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On the origin of the DNA sequence selectivity of the azinomycins

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Received 11th February 2005, Accepted 21st February 2005
First published as an Advance Article on the web 1st March 2005

Simplified synthetic azinomycins preferentially induce *in vitro* DNA interstrand cross-links at the same 5′-d(GCC)-3′ site as the natural products revealing that non-covalent interactions are relatively unimportant in defining sequence specificity.

The antitumour antibiotics azinomycin A (**1**) and B (**2**) are structurally unique natural products containing the strained 1-azabicyclo[3.1.0]hexane ring system (Fig. 1).[1] These compounds possess potent *in vitro* cytotoxic activity, significant *in vivo* anti-tumour activity and appear to act by disruption of cellular DNA replication by interstrand cross-link (ISC) formation.[2] Since the initial studies of Lown, which demonstrated that azinomycin B (*née* carzinophilin) induces ISCs without activation,[3] a number of studies have attempted to address the details of its molecular mechanism of action.[4–8] This work was hampered by the limited availability and poor chemical stability of these natural products, but in spite of these obstacles, considerable information has been accrued. Azinomycin B interacts with duplex DNA in the major groove.[4] Covalent ISCs arise from nucleophilic opening of the aziridine at C-10 and the epoxide at C-21 by the N-7 position of purine residues (A or G) located on the two DNA strands.[4,5] The highest levels of covalent ISCs have been observed with the 5′-d(*G*CC)-3′/3′-d(CG*G*)-5′ base sequence (italicised bases indicate sites of reaction) and kinetic experiments suggest that initial alkylation occurs on the more nucleophilic 3′-d(CG*G*)-5′ strand.[7] From analysis of this and the other sequence data, Coleman has put forward the idea that the sequence specificity of these natural products is related more to the nucleophilicity of the DNA sequence rather than to any specific recognition elements between the drug and DNA duplex.[7] If correct, this is an intriguing observation in view of the high density of functionalities (*e.g.* N-5 amide side chain, C-12 hydroxyl group, C-13 acetate) contained within the azinomycin skeleton that are capable of interacting with duplex DNA. Herein, we provide new evidence, obtained using synthetic analogues, that substantiates the Coleman hypothesis.

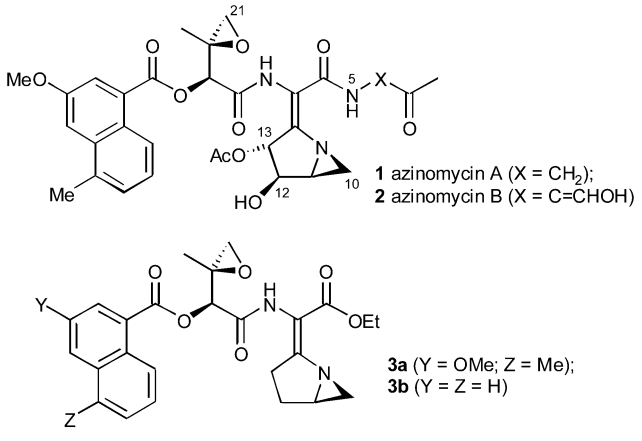

Fig. 1 The structures of azinomycin A and B, and the synthetic epoxyaziridines.

In earlier work, we have used simplified synthetic analogues, such as epoxyaziridine **3**, to show the involvement of the epoxide and the aziridine in the ISC process,[9] and to establish that ISC efficiency is dependent on the nature of the naphthalene substitution pattern.[10] Being bereft of many of the functional groups not directly involved in the ISC process, we reasoned that epoxyaziridine **3** would be an ideal substrate with which to explore the influence of non-covalent interactions on DNA sequence specificity. If such interactions are unimportant, as proposed by Coleman,[7] **3a** and **3b** would be expected to display similar specificity to azinomycin B itself.

Fresh samples of epoxyaziridines **3a** and **3b** were resynthesised according to published methods.[9,10] Interstrand cross-linking activities were quantified using a series of synthetic DNA duplexes (**4**–**9**) containing a reactive triplet sequence located within an A–T tract. Identical DNA 15-mer duplexes were used by Coleman to study the sequence selectivity of azinomycin B.[7] The cross-linking studies were performed with each strand of the duplex, in turn, 5′-^{32}P end labelled. After gel electrophoresis upon a denaturing 20% poly(acrylamide), two new bands of lower mobility were detected (Fig. 2). The first, running close to the starting DNA, being assigned to monoalkylation, the second, of much lower motility, to the ISC product. The extent of monoalkylation and ISC for each drug was quantified by phosphorimage analysis and the results are summarised in Table 1. The highest levels of ISC formation were observed with duplex **4** containing the 5′-d(GCC)-3′ sequence. For each strand of this duplex, the site of reaction was confirmed as G by fragmentation of the oligomer by treatment with hot

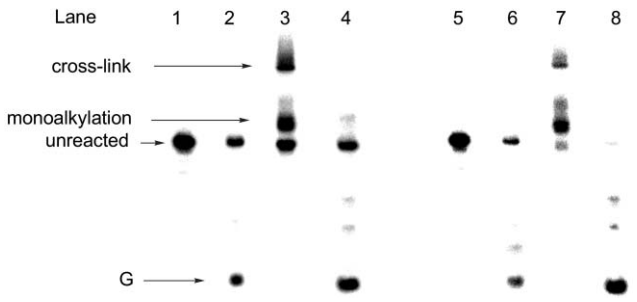

Fig. 2 Representative poly(acrylamide) gel showing extent of alkylation and ISC formation by azinomycin analogue **3a**. Sequence used: 5′-d(TATTATGCCATTATT)-3′/3′-d(ATAATACGGTAATAA)-5. Lanes 1–4 5′-^{32}P end labelled strand contains 5′-d(GCC)-3′ triplet. Lanes 5–8 5′-^{32}P end labelled strand contains 5′-d(CGG)-3′ triplet. Lane 1, DNA only; lane 2, DNA treated with Me_2SO_4 then hot piperidine; lane 3, DNA + **3a**; lane 4, DNA + **3a** then hot piperidine; lane 5, DNA only; lane 6, DNA treated with Me_2SO_4 then hot piperidine; lane 7, DNA + **3a**; lane 8, DNA + **3a** then hot piperidine; reaction conditions: 1000 μM drug, 10 μM duplex DNA, 45 mM sodium cacodylate (pH 7), *v*/*v* 10% DMSO, 8 °C, 72 h.

DOI: 10.1039/b502188j

Table 1 *In vitro* DNA alkylation and ISC efficiencies of azinomycin analogues **3a** and **3b** determined against six oligonucleotide duplexes (**4**–**9**)

Drug	DNA duplex	5′-^{32}P labelled DNA strand[a]	DNA cross-link (%)	Monoalkylation (%)	Unreacted DNA (%)
3a	**4**	5′-d(TATTAT**GCC**ATTATT)-3′	30	24	46
3a		3′-d(ATAATA**CGG**TAATAA)-5′	30	50	20
3a	**5**	5′-d(TATTAT**GTC**ATTATT)-3′	15	40	45
3a		3′-d(ATAATA**CAG**TAATAA)-5′	12	47	41
3a	**6**	5′-d(TATTAT**GCT**ATTATT)-3′	7	35	58
3a		3′-d(ATAATA**CGA**TAATAA)-5′	6	44	50
3a	**7**	5′-d(TATTAT**GTT**ATTATT)-3′	2	62	36
3a		3′-d(ATAATA**CAA**TAATAA)-5′	1	39	60
3a	**8**	5′-d(TATTAT**ATC**ATTATT)-3′	2	24	74
3a		3′-d(ATAATA**TAG**TAATAA)-5′	2	63	35
3a	**9**	5′-d(TATTAT**ACC**ATTATT)-3′	5	27	68
3a		3′-d(ATAATA**TGG**TAATAA)-5′	4	86	10
3b	**4**	5′-d(TATTAT**GCC**ATTATT)-3′	7	26	67
3b		3′-d(ATAATA**CGG**TAATAA)-5′	8	41	51

[a] For all six duplexes (**4**–**9**), each strand was 5′ end labelled in turn. ^{32}P labelling was performed with T4 polynucleotide kinase and [^{32}P]ATP prior to annealing with the unlabelled complementary strand. Reaction conditions: 1000 μM drug, 10 μM duplex DNA, 45 μM sodium cacodylate (pH 7), *v*/*v* 10% DMSO, 8 °C, 20 h. Experiments were performed in triplicate (mean values reported).

piperidine (Fig. 2, Lanes 4 and 8) and comparison with G specific cleavage induced by dimethyl sulfate[11] (Fig. 2, Lanes 2 and 6).† For the 3′-d(CGG)-5′ strand, alkylation occurs selectively on the 5′-G. Selective reaction at this site has been observed for azinomycin B.[7] Appreciable levels of ISC formation were also witnessed with duplex **5** containing the 5′-d(GTC)-3′ sequence. Lower (but measurable) levels of ISC were seen with 5′-d(GCT)-3′ (**6**) and 5′-d(ACC)-3′ (**9**). For epoxyaziridine **3b**, the levels of DNA ISC activity were reduced (*ca.* 4–5 fold), but the preference for the 5′-d(GCC)-3′ sequence retained (Table 1).‡

For azinomycin B, three sequences, namely 5′-d(GCC)-3′, 5′-d(GTC)-3′ and 5′-d(GCT-3′, are known to produce appreciable levels of ISC formation.[4,5,7] Of these, the 5′-d(GCC)-3′ sequence produces the most cross-links.[7] Thus, there is good correlation between the DNA sequence specificity of epoxyaziridine **3**, devoid of many of the functional groups contained within natural products, and azinomycin B itself. These findings support the hypothesis originally put forward by Coleman,[7] that the azinomycins are rather reactive cross-linking agents whose preferred position of attack on the DNA duplex is determined primarily by the relative nucleophilicity of the different bases. Of course, the distance between the epoxide and aziridine groups must play an additional role in defining what cross-links are permitted.

If the sequence specificity of the azinomycins is largely unaffected by the functional groups that 'decorate' the azinomycin skeleton, then the question arises: what is their role? It has been established that the methoxy and methyl groups of the naphthalene ring increase the efficiency of ISC formation in a linearised plasmid.[10] A similar trend was seen herein, where the level of DNA ISC formation produced by **3b** was 4–5 fold lower than for **3a**. Other functional groups within the skeleton of the natural products may also serve to enhance the efficiency of ISC formation. For example, it is known that a free hydroxyl group at C-12 renders azinomycin B more reactive.[12] Furthermore, a hydrogen bond between the aziridine nitrogen and the N-5 amide hydrogen is observed in the ^{1}H NMR spectrum (δ = 10.09) of azinomycin B.[1b] This pre-protonation might be expected to help facilitate aziridine rupture by the DNA bases, and hence increase the yield of alkylation products. Efforts to provide experimental evidence in support of these proposals are ongoing in our laboratories.

Acknowledgements

We are indebted to BBSRC (B15997) for financial support of this work.

Notes and references

† The involvement of guanine N-7 in the ISC process was confirmed by the following experiment. Two self-complementary ^{32}P 5′-end labelled 14-mer DNA duplexes containing the 5′-d(GCT)-3′ triplet (*c.f.* **6**) were synthesised from 5′-d(TATATAGCTATATA)-3′ (**10**) and 5′-d(TATATAG*CTATATA)-3′ (**11**) (G* = 7-deazaguanine). When duplex **10** was incubated with **3a** for 70 h, both monoalkylation (58%) and ISC formation (25%) were observed. In contrast, using duplex **11**, monoalkylation (53%) was detected, but no ISCs.

‡ Epoxyaziridine **3b** produced ≤3% ISCs with DNA duplexes **5**–**9**.

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