m, 4H, $2CH_2$), 2.32 (m, 4H, $2CH_2$), 2.36 (*s*, 6H, $2CH_3$), 2.84 (m, 8H, $4CH_2$), 3.87 (*m,* 4H, $2CH_2$), 4.20 (br m, 2H, 2CH), 7.25–7.38 (*m,* 4H, 4ArH), 7.64 (m, 2H, 2ArH), 7.94 (br, 2H, 2ArH), 8.11 (d, 2H, $J = 8.6$, 2ArH), 8.26 (d, 2H, $J = 8.6$, 2ArH), 8.84 (br s, 2H, 2ArH), 12.54 (br s, 2H, 2CONH). MALDI-MS: 778.47 (M^+). The hexahydrochloride salt was obtained as a yellow solid, mp 232.5–234 °C (Found: C, 50.70; H, 6.96; N, 12.93; $C_{46}H_{56}N_{10}O_2{\cdot}6HCl{\cdot}5H_2O$ requires: C, 50.69; H, 6.73; N, 12.64).

5.12. *N*,*N*-Bis{4-[*N*-(*N*-methylpiperidin-4-yl)carbamoyl]acridin-9-yl}-[1,4-bis(2-aminoethyl)-piperazine] (C2pipC2 NMP)

This was prepared in the manner of the C8 NMP dimer, from *N*-(*N*-methylpiperidin-4-yl)-9-chloroacridine-4-carboxamide (0.177 g, 0.5 mmol) and 1,4-bis(2-aminoethyl)piperazine (0.043 g, 0.25 mmol), and purified by column chromatography (alumina, ethyl acetate/di(isopropyl)amine (v/v = 10/1) then methanol/chloroform). The product obtained contains trace amounts of impurities which were removed by repeated washing with hot ethyl acetate, and recrystallization from hot acetonitrile to give a yellow powder (0.07 g, 35%). ^{1}H NMR: 1.55–1.70 (m, 4H, $2CH_2$), 1.82 (m, 4H, $2CH_2$), 2.11 (m, 4H, $2CH_2$), 2.28 (m, 4H, $2CH_2$), 2.30 (*s*, 6H, $2CH_3$), 2.42 (m, 8H, $4CH_2$), 2.49 (m, 4H, $2CH_2$), 2.62 (m, 4H, $2CH_2$), 2.80 (m, 4H, $2CH_2$), 4.14 (br s, 2H, 2CH), 5.69 (br s, 1H, 1NH), 6.68 (br s, 1H, 1NH), 7.34–7.39 (m, 4H, 4ArH), 7.63 (*m,* 2H, 2ArH), 7.80–8.15 (m, 6H, 6ArH), 8.81 (d, 2H, $J = 8.6$ Hz, 2ArH), 12.53 (br s, 2H, 2CONH). MALDI-MS: 804.48 (M^+). The hexahydrochloride salt was obtained as a yellow solid, mp 233.5–235 °C (Found: C, 50.71; H, 6.72; N, 12.35; $C_{48}H_{58}N_{10}O_2{\cdot}5.5H_2O$ requires: C, 51.25; H, 6.72; N, 12.45).

5.13. 1,8-Bis{4-[*N*-(2-piperidinylethyl)carbamoyl]acridin-9-yl}-diaminooctane (C8 Pip)

This was prepared as for the C8 NMP dimer, using *N*-[2-(*N*-piperidine)ethyl]-9-chloroacridine-4-carboxamide and 1,8-diaminooctane. The pure product was obtained as a yellow solid following flash chromatography on alumina (ethyl acetate then methanol/chloroform, v/v = 1/1) in 57% yield, mp 112–113 °C (Found: C, 70.99; H, 7.81; N, 13.24; $C_{50}H_{62}N_8O{\cdot}2H_2O$ requires: C, 71.23; H, 7.89; N, 13.29). ^{1}H NMR: 1.25–1.37 (m, 8H, $4CH_2$), 1.50–1.53 (m, 4H, $2CH_2$), 2.69 (t, 8H, $J = 5.6$ Hz, $4CH_2$), 2.85 (t, 4H, $J = 6.2$ Hz, $2CH_2$), 3.78 (t, 8H, $J = 6.2$ Hz, $4CH_2$), 5.22 (br s, 2H, 2NH), 7.35 (t, 4H, $J = 7.5$ Hz, 4ArH), 7.67 (t, 2H, $J = 7.5$ Hz, ArH), 7.99 (d, 2H, $J = 8.6$ Hz, 2ArH), 8.11 (d, 2H, $J = 8.6$ Hz, 2ArH), 8.27 (d, 2H, $J = 8.3$ Hz, 2ArH), 8.69 (d, 2H, $J = 7.2$ Hz, 2ArH), 11.81 (br s, 2H, 2CONH). MALDI-MS: 807.62 (M^+). The tetrahydrochloride salt was obtained as a yellow solid, mp 199–200 °C.

5.14. 1,6-Bis{4-[*N*-(2-piperidinylethyl)carbamoyl]acridin-9-yl}-diaminohexane (C6 Pip)

This was prepared in the manner of the C8 Pip dimer using the same chloro-precursor and hexamethylenediamine, followed by flash chromatography on alumina (ethyl acetate then methanol/chloroform) to give the product as a yellow solid (60% yield), mp 171–172 °C (Found: C, 71.72; H, 7.52; N, 13.73; $C_{48}H_{58}N_8O{\cdot}1.5H_2O$ requires: C, 71.52; H, 7.63; N, 13.90). ^{1}H NMR: 1.46–1.50 (m, 8H, $4CH_2$), 1.65–1.70 (m, 12H, $6CH_2$), 2.58–2.61 (*m,* 8H, $4CH_2$), 2.69–2.73 (m, 4H, $2CH_2$), 3.77–3.84 (m, 8H, $4CH_2$), 5.20 (br s, 2H, 2NH), 7.37–7.43 (*m,* 4H, 4ArH), 7.62–7.68 (m, 2H, 2ArH), 8.02–8.22 (m, 6H, 6ArH), 8.85 (br s, 2H, 2ArH), 12.39 (br s, 2H, 2CONH). MALDI-MS: 779.53 (M^+). The tetrahydrochloride salt was obtained as a yellow solid, mp 234.5–236 °C.

5.15. 1,7-Bis{4-[*N*-(2-piperidinylethyl)carbamoyl]acridin-9-yl}-1,4,7-triazaheptane (C3NC3 Pip)

This was prepared in the manner of the C8 Pip dimer using the same chloro-precursor and *N*-(3-aminopropyl)-1,3-propane-diamine, followed by flash chromatography on alumina (ethyl acetate/di(isopropyl)amine, v/v = 10/1, then methanol/di(isopropyl)amine, v/v = 5/1). The product so obtained was impure, purification being achieved by converting the free base to its pentahydrochloride salt followed by re-basification with 3% sodium hydroxide. The free base was then recrystallized from chloroform/hexane as a yellow powder, mp 102–103 °C (Found: C, 71.21; H, 7.52; N, 15.39; $C_{48}H_{59}N_9O_2{\cdot}H_2O$ requires: C, 70.99; H, 7.57; N, 15.52). ^{1}H NMR: 1.48–1.51 (m, 4H, $2CH_2$), 1.67–1.72 (m, 8H, $4CH_2$), 2.08–2.12 (*m,* 4H, $2CH_2$), 2.68–2.71 (m, 8H, $4CH_2$), 2.85 (*t,* 4H, $J = 6.2$ Hz, $2CH_2$), 2.96–3.01 (m, 4H, $2CH_2$), 3.76–3.79 (m, 4H, $2CH_2$), 3.95–3.97 (m, 4H, $2CH_2$), 6.63 (br s, 2H, 2NH), 7.16–7.23 (m, 4H, 4ArH), 7.56 (t, 2H, $J = 7.7$ Hz, 2ArH), 7.74 (d, 2H, $J = 8.2$ Hz, 2ArH), 8.12 (d, 2H, $J = 8.2$ Hz, 2ArH), 8.27 (d, 2H, $J = 7.9$ Hz, 2ArH), 8.45 (d, 2H, $J = 7.2$ Hz, 2ArH), 11.27 (br s, 2H, 2CONH). MALDI-MS: 794.23 (M^+). The pentahydrochloride salt was obtained as a yellow solid, mp 199.5–201 °C (some sublimation at >165 °C).

5.16. 1,10-Bis{4-[*N*-(2-piperidinylethyl)carbamoyl]acridin-9-yl}-1,5,10-triazadecane (C3NC4 Pip)

This was prepared as for the C8 Pip dimer, from *N*-[2-(*N*-piperidine)ethyl]-9-chloroacridine-4-carboxamide (0.50 mmol) and spermidine (0.25 mmol). The product

was purified by chromatography on an alumina column (ethyl acetate then methanol) and recrystallized from chloroform/hexane. The dimer was obtained as a yellow solid (0.145 g, 35%) (Found: C, 72.83; H, 7.61; N, 15.60; $C_{49}H_{61}N_{19}O_2{\cdot}0.5H_2O$ requires: C, 70.47; H, 7.52; N, 17.12). ^{1}H NMR: 1.47–1.49 (m, 4H, $2CH_2$), 1.63–1.66 (m, 8H, $4CH_2$), 2.55 (m, 4H, $2CH_2$), 2.72 (m, 8H, $4CH_2$), 2.88 (m, 8H, $4CH_2$), 2.98 (m, 2H, CH_2), 3.76–3.78 (m, 4H, $2CH_2$), 3.83–3.87 (m, 2H, CH_2), 3.99–4.09 (m, 2H, CH_2), 7.21–7.30 (m, 4H, 4ArH), 7.57–7.65 (m, 2H, 2ArH), 8.01–8.24 (m, 6H, 6ArH), 8.79 (br s, 2H, 2ArH), 12.41 (br s, 2H, 2CONH). MALDI-MS: 805.50 (M^+). The hexahydrochloride salt was obtained as a yellow solid, mp 201–202.5 °C.

5.17. 1,10-Bis{4-[*N*-(2-piperidinylethyl)carbamoyl]acridin-9-yl}-1,4,7,10-tetraaza-decane (C6N2 Pip)

This was prepared as for the C8 Pip dimer, using *N*-[2-(*N*-piperidine)ethyl]-9-chloroacridine-4-carboxamide (0.50 mmol) and triethylenetetramine. Following alumina column chromatography (ethyl acetate then methanol/chloroform) the product was still impure, the clean free base finally being obtained by re-basifying its hydrochloride salt, followed by recrystallization from acetonitrile. The dimer was obtained as a yellow solid in 39% yield, mp 119–121 °C (Found: C, 70.78; H, 7.40; N, 17.27; $C_{48}H_{60}N_{10}O_2{\cdot}0.5H_2O$ requires: C, 70.47; H, 7.52; N, 17.12). ^{1}H NMR: 1.47–1.49 (m, 4H, $2CH_2$), 1.63–1.66 (m, 8H, $4CH_2$), 2.52–2.55 (m, 12H, $6CH_2$), 2.67–2.70 (*m,* 4H, $2CH_2$), 2.93–2.95 (m, 4H, $2CH_2$), 3.74–3.77 (m, 4H, $2CH_2$), 3.83–3.86 (m, 4H, $2CH_2$), 7.26–7.34 (m, 4H, 4ArH), 7.59–7.64 (m, 2H, 2ArH), 8.03-8.23 (m, 6H, 6ArH), 8.80 (br s, 2H, 2ArH), 12.42 (*br s,* 2H, 2CONH). The hexahydrochloride salt was obtained as a yellow solid, mp 239.5–241 °C.

5.18. *N*,*N*-Bis{4-[*N*-(2-piperidinylethyl)carbamoyl]acridin-9-yl}-[1,4-bis(2-aminoethyl)-piperazine] (C2pipC2 Pip)

This was prepared as for the C8 Pip dimer, using *N*-[2-(*N*-piperidine)ethyl]-9-chloroacridine-4-carboxamide (0.50 mmol) and 1,4-bis(2-aminoethyl)piperazine, and purified by column chromatography (alumina, ethyl acetate/di(isopropyl)amine, v/v = 10/1, then methanol/chloroform). The product obtained this way still contains trace amounts of impurities which were removed by repeated washing with hot ethyl acetate. Recrystallization from chloroform/hexane gave the pure dimer as a yellow powder, mp 119.5–121 °C (Found: C, 69.64; H, 7.51; N, 16.48; $C_{50}H_{62}N_{10}O_2{\cdot}1.5H_2O$ requires: C, 69.66; H, 7.60; N, 16.25). ^{1}H NMR: 1.46–1.51 (m, 4H, $2CH_2$), 1.65–1.72 (m, 8H, $4CH_2$), 2.55–2.78 (m, 24H, $12CH_2$), 3.79 (*q*, 4H, $J = 5.7$ Hz, $2CH_2$), 3.94 (t-like, 4H, $2CH_2$), 6.68 (br s, 2H, 2NH), 7.37–7.46 (m, 4H, 4ArH), 7.71 (t, 2H, $J = 7.2$ Hz, 2ArH), 8.16–8.20 (m, 4H, 4ArH), 8.30 (*d,* 2H, $J = 7.9$ Hz, 2ArH), 8.83 (d, 2H, $J = 7.2$ Hz, 2ArH), 12.41 (br s, 2H, 2CONH). MALDI-MS: 835.31 (M^+). The hexahydrochloride salt was obtained as a yellow solid, mp 217.5–219 °C.

5.19. DNA helix unwinding assay

Helix unwinding angles were measured using agarose gels as previously described.[8] Briefly, supercoiled pBR322 DNA was purchased from Progen Industries Ltd as a 0.5 mg per ml solution in 10 mM Tris–HCl buffer, pH 7.5. Solutions of both series of dimers were prepared in 40 mM TAE buffer [40 mM tris(hydroxymethyl)aminomethane, 30 mM glacial acetic acid, 1 mM EDTA, pH 7.5] at the required concentrations, and 5 μl of solution was mixed with an equal volume of a 30 μg per ml solution of DNA (150 ng of DNA, 23.6 μM in base pairs) in TAE buffer pH 7.5. NMP dimer–DNA complexes were also prepared in 10 mM TAE buffer [10 mM tris(hydroxymethyl)aminomethane, 7.5 mM glacial acetic acid, 1 mM EDTA, pH 7.5]. Complexes were incubated in the dark for 1 h at room temperature prior to application to a 0.8% agarose gel. Each titration was repeated at least 3 times. Following incubation, 3 μl of loading buffer [0.25%(w/v) bromophenol blue and 30%(w/v) glycerol] was mixed with the complex samples and the samples electrophoresed for 1 h and 15 min at 74 V (5 V/cm) in either 10 or 40 mM tris buffer at room temperature. After electrophoresis, the gel was stained with ethidium bromide and visualized by fluorescence using a CCD camera coupled to a Bio-Rad Gel Doc™ 2000 apparatus.

5.20. Growth inhibition assay and flow cytometry

Growth inhibition and flow cytometry were performed as previously described.[8] Briefly, for growth inhibition measurements, 1×10^5 CCFR-CEM cells/ml were incubated in 24-well plates with test agents for 72 h at 37 °C in RPMI medium 1640 (Gibco BRL) supplemented with 10% foetal bovine serum (Trace). Cells numbers were determined using a Coulter Counter, each drug concentration being measured in duplicate, and the experiment repeated at least 3 times. IC_{50} values were obtained using GraphPad Prism software. For flow cytometry, cells were seeded at a density of 1×10^5 per ml in 25 ml flasks and incubated for 24 h before addition of drugs. Drugs were added at 1×, 2×, 5×, and 10× the measured IC_{50} concentrations, and the cells incubated for a further 24 h. The cells were then washed in phosphate-buffered saline and fixed in ice-cold ethanol, stained with propidium iodide, and analysed on a Becton–Dickson FACSort using CellQuest software. Suitable control cells were prepared in a similar manner.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.02.063.

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