Targeting of Cytotoxic Agents by Polyamines: Synthesis of a Chlorambucil–Spermidine Conjugate

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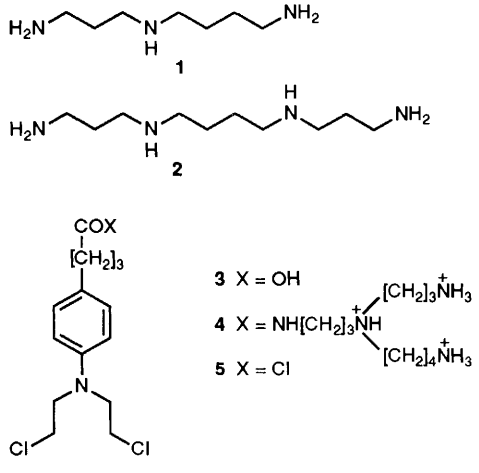
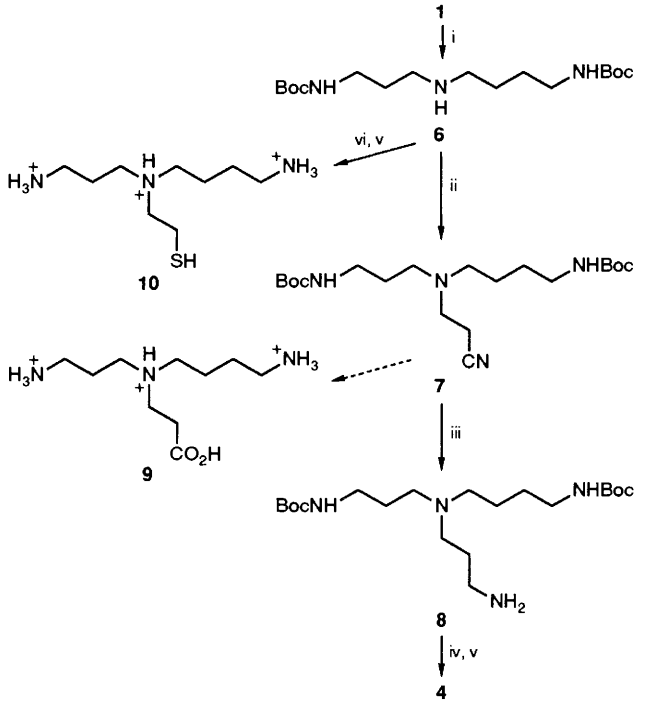
An efficient synthesis of the chlorambucil–spermidine conjugate **4** has been achieved *via* N^1,N^8-bis-Boc-spermidine and **4** has been shown to crosslink DNA 10^4 times more efficiently than chlorambucil itself.

The site of action of many cytotoxic agents is intracellular and as such these agents must first cross the cell membrane and having done so they must recognise and interact selectively with their cellular targets. The development of novel strategies for drug delivery and targeting therefore presents a major challenge. Polyamines **1** and **2** represent attractive candidates for the targeting of drugs whose site of action is nuclear DNA[1,2] because of two unique features: (*i*) the nature of their interaction with DNA, and (*ii*) the existence of an active uptake system for polyamines in a variety of cell types.

Polyammonium salts bind to DNA through an electrostatic interaction in an essentially non-sequence-specific manner, with relatively high affinities (typically in the range 10^3 to 10^6 dm^3 mol^{-1} for ligands bearing 3+ to 4+ charges[3,4]). Importantly, it has recently been shown that whilst constrained to remain close to DNA the polyammonium cations retain a high degree of freedom of motion within the polycation–DNA complex.[5,6] Thus, conjugation of a drug to a polyammonium cation will confer a high DNA affinity but the mobility will allow drugs with high specificity to locate appropriate sites on DNA. The strength of binding of these polyammonium cations will of course be dependent on the ionic strength. Polyamine active transport systems have been characterised in a variety of cell types[7,8] and may be exploited both in drug delivery and tissue targeting.[8] The extent of polyamine uptake





Scheme 1 *Reagents and conditions*: i, Boc-ON [Bu^{t}OC(:O)ON=CPhCN; 2 equiv]; ii, CH_2=CHCN; iii, H_2 Raney nickel; iv, compound **5**; v, H^+, H_2O; vi, thiirane

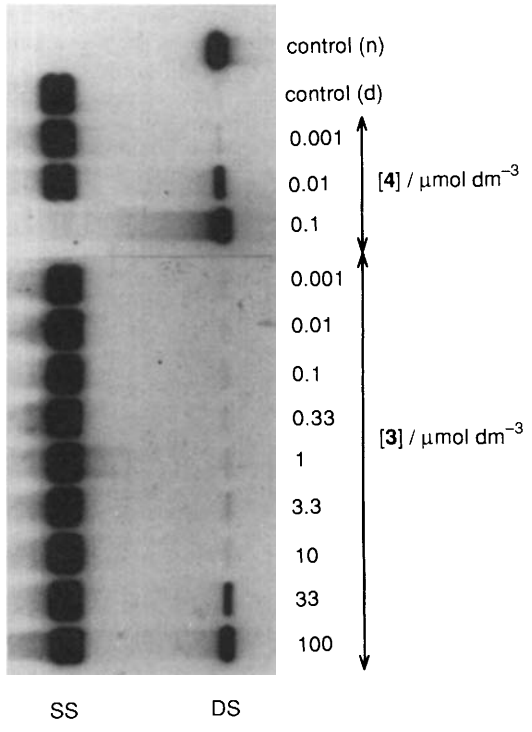


Fig. 1 Neutral agarose gel electrophoresis of linear double-stranded DNA derived from the plasmid pBR322 after exposure to chlorambucil **3** or the spermidine–chlorambucil conjugate **4** followed by complete denaturation (d) to single-stranded DNA (SS). The double-stranded DNA (DS) is observed in the control, which was not exposed to the denaturation conditions, and in the tracks where the presence of interstrand crosslinks promotes renaturation to double-stranded DNA.

from extracellular pools varies between tissues and can be further manipulated by inhibition of ornithine decarboxylase[9] thus offering opportunities for selectivity. To test these ideas we have initially selected chlorambucil **3**, a well known aromatic nitrogen mustard widely used in the treatment of chronic lymphocytic leukaemia, lymphomas and ovarian carcinoma. We report here the syntheses of a number of possible polyammonium carriers together with the synthesis and preliminary biological data for the spermidine–chlorambucil conjugate **4**.

Compound **4** has structural features designed to optimise both DNA affinity and cellular uptake. These important features are: (*i*) a minimum of three positive charges on the ligand to ensure DNA affinity, (*ii*) an appropriate distance between the two primary amines necessary for efficient transport,[2,8] (*iii*) the attachment of the drug *via* a stable amide linkage to a flexible linker attached to a site within the polyamine chain.

Many approaches to the selective and differential protection of polyamines have been reported.[10–14] However, for our purposes the choice of protecting group was limited by the sensitive nature of the nitrogen mustard functionality of chlorambucil. The reactivity of the β-chloroethylamine groups towards nucleophiles clearly required that the spermidine moiety be maintained in its fully protonated state, and hence an acid-labile protecting group was chosen. The synthesis of **4** is shown in Scheme 1. The key differentially protected polyamine, N^1,N^8-bis-Boc-spermidine **6**, can be obtained in one step from spermidine using 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON) [72% yield; m.p. 85–86 °C (lit;[15] m.p. 86.5–87.5 °C)]. In passing it is worthy of note that two syntheses of N^1,N^8-bis-Boc-spermidine **6** have been previously reported.[14,15] The report from Ragnarrson and coworkers observes that mixtures were obtained on reaction of spermidine directly with Boc anhydride and dismisses as unsatisfactory the direct selective protection of spermidine. The synthesis reported by Bergeron can be achieved in good overall yield but involves six steps. To demonstrate the generality of the selectivity of Boc-ON for primary over secondary amines we have also prepared N^1,N^{12}-bis-Boc-spermine (60% yield, m.p. 92.5–95.5 °C) directly from spermine.†

Reaction of **6** with acrylonitrile led to smooth introduction of the cyanoethyl group which could be hydrogenated to give **8**. Instead of reducing the nitrile it is also possible in principle to hydrolyse this to give the corresponding carboxylate **9**. Alternatively reaction of **6** with thiirane led to the thiol **10** where selective alkylation of the thiol functionality has also been used to prepare drug polyamine conjugates. Reaction of the primary amino group of **8** with the acid chloride of chlorambucil **5** (generated *in situ* from **3** and $SOCl_2$) gave the bis-Boc-derivative of **4** in good yield which was deprotected to give the spermidine–chlorambucil conjugate **4**.†

The efficacy of the drug chlorambucil is related to its ability to act as a DNA crosslinking agent. Previous *in vitro* studies of the sequence specificity of simple nitrogen mustards have demonstrated a preference for the initial monoalkylation event at 5′-(G)$_n$-3′ sequences where $n \geq 3$,[16,17] whereas interstrand crosslinking occur preferentially at 5′-GNC-3′

† All new compounds were fully characterised by ^{1}H and ^{13}C NMR spectroscopy and high-resolution mass spectrometry. *N*-{3-[*N*-(3-aminopropyl)-*N*-(4-aminobutyl)]aminopropyl}-4-[*p*-bis(2-chloroethyl)amino]phenylbutanamide **4**: δ_H (300 MHz, D_2O); 7.51, 7.49, 7.40, 7.37 (4H, aromatic AB system); 4.01 (t, 4H, *J* 6.0 Hz, $ArNCH_2$); 3.51 (t, 4H, *J* 6.0 Hz, CH_2Cl); 3.26–3.12 (m, 8H, H-1, H-3, H-1′, H-1″); 3.09–3.00 (m, 4H, overlapping t, H-3′, H-4″), 2.61 (t, 2H, *J* 7.4 Hz, $ArCH_2$); 2.20 (t, 2H, *J* 7.4 Hz, $COCH_2$); 2.12–2.02 (m, 2H, H-2′); 1.93–1.63 (m, 8H, H-2, H-2″, H-3″, $ArCH_2CH_2$). δ_C (75 MHz, D_2O): 179.1 (C=O); 147.9 (C_{Ar}-N); 134.8 (C_{Ar}-C); 133.4, 124.8 (C_{Ar}–H); 61.7 ($ArNCH_2$); 54.9 (C-1′); 53.4 (C-3); 52.5 (C-1″); 41.46 (C-3′); 39.9 (CH_2Cl); 39.2, 38.8 (C-4″, C-1); 37.6, 36.6 ($COCH_2$, $ArCH_2$); 29.2 (C-2′); 26.5, 25.9, 24.3, 23.2 (C-2, C-2″, C-3″, $ArCH_2CH_2$). FABS MS (nitrobenzyl alcohol matrix): *m/z* 489, 491, 493 (ratio 100 : 67 : 18, as expected for $C_{24}H_{45}Cl_2N_5O$).

sequences.[18] Notwithstanding our reasons for attachment of polyamines, in principle, the presence of the polyamine could adversely affect the reactivity of the chlorambucil moiety by virtue of the binding preference of the polyammonium 'arm', for example directing it to the minor groove. We have investigated the relative reactivity of the conjugate using the crosslinking assay that has been previously described[19] (0.025 mol dm^{-3} triethanolamine HCl buffer, 1 mmol dm^{-3} ethylenediaminetetraacetic acid, pH 7.2, 37 °C). Fig. 1 shows the progress of the crosslinking reaction of chlorambucil and spermidine-conjugated chlorambucil **4** with linear double-stranded DNA derived from the plasmid pBR322 (*ca.* 4000 base pairs, linearised with *Hind III* and ^{31}P-end-labelled) following complete denaturation (d) to single stranded form (SS). The presence of an interstrand crosslink results in renaturation to double-stranded DNA (DS) on neutral agarose gel electrophoresis. It is clear that crosslinking induced by the conjugate **4** occurs in the 10^{-9}–10^{-8} mol dm^{-3} range whilst chlorambucil shows comparable reactivity only at 10^{-5}–10^{-4} mol dm^{-3} [control reactions in which an equimolar mixture of chlorambucil and spermidine was added to DNA showed results similar to chlorambucil alone (data not shown)]. Gratifyingly the conjugate appears to be four orders of magnitude more efficient at crosslinking than the parent chlorambucil. Further details of the DNA reactivity and sequence selectivity together with results of *in vivo* studies will be published elsewhere.

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