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Synthesis of novel DNA cross-linking antitumour agents based on polyazamacrocycles

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Abstract—We are seeking to develop more effective alkylating agents as antitumour agents. In previous work conformationally restricted nitrogen mustards were synthesised containing piperidine or pyrrolidine rings. The free bases were designed to be bifunctional alkylating agents via aziridinium ion formation and the effects of varying the distances between the two alkylating sites were studied. Some efficient cross-linkers of naked DNA were prepared but few of these compounds exhibited significant cytotoxicity in human tumour cells in vitro. We have extended this work by making tri- and tetra-azamacrocyclic compounds containing two to four potential alkylating sites. Most of these compounds were powerful DNA alkylating agents and showed cytotoxicity (IC_{50} values 6–100 μM) comparable with chlorambucil (45 μM) and melphalan (8.5 μM). In particular the cyclen derivative **2a** was more than 10^4 times more effective at cross-linking DNA (**2a** $XL_{50} \ll 10$ nM) than chlorambucil (XL_{50} 100 μM), and showed significant cytotoxicity in human tumour cells in vitro.

1. Introduction

The nitrogen mustards chlorambucil, melphalan, cyclophosphamide and ifosfamide are still among the most useful clinical agents for the treatment of a number of cancers.[1] They are bifunctional alkylating agents, which alkylate DNA primarily at the N-7 position of guanine bases in the major groove after the formation of aziridinium ions.[2,3] These compounds have been reported to have limited sequence specificity with alkylation occurring preferentially in the middle guanine N-7 position of runs of guanines.[4,5]

The critical event caused by bifunctional agents of this type is the DNA interstrand cross-link.[2,3] For most nitrogen mustards, however, these cross-links constitute a minority of the total DNA adducts. The majority of these adducts are monoadducts, which are much less cytotoxic than cross-links yet they appear to be the primary mutagenic lesion produced.[6] A therapeutic advance in this area might be possible if more efficient and selective DNA interstrand cross-linking agents could be developed.

We recently prepared a series of bifunctional *N*-mustard derivatives, including 2,6-disubstituted *N*-methylpiperidines,[7] bispiperidines[8] and homochiral bispyrrolidines.[9] These compounds were bifunctional alkylating agents via aziridinium ion formation, and offered an insight into structure/activity relationships for compounds with carbon chains having different lengths bridging the two nitrogens. A bridging distance between the nitrogen atoms of two carbon atoms usually produced the most efficient cross-linking of DNA.[8]

Although these results provided information about optimum carbon linker lengths required for DNA interstrand cross-linking,[9] the cytotoxicities observed were variable. Some efficient alkylating agents were virtually noncytotoxic (possibly due to their facile hydrolysis in aqueous solution to form nontoxic 2-hydroxyethyl-substituted compounds). Additionally, the results from attempts to convert the cytotoxic mustards into bioreducible prodrugs via *N*-oxide formation were not

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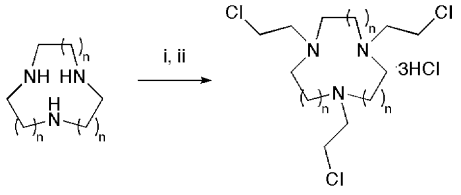
promising. Our search for new *N*-mustard compounds that would provide highly efficient DNA cross-linking and allow the development of alternative strategies for prodrug formation led us to prepare a range of polyfunctional alkylating agents based on polyazamacrocycles.

Polyazamacrocycles provide the possibility for two or more alkylating moieties to be present in the same molecule, and the distance between them can be varied by changing the number of carbons in the ring. Their coordination chemistry is well-documented,[10–14] providing an opportunity for prodrug formation through complexation with metal ions.[15] Here we report our progress in the synthesis of this new class of mustard drugs, as well as their biological evaluation as DNA cross-linking and antitumour agents.

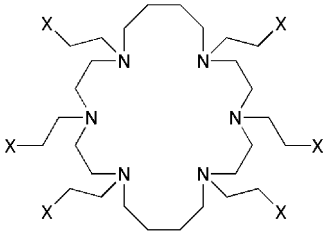
2. Results and discussion

2.1. Chemistry

The parent triazamacrocycles were synthesised through (2-trimethylsilyl)ethanesulfonamide (SES-amide) intermediates as previously described.[16,17] The tetraazamacrocycles were obtained commercially. The mustard derivatives (compounds **2** and **4**) were obtained from the azamacrocycles in good yield via the corresponding *N*-(2-hydroxyethyl) compounds **1** and **3**, as illustrated in Scheme 1. The bifunctional triazamacrocyclic mustard **4d** was obtained from the *N*,*N*′-bis(2-hydroxyethyl) triazamacrocycle, isolated from an attempted synthesis of **3e**. Additionally, the hexaazamacrocyclic mustard **4j** was synthesised (Fig. 1). Compound details and yields are given in Tables 1 and 2.



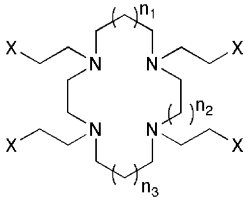
Scheme 1. General synthesis of polyazamacrocyclic nitrogen mustards. Reagents and conditions: (i) ethylene oxide (excess), EtOH, 5 °C, 18 h; (ii) $SOCl_2$, 50 °C, 18 h.



3j: X = OH
4j: X = Cl, 6HCl

Figure 1. Hexaazamacrocyclic derivatives **3j** and **4j** (see Table 2 for yields).

Table 1. Structural details and yields for tetraazamacrocyclic *N*-mustards

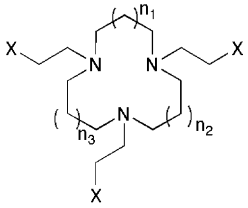


1a-c, 2a-c

n_1, n_2, n_3	Ring size[a]	X = OH yield (%)	X = Cl yield (%) (as HCl salt)
0, 1, 0	[2, 2, 2, 2]	**1a**[b]	**2a**[b]
1, 1, 1	[2, 3, 2, 3]	**1b**[b]	**2b**[b]
1, 2, 1	[2, 3, 3, 3]	**1c**: 86	**2c**: 74

[a] 'Ring size' indicates the number of carbons between nitrogens in the parent ring.
[b] Synthesis reported elsewhere.[18]

Table 2. Structural details and yields for triazamacrocyclic *N*-mustards



3a-j, 4a-j

n_1, n_2, n_3	Ring size[a]	X = OH yield (%)	X = Cl yield (%) (as HCl salt)
0, 0, 0	[2, 2, 2]	**3a**[b]	**4a**[b]
1, 1, 1	[3, 3, 3]	**3b**: 88	**4b**: 61
1, 1, 2	[3, 3, 4]	**3c**: 44	**4c**: 89
1, 1, 3	[3, 3, 5]	(Bis) **3d**: 13	(Bis) **4d**: 99
1, 1, 3	[3, 3, 5]	(Tris) **3e**: 57	(Tris) **4e**: 93
1, 2, 2	[3, 4, 4]	**3f**: 68	**4f**: 85
1, 2, 3	[3, 4, 5]	**3g**: 96	**4g**: 99
2, 3, 3	[4, 5, 5]	**3h**: 99	**4h**: 99
2, 4, 4	[4, 6, 6]	**3i**: 99	**4i**: 99
2(1, 1, 2)	[2, 2, 4, 2, 2, 4]	**3j**: 74	**4j**: 83

[a] As in Table 1.
[b] Synthesis reported elsewhere.[18]

2.2. Biological assays

The cytotoxicities of the novel nitrogen mustards were determined against the human chronic myeloid leukemia cell line K562 using the MTT cell proliferation assay[19] after a 1 h exposure to each drug. We have found that for agents of this type (which have short half lives in aqueous media), 1 h exposure times are most useful. This allows sufficient time for the active agent to enter cells. Due to the rapid hydrolysis of 2-chloroethyl derivatives to unreactive 2-hydroxyethyl derivatives in aqueous solution, longer exposure times would not provide any additional activity since only the unreactive compound would remain. For the cross-linking

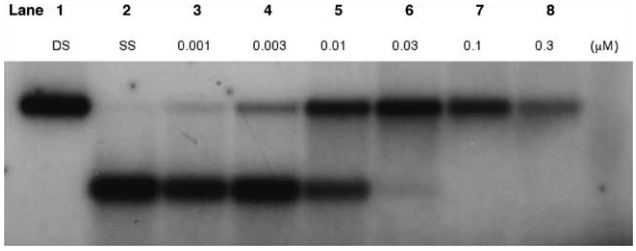


Figure 2. Representative gel for **4d**. Lanes 1 and 2 are control lanes showing double-stranded (DS) and single-stranded (SS) DNA. Lanes 3–8 show the extent of cross-linking from SS to DS at different concentrations of drug (μM).

assays, the drugs were each allowed to react with ^{32}P-end labelled linearised plasmid DNA for two hours, then the DNA was precipitated and incubated with strand separation buffer. Our experience indicates that two hours of exposure in the DNA cross-link assay is best to compare different interstrand cross-linkers for their ability to crosslink naked DNA. By this time most reactive agents have produced a peak of cross-linking. Gel electrophoresis was performed against single-stranded and double-stranded DNA as controls, resulting in the migration of the cross-linked DNA with the double-stranded control. The percent double-stranded DNA in each lane was determined from densitometry of autoradiographed images of the gels. A representative gel (from the assay of **4d**) is shown in Figure 2. The cross-linking activity is expressed as XL_{50}: the concentration of drug, which resulted in 50% cross-linked DNA.

The cytotoxicities vary widely, and don't show an obvious relationship to the cross-linking efficiency. This may be due to hydrolytic decomposition of the mustard drugs in cell growth medium, but could also result from differences in uptake of the drugs. Nonetheless, the cytotoxicities for the novel mustard drugs are comparable to those for chlorambucil and melphalan under the same assay conditions (Table 3).

Meaningful cross-linking data could not be obtained for two of the compounds, **4f** and **4g**. Compound **4f** did not cross-link DNA under any of the conditions used. Compound **4g** caused aggregation of the DNA, so that individual data points could not be observed—however, it was determined to cause 100% cross-linking at 100 nM. With the exception of the hexaazamacrocyclic mustard **4j** (which is probably too sterically bulky for efficient cross-linking), the rest of the compounds are remarkably potent DNA cross-linking agents, with XL_{50} activities in the nanomolar range (Table 3). Increased cross-linking efficiency was observed for the bis-substituted mustard **4d** over the tris-substituted compound **4e**. All of the tetrafunctional mustards had XL_{50} values below 10 nM—probably as a result of having the additional alkylating functionality.

It is not possible to determine, which bifunctional cross-links are being formed from the tetra-substituted mustards—that is between '*cis*-' or '*trans*-ring' alkylating substituents (Fig. 3). Bifunctional *N*-mustard drugs usually form 'diagonal' cross-links between nonadjacent guanines (5′-**G**NC-3′/3′-CN**G**-5′), rather than forming the 5′-**GC**-3′/3′-C**G**-5′ cross-link (Fig. 4). This is thought to be due to distortion of the classical solution B-DNA structure induced by the initial alkylation to the monofunctional adduct.[20] All of the macrocyclic parent ring structures are conformationally flexible, so it is likely that nearly all the sterically possible cross-links are being formed. The high efficiency of these compounds for cross-linking DNA is worthy of further investigation.

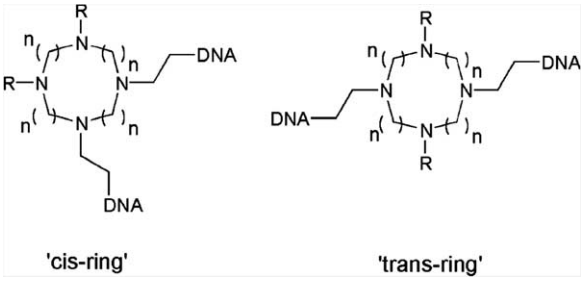


Figure 3. Possible outcomes for bifunctional alkylation.

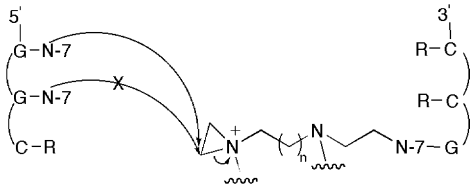


Figure 4. 'Diagonal' cross-linking by bifunctional mustards. 'G' and 'C' represent the nucleobases guanine and cytosine. N-7 is the nucleophilic nitrogen from the guanine ring.

Table 3. Characterisation of biological activity for compounds **2a–c** and **4a–j**

Compound [ring size]	**2a** [2,2,2,2]	**2b** [2,3,2,3]	**2c** [2,3,3,3]	**4a** [2,2,2]	**4b** [3,3,3]	**4c** [3,3,4]	**4d** [3,3,5]-Bis	**4e** [3,3,5]-Tris	**4f** [3,4,4]	**4g** [3,4,5]	**4h** [5,5,4]	**4i** [6,6,4]	**4j** $[2,2,4]_2$	C_1[c]	C_2[c]
IC_{50}[a](μM)	22	7.5	9	10.5	6	25	6.3	25	>100	21	>100	35	100	45	8.5
XL_{50} (nM)	≪10	30	<10	60	90	45	10	35	Nd[b]	Nd[b]	35	100	>300	10^5	2×10^4
# of atoms in ring	12	14	15	9	12	13	14	14	14	14	17	20	22	Na	Na

[a] Against human chronic myeloid leukaemia cell line K562.

[b] Unable to acquire meaningful cross-linking data.

[c] Controls: C_1 = chlorambucil, C_2 = melphalan.

3. Conclusion

Twelve novel macrocyclic nitrogen mustards, as well as a previously reported compound (**2b**) whose biological activity had not been discussed, were synthesised and assessed for their DNA cross-linking efficiency and cytotoxicity against the human leukaemia cell line K562. Most of the compounds exhibited highly efficient DNA cross-linking activity (up to 10^4 times greater than that for the anticancer alkylating agent chlorambucil and 2000 times greater than melphalan) and comparable cytotoxicity to these clinically-relevant drugs. Other than the observation that additional alkylating functionality tended to provide greater cross-linking efficiency, no clear structure–activity relationship was evident. However, nearly all of the compounds were highly promising as DNA cross-linking agents, and represent a novel approach to the design of polyfunctional alkylating agents. The most efficient cross-linker **2a** reported here can be converted into a stable bioreducible prodrug,[18] and is a lead compound for the development of new hypoxia-selective cytotoxins.

4. Experimental

4.1. Chemistry

4.1.1. General methods. Chemicals were purchased from Aldrich Chemical Company (Gillingham, Dorset, UK) or Lancaster Synthesis Ltd (Morecambe, Lancs, UK) and used without further purification. Melting points were measured with a Gallenkamp apparatus and are uncorrected. ^{1}H and ^{13}C NMR were recorded on a Bruker DPX 400 spectrometer, with chemical shift values reported on the δ scale (TMS = 0) relative to residual chloroform (δ_H = 7.27 or δ_C = 77.2) as internal standard unless otherwise stated. Coupling constants (*J*) are reported in Hertz (Hz). Mass spectrometry was performed on a JEOL JMS-700 spectrometer.

4.1.2. General synthesis of poly-*N*-(2-hydroxyethyl)polyazamacrocycles (1a–c, 3a–j). The polyazamacrocycle free base[17] (1 equiv) was dissolved in EtOH (10 mL/100 mg polyazamacrocycle) in a round bottom flask fitted with a cold finger and cooled to 5 °C with a circulating cooling bath. Ethylene oxide (excess of approximately 100 equiv) was added dropwise by condensing on the cold finger. The mixture was stirred and held at 0 °C overnight, after which the solvent was removed by rotary evaporation. The residue was typically pure, but could be purified by recrystallisation or Kugelrohr distillation if necessary.

4.1.3. 1,4,8,12-Tetra(2-hydroxyethyl)-1,4,8,12-tetraazacyclopentadecane (1c). Using 1,4,8,12-tetraazacyclopentadecane (0.2 g, 0.9 mmol), **1c** was prepared according to the above method as a clear oil (0.30 g, 86%); δ_H ($CDCl_3$) 1.02–1.33 (6H, m), 1.98–2.70 (24H, m), 3.00–3.20 (8H, m); δ_C ($CDCl_3$) 25.0, 26.6, 53.4, 54.4, 54.7, 58.9, 59.6 and 63.8; *m/z* (CI+) 391.3 ($[M+H]^+$, 100%). Found $[M+H]^+$, 391.3284. $C_{19}H_{27}N_2O_4$ requires 391.3284.

4.1.4. 1,5,9-Tris(2-hydroxyethyl)-1,5,9-triazacyclododecane (3b). Using 1,5,9-triazacyclododecane (0.050 g, 0.29 mmol), **3b** was prepared according to the above procedure as a clear oil (0.079 g, 88% yield); δ_H ($CDCl_3$) 1.74 (6H, quintet, J = 6.0), 2.35–2.65 (18H, m), 3.65 (6H, t, J = 5.0); δ_C ($CDCl_3$) 24.1, 51.0, 56.0, 58.7.

4.1.5. 1,5,9-Tris(2-hydroxyethyl)l-1,5,9-triazacyclotridecane (3c). Using 1,5,9-triazacyclotridecane (0.040 g, 0.22 mmol), **3c** was prepared according to the above procedure and purified by Kugelrohr distillation to give a clear oil (0.030 g, 44% yield); δ_H ($CDCl_3$) 1.62–1.65 (4H, m), 1.67–1.71 (4H, m), 2.49–2.58 (18H, m), 3.59–3.63 (6H, m); δ_C ($CDCl_3$) 23.6, 24.3, 51.7, 52.7, 53.3, 55.1, 56.4, 57.7, 57.9.

4.1.6. 1,9-Bis(2-hydroxyethyl)-1,5,9-triazacyclotetradecane (3d). Using 1,5,9-triazacyclotetradecane (0.075 g, 0.38 mmol), **3d** was isolated from an attempted preparation of **3e** according to the above procedure and purified by Kugelrohr distillation to give a clear oil (0.014 g, 13% yield); δ_H ($CDCl_3$) 1.40–1.57 (6H, m), 1.76 (4H, quintet, J = 5.6), 2.42–2.64 (12H, m), 2.65–2.71 (4H, m), 3.60–3.64 (4H, m); δ_C ($CDCl_3$) 23.1, 25.4, 26.7, 49.1, 51.3, 54.1, 56.8, 60.4; *m/z* (FAB+ mode) 288.2 ($[M+H]^+$, 100%), 286.2 (10), 128.1 (5), 98.4 (7), 73.7 (17). Found $[M+H]^+$ 288.2653, $C_{15}H_{34}N_3O_2$ requires 288.2651.

4.1.7. 1,5,9-Tris(2-hydroxyethyl)-1,5,9-triazacyclotetradecane (3e). Using 1,5,9-triazacyclotetradecane (0.060 g, 0.30 mmol), **3e** was prepared according to the above procedure as a clear oil (0.057 g, 57% yield); δ_H ($CDCl_3$) 1.55–1.78 (10H, m), 2.42–2.71 (18H, m), 3.54–3.67 (6H, m); δ_C ($CDCl_3$) 23.2, 24.5, 25.1, 25.5, 26.3, 49.1, 51.0, 52.2, 52.5, 53.3, 53.4, 56.4, 56.7, 57.5, 58.9, 59.1 and 59.7; *m/z* (CI+ mode/isobutane) 388.4 (10%), 349.2 (33), 332.3 ($[M+H]^+$, 43), 288.3 (100), 279.2 (22), 270.3 (10), 244.3 (6), 195.2 (4), 151.2 (7), 107.1 (8), 91.1 (4). Found $[M+H]^+$ 332.2907, $C_{17}H_{38}N_3O_3$ requires 332.2913.

4.1.8. 1,5,10-Tris(2-hydroxyethyl)-1,5,10-triazacyclotetradecane (3f). Using 1,5,10-triazacyclotetradecane (0.041 g, 0.21 mmol), **3f** was prepared according to the above procedure as a clear oil (0.046 g, 68% yield); δ_H (D_2O) 1.38–1.89 (10H, m), 2.36–2.59 (18H, m), 3.44–3.58 (6H, m); δ_C (D_2O; ref. to internal MeOH = 38.0) 21.3, 27.2, 27.8, 55.4, 56.8, 57.0, 57.4, 59.7, 60.8 and 63.6; *m/z* (CI+ mode/isobutene) 388.4 (20%), 332.3 ($[M+H]^+$, 100), 330.3 (10), 288.3 (7). Found $[M+H]^+$ 332.2909, $C_{17}H_{38}N_3O_3$ requires 332.2913.

4.1.9. 1,5,10-Tris(2-hydroxyethyl)-1,5,10-triazacyclopentadecane (3g). Using 1,5,10-triazacyclopentadecane (0.040 g, 0.19 mmol), **3g** was prepared according to the above procedure as a clear oil (0.063 g, 96% yield); δ_H ($CDCl_3$) 1.35–1.66 (12H, m), 2.37–2.57 (18H, m), 3.53–3.57 (6H, m); δ_C ($CDCl_3$) 25.7, 26.0, 27.7, 27.9, 53.9, 54.1, 54.2, 55.1, 55.4, 56.0, 56.6, 57.5, 57.8, 59.3, 59.4; *m/z* (FAB+ mode) 346.3 ($[M+H]^+$, 100%), 344.3 (20), 302.3 (10), 128.1 (15), 98.4 (14), 84.6 (13), 73.7 (11), 56.9 (6). Found $[M+H]^+$ 346.3072, $C_{18}H_{40}N_3O_3$ requires 346.3070.

4.1.10. 1,6,12-Tris(2-hydroxyethyl)-1,6,12-triazacycloheptadecane (3h). Using 1,6,12-triazacycloheptadecane (0.040 g, 0.17 mmol), **3h** was prepared according to the above procedure as a clear oil (0.061 g, >99% yield); δ_H ($CDCl_3$) 1.32–1.49 (16H, m), 2.28–2.48 (18H, m), 3.45–3.48 (6H, m); δ_C ($CDCl_3$) 25.6, 26.5, 27.8, 28.0, 54.3, 54.4, 54.6, 56.4, 58.4, 58.9 and 59.0; *m/z* (FAB+ mode/NOBA) 374.6 ($[M+H]^+$, 40%), 372.6 (12), 327.2 (5), 281.2 (4), 207.1 (7), 147.1 (14), 98.5 (9), 73.7 (100), 44.1 (8). Found $[M+H]^+$ 374.3378, $C_{20}H_{44}N_3O_3$ requires 374.3383.

4.1.11. 1,6,13-Tris(2-hydroxyethyl)-1,6,13-triazacyclononadecane (3i). Using 1,6,13-triazacyclononadecane (0.040 g, 0.15 mmol), **3i** was prepared according to the above procedure as a clear oil (0.060 g, >99% yield); δ_H ($CDCl_3$) 1.26–1.51 (20H, m), 2.42–2.61 (18H, m), 3.49–3.55 (6H, m); δ_C ($CDCl_3$) 25.8, 27.1, 27.2, 27.5, 27.6, 53.8, 54.1, 54.3, 56.7, 56.9, 58.6, 58.7 and 77.5; *m/z* (EI+ mode) 401.7 ($[M+H]^+$, 7%), 371.6 (84), 370.6 (83), 324.6 (29), 323.6 (28), 255.4 (14), 225.4 (15), 170.3 (16), 142.2 (38), 112.2 (96), 84.1 (100), 55.1 (100). Found $[M+H]^+$ 401.3616, $C_{22}H_{47}N_3O_3$ requires 401.3617.

4.1.12. 1,5,9,12,17,20-Hexa(2-hydroxyethyl)-1,5,9,12,17,20-hexaazacyclodocosane (3j). Using 1,5,9,12,17,20-hexaazacyclodocosane (0.040 g, 0.13 mmol), **3j** was prepared according to the above procedure as a clear oil (0.055 g, 74% yield); δ_H ($CDCl_3$) 1.47–1.56 (8H, m), 2.45–2.67 (36H, m), 3.54–3.60 (12H, m); δ_C ($CDCl_3$) 25.3, 52.1, 52.5, 55.3, 56.3, 57.6, 59.5, 59.7; *m/z* (FAB+ mode) 579.5 ($[M+H]^+$, 37%), 535.4 (35), 491.4 (17), 393.3 (10), 322.2 (13), 290.2 (8), 171.1 (22), 114.2 (80), 84.6 (100), 73.7 (87). Found $[M+H]^+$ 579.4817, $C_{28}H_{63}N_6O_6$ requires 579.4809.

4.1.13. General synthesis of nitrogen mustard derivatives (2a–c, 4a–j). The poly-*N*-(2-hydroxyethyl) derivative **1** or **3** was stirred with $SOCl_2$ (5 mL/100 mg **1** or **3**) while heating to 50 °C overnight. The excess thionyl chloride was removed in vacuo leaving the hydrochloride salt, which was usually sufficiently pure for analysis and testing. If necessary, the salt could be crystallised from MeOH or 2-PrOH.

4.1.14. 1,4,8,12-Tetra(2-chloroethyl)-1,4,8,12-tetraazacyclopentadecane dihydrochloride (2c). Using **1c** (0.30 g, 0.8 mmol), **2c** was prepared according to the above procedure to give a yellow solid (0.26 g, 74%); δ_H (D_2O) 1.81–2.10 (6H, m), 2.85–3.55 (24H, m), 3.62–3.81 (8H, m); δ_C (D_2O; ref. to internal MeOH = 38.0) 25.5, 26.9, 41.2, 53.7, 54.8, 54.9, 59.3 and 59.9; *m/z* (CI+) 463.2 ($[M+H]^+$, 100%). Found $[M+H]^+$ 463.1927, $C_{19}H_{39}N_4{}^{35}Cl_4$ requires 463.1929.

4.1.15. 1,5,9-Tris(2-chloroethyl)-1,5,9-triazacyclododecane trihydrochloride (4b). Using **3b** (0.032 g, 0.11 mmol), **4b** was prepared according to the above procedure as a cream solid (0.030 g, 61% yield); δ_H (D_2O) 1.90–1.93 (6H, m), 3.04–3.07 (12H, m), 3.16–3.19 (6H, m), 3.62–3.65 (6H, m); δ_C (D_2O; ref. to internal MeOH = 38.0) 18.2, 38.7, 48.9 and 55.5; *m/z* (FAB+, glycerol) 358.1 ($[(M-3HCl)+H]^+$, 100%), 340.2 (13), 324.2 (6), 296.1 (4), 232.1 (4), 132.0 (13), 106.3 (33), 84.6 (12), 70.8 (11), 58.9 (7). Found $[(M-3HCl)+H]^+$ 360.1553, $C_{15}H_{31}N_3{}^{35}Cl_2{}^{37}Cl$ requires 360.1556.

4.1.16. 1,5,9-Tris(2-chloroethyl)-1,5,9-triazacyclotridecane trihydrochloride (4c). Using **3c** (0.030 g, 0.094 mmol), **4c** was prepared according to the above procedure as a yellow foamy solid (0.041 g, 89% yield); δ_H (D_2O) 1.85–1.98 (4H, m), 2.132.19 (4H, m), 3.33–3.61 (18H, m), 3.91–3.98 (6H, m); δ_C (D_2O; ref. to internal MeOH = 38.0) 17.7, 20.4, 37.8, 38.0, 48.8, 49.4, 49.7, 51.4, 56.7, 56.8, 56.9; *m/z* (FAB+, NOBA) 372.3 ($[(M-3HCl)+H]^+$, 12%), 357.3 (5), 310.3 (10), 238.3 (5), 169.1 (54), 85.6 (100), 84.6 (15), 66.8 (5). Found $[(M-3HCl)+H]^+$ 372.1738, $C_{16}H_{33}N_3{}^{35}Cl_3$ requires 372.1740.

4.1.17. 1,9-Bis(2-chloroethyl)-1,5,9-triazacyclotetradecane trihydrochloride (4d). Using **3d** (0.014 g, 0.042 mmol), **4d** was prepared according to the above procedure as a yellow foamy solid (0.022 g, >99% yield); δ_H (D_2O) 1.59 (2H, broad m) 1.73 (4H, broad m), 2.01–2.09 (4H, broad m), 3.26–3.38 (12H, m), 3.59–3.64 (4H, m), 3.91–3.94 (4H, m); δ_C (D_2O; ref. to internal MeOH = 38.0) 16.9, 18.5, 20.2, 37.8, 40.1, 47.3, 49.6, 55.9; *m/z* (FAB+) 324.4 ($[(M-3HCl)+H]^+$, 100%), 288.3 (22), 262.3 (11), 222.3 (4), 130.2 (38), 102.3 (36), 98.3 (22). Found $[(M-3HCl)+H]^+$ 324.1970, $C_{17}H_{35}N_3{}^{35}Cl_3$ requires 324.1973.

4.1.18. 1,5,9-Tris(2-chloroethyl)-1,5,9-triazacyclotetradecane trihydrochloride (4e). Using **3e** (0.057 g, 0.17 mmol), **4e** was prepared according to the above procedure as a yellow foamy solid (0.079 g, 93% yield); δ_H (D_2O) 1.28 (2H, broad m), 1.43–1.44 (4H, broad m), 1.74–1.75 (4H, broad m), 2.93–3.18 (12H, m), 3.29–3.39 (6H, m), 3.60–3.63 (6H, m); δ_C (D_2O; ref. to internal MeOH = 38.0) 19.9, 20.6, 35.2, 37.0, 37.7, 50.4, 52.0, 56.2; *m/z* (FAB+) 386.3 ($[(M-3HCl)+H]^+$, 63%), 352.4 (10), 324.3 (100), 288.3 (21), 262.3 (20), 210.3 (12), 167.2 (5), 106.2 (35). Found $[(M-3HCl)+H]^+$ 386.1902, $C_{17}H_{35}N_3{}^{35}Cl_3$ requires 386.1897.

4.1.19. 1,5,10-Tris(2-chloroethyl)-1,5,10-triazacyclotetradecane trihydrochloride (4f). Using **3f** (0.040 g, 0.12 mmol), **4f** was prepared according to the above procedure as a tan foamy solid (0.051 g, 85% yield); δ_H (D_2O) 1.51–1.98 (10H, broad m), 3.05–3.22 (12H, broad m), 3.30–3.39 (6H, m), 3.58–3.67 (6H, m); (D_2O; ref. to internal MeOH = 38.0) 19.9, 20.6, 35.2, 37.0, 37.6, 37.7, 50.4, 52.0, 56.2, 56.3; *m/z* (FAB+) 387.2 ($[(M-3HCl)+H]^+$, 18%), 326.2 (6), 289.2 (4), 240.0 (4), 191.1 (28), 135.0 (25), 97.4 (100), 96.4 (93), 77.6 (44), 56.0 (32). Found $[(M-3HCl)+H]^+$ 386.1903; $C_{17}H_{35}N_3{}^{35}Cl_3$ requires 386.1897.

4.1.20. 1,5,10-Tris(2-chloroethyl)-1,5,10-triazacyclopentadecane trihydrochloride (4g). Using **3g** (0.053 g, 0.15 mmol), **4g** was prepared according to the above procedure as a yellow foamy solid (0.079 g, >99% yield); δ_H (D_2O) 1.42–1.99 (10H, broad m), 2.05–2.25 (2H,

broad m), 3.20–3.53 (12H, broad m), 3.58–3.67 (6H, m), 3.873.94 (6H, m); (D_2O; ref. to internal MeOH = 38.0) 20.6, 21.2, 21.6, 21.8, 35.3, 37.6, 37.8, 48.5, 50.9, 51.3, 52.2, 56.0, 56.2, 56.4; *m/z* (FAB+) 401.2 ([(M−3HCl)+H]$^+$, 48%), 400.2 (13), 367.2 (11), 340.2 (8), 303.2 (5), 190.1 (35), 189.1 (23), 146.0 (22), 96.4 (100), 95.4 (53), 77.6 (33), 56.0 (19). Found [(M−3HCl)+H]$^+$ 402.2221; $C_{18}H_{39}N_3{}^{35}Cl_3$ requires 402.2210.

4.1.21. 1,6,12-Tris(2-chloroethyl)-1,6,12-triazacycloheptadecane trihydrochloride (4h). Using **3h** (0.055 g, 0.15 mmol), **4h** was prepared according to the above procedure as a yellow solid (0.080 g, >99% yield); δ_H (D_2O) 1.42–1.54 (4H, broad m), 1.711.90 (12H, broad m), 3.15–3.98 (24H, broad m); δ_C (D_2O; ref. to internal MeOH = 38.0) 20.6, 21.3, 22.0, 22.3, 51.6, 52.0, 52.5, 52.7, 53.1, 54.8, 55.1; *m/z* (FAB+ mode) 428.5 ([(M−3HCl)+H]$^+$, 100%) 394.5 (43), 392.5 (25), 366.4 (18). 253.2 (19), 202.2 (22), 148.2 (52), 98.3 (95). Found [(M−3HCl)+H]$^+$ 428.2360, $C_{20}H_{41}N_3{}^{35}Cl_3$ requires 428.2366.

4.1.22. 1,6,13-Tris(2-chloroethyl)-1,6,13-triazacyclononadecane trihydrochloride (4i). Using **3i** (0.048 g, 0.12 mmol), **4i** was prepared according to the above procedure as a yellow solid (0.068 g, >99% yield); δ_H (D_2O) 1.33–1.57 (8H, broad s), 1.611.87 (12H, broad m), 3.12–3.98 (24H, m); δ_C (D_2O; ref. to internal MeOH = 38.0) 20.3, 21.4, 21.6, 22.2, 22.6, 51.5, 51.9, 52.3, 52.5, 53.3, 55.0, 55.2; *m/z* (FAB+ mode) 456.6 ([(M−3HCl)+H]$^+$, 100%), 359.5 (10), 329.5 (9), 271.3 (8), 216.3 (15), 190.2 (35), 112.3 (32), 96.5 (100), 56.0 (90). Found [(M−3HCl)+H]$^+$ 456.2680, $C_{22}H_{45}N_3{}^{35}Cl_3$ requires 456.2679.

4.1.23. 1,5,9,12,17,20-Hexa(2-chloroethyl)-1,5,9,12,17,20-hexaazacyclodocosane hexahydrochloride (4j). Using **3j** (0.055 g, 0.095 mmol), **4j** was prepared according to the above procedure to give a tan powder (0.071 g, 83% yield); δ_H (D_2O) 1.82–1.95 (8H, broad m), 2.94–3.97 (48H, broad m); δ_C (D_2O; ref. to internal MeOH = 38.0) 20.5, 20.6, 37.8, 48.8, 53.1, 55.2, 55.6, 56.0.

4.2. Cytotoxicity

The cytotoxic effects of the agents studied were measured against the human chronic myeloid leukaemia cell line K562. Cells were maintained as a suspension in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) and 2 mM glutamine (Gln) at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. The IC_{50} values of the series of analogues following a 1 h exposure to drug were determined using the MTT assay[19] as has been previously described.[21]

4.3. DNA cross-linking activity

4.3.1. Agarose gel cross-link assay. This method was previously described.[22] pBR322 plasmid DNA was linearised by digestion with HinDIII and dephosphorylated by treatment with bacterial alkaline phosphatase. The DNA was 5′-end-labelled using T4 polynucleotide kinase and [γ-^{32}P] ATP (5000 Ci/mmol, Amersham, UK.). Following precipitation and removal of unincorporated ATP, the DNA was resuspended in sterile double-distilled water at 1 mg/mL. Approximately 10 ng of labelled DNA were used for each experimental point. Reactions with drug were performed in 25 mM triethanolamine, 1 mM EDTA (pH 7.2) at 37 °C for 2 h. Reactions were terminated by the addition of a greater than equal volume of stop solution (0.6 M sodium acetate, 20 mM EDTA, 100 μg/mL tRNA) and the DNA was precipitated by the addition of 3 vols 95% ethanol. Following centrifugation and removal of supernatant, the DNA pellet was dried by lyophilisation. Samples were dissolved in 10 μL strand separation buffer (30% dimethyl sulfoxide, 1 mM EDTA, 0.04% bromophenol blue, 0.04% xylene cyanol), heated at 90 °C for 2 min and chilled immediately in an ice-water bath prior to loading. Control undenatured samples were dissolved in 10 μL 6% sucrose, 0.04% bromophenol blue and loaded directly. Electrophoresis was performed on 20 cm long 0.8% submerged horizontal agarose gels at 40 V for 16 h. The gel and running buffer were 40 mM Tris, 20 mM acetic acid and 2 mM EDTA (pH 8.1). Gels were dried at 80 °C onto one layer of Whatman 3 MM and one layer of DE81 filter papers on a Bio-Rad Model 583 gel drier connected to a vacuum. Autoradiography was performed with Hyperfilm MP (Amersham, UK) at −70 °C using a DuPont-Cronex Lightening-plus intensifying screen. Sharper images were obtained by overnight exposure without the intensifying screen. The percentage double stranded (cross-linked) DNA was obtained in each lane using a BioRad Imaging Densitometer.

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