Sequence Specificity of Alkylation for a Series of Nitrogen Mustard-Containing Analogues of Distamycin of Increasing Binding Site Size: Evidence for Increased Cytotoxicity with Enhanced Sequence Specificity†

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ABSTRACT: The covalent sequence specificity of a series of nitrogen mustard-containing analogues of distamycin was determined using modified sequencing techniques. The analogues tether benzoic acid mustard (BAM) and possess either one, two, or three pyrrole-amide units. Previous characterization of the biological profile of the series revealed an increase in cytotoxicity for each corresponding increase in the number of pyrrole units, while showing poor cross-link formation in isolated and cellular DNA. Examination of the sequence specificity revealed that BAM produced guanine-N7 lesions in similar manner to other conventional nitrogen mustards. The monopyrrole BAM conjugate also produced guanine-N7 alkylation in a similar pattern to BAM. However, alkylation of adenines was also seen that was found to be minor groove adenine-N3 lesions. The dipyrrole and tripyrrole conjugates did not produce detectable guanine-N7 alkylation but only alkylated in AT tracts. In addition, the tripyrrole conjugate preferentially alkylated only a subset of those sites alkylated by the monopyrrole and dipyrrole conjugates. Two sites, 5′-TTTTGG and 5′-TTTTGA, confirmed as guanine-N3 and adenine-N3 lesions, respectively, were strongly alkylated by the tripyrrole conjugate in preference to other similar sites including three occurrences of 5′-TTTTAA. Footprinting studies comparing distamycin and the tripyrrole conjugate showed identical non-covalent recognition of AT-rich sites. Hence, the drug that possessed the most enhanced sequence specificity for alkylation was also the most cytotoxic of this series.

The minor groove of DNA has become a site of great interest for targeting by sequence-specific binding agents, in large part due to the precedents provided by the two naturally occurring ligands netropsin and distamycin. These peptide antibiotics bind non-covalently in the minor groove of AT-rich sequences, and their interactions with DNA have been extensively studied [for reviews, see Zimmer and Wahnert (1986), Gilbert and Feigon (1991), and Krugh (1994)]. The footprinting studies on distamycin demonstrated binding to A tracts and alternating AT tracts of four or more base pairs while tolerating a single GC base pair in the binding site (Van Dyke et al., 1982; Fox & Waring, 1984; Harshman & Dervan, 1985).

Alkylation in the minor groove is achieved by natural products such as mitomycin C (Tomasz et al., 1986), anthramycin (Petrusek et al., 1981), and CC-1065 (Hurley et al., 1984). The antibiotic CC-1065 consists of three repeating pyrroloindole units, the first of which possesses a reactive cyclopropane functionality, and it has been shown to alkylate the N3 position on adenine within the sequences 5′-PuNTTA or 5′-AAAAA (Hurley et al., 1984; Reynolds et al., 1985).

There are several examples of agents that utilize the pyrrole-amide framework of distamycin as a DNA binding vector that tethers an alkylating species. DNA binding agents of this type are known as lexitropsins or information reading oligopeptides (Lown, 1988). An electrophilic analogue, *N*-bromoacetyl distamycin, was shown to bind non-covalently to several AT sequences on a 157 base pair fragment identical to distamycin (Baker & Dervan, 1989). Alkylation, however, occurred slowly in only one of the sites, at the adenine opposite the 3′-T in the sequence 5′-AGTTTA (Baker & Dervan, 1989). Dipyrrole lexitropsins were used to tether a chloroethyl nitrosourea group (Church et al., 1990), but the majority of the alkylation for the conjugates corresponded to those seen for chloroethyl nitrosourea itself. More recently, uncharged dipyrrole lexitropsins tethering sulfonate ester groups were reported (Zhang et al., 1993), which showed methylation at adenine sites in AT tracts. A pyrrole−oligopeptide conjugate was shown to efficiently cross-link an oligonucleotide of the core sequence CGAATTGC at the guanine-2-NH_2 positions (Sigurdsson et al., 1993).

Tallimustine (FCE 24517, Figure 1) is a benzoic acid mustard derivative of distamycin that is undergoing clinical evaluation (Arcamone et al., 1989). Studies on its mechanism of action revealed alkylation in the minor groove at selected adenines while showing no major groove guanine-N7 alkylation, which is characteristic of conventional nitrogen mustards (Broggini et al., 1991). A footprinting study demonstrated identical binding sites to distamycin, which indicates that tallimustine has retained the preference

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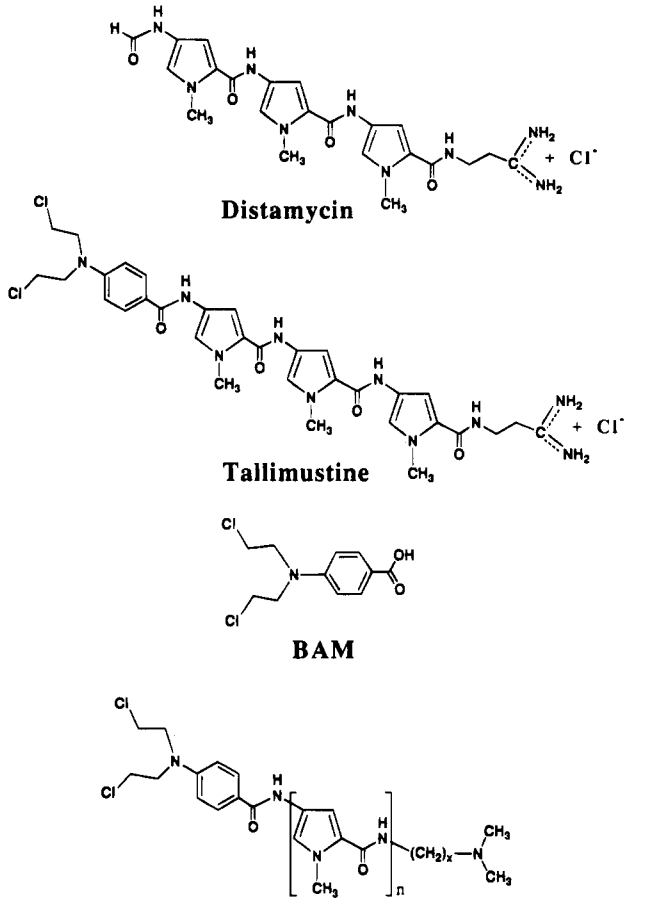

FIGURE 1: Structures of distamycin, tallimustine, benzoic acid mustard (BAM), and the BAM pyrrole conjugates.

for AT-rich sequences (Broggini et al., 1989). Very recently, it was found that tallimustine only alkylated the 3′-adenine within the sequence 5′-TTTTPuA (Broggini et al., 1995) and a single base pair change in the consensus sequence prevented alkylation.

A series of nitrogen mustard-tethered lexitropsins, which incorporate a dimethylamino group on the C-terminus, has been evaluated for their cross-linking ability and cytotoxicity (Wyatt et al., 1994). The lexitropsins consist of either one, two, or three pyrrole-amide units and tether a benzoic acid mustard (BAM) (Figure 1). It was found that the analogues were more cytotoxic than BAM itself and the cytotoxicity and non-covalent DNA binding affinity increased as the number of pyrrole units increased. In contrast to conventional nitrogen mustards, however, the increased cytotoxicity did not correlate with increased cross-linking ability.

Determination of the covalent sequence specificity of alkylation for each of the analogues was undertaken to further elucidate the mechanism of action of this series of conjugates. Analysis of the sequence specificity of alkylation in comparison to BAM itself allows for determination of the influence of the lexitropsin portion on the reactivity and specificity of the nitrogen mustard functionality.

MATERIALS AND METHODS

The synthesis of the pyrrole BAM conjugates has been reported (Wyatt et al., 1994). BAM was a gift from Dr. Philip Burke, CRC Department of Medical Oncology, Charing Cross Hospital. The regions of pBR322 DNA are referred to by sequence number as listed (Maniatis et al., 1982). The GC-rich *Bam*H1/*Sal*1 region (375−640) is 265 base pairs long with a GC content of 65% and 18 tracts of four or more GC base pairs. The 213 base pair region defined between base pair 3090 and 3303 has a GC content of 36% and 11 tracts of four or more AT base pairs.

Drug/DNA Reactions. All drug/DNA reactions were performed in 25 mM triethanolamine and 1 mM EDTA, pH 7.2, at 37 °C for 5 h and terminated by the addition of an equal volume of stop solution (0.6 M sodium acetate, 20 mM EDTA, 100 μg/mL tRNA). The samples were precipitated with 3 volumes of ethanol and washed with 70% ethanol. Each drug was examined over a wide dose range initially to determine a drug dose that would ensure single hit kinetics, i.e., that a proportion of the DNA remained undamaged.

Taq Polymerase Stop Assay. The procedure employed was an application of a previously described method (Ponti et al., 1991). Prior to drug/DNA incubation, plasmid pBR322 DNA was linearized with an appropriate restriction enzyme to provide a stop for the *Taq* downstream from the primer. The oligodeoxynucleotide primers were 5′-end labeled prior to amplification using T4 polynucleotide kinase and [γ-^{32}P]-ATP (5000 Ci/mmol, Amersham). The labeled primers were purified by elution through Bio-Rad BioSpin columns. The synthetic primer used for amplification of the *Bam*H1/*Sal*1 region has been reported (Ponti et al., 1991). The synthetic primer 5′-GCAGCAGATTACGCGCAGAA-3′, identified as SCA, binds to the complementary strand at position 3090−3109 and was used to examine alkylation of the bottom strand. The primer 5′-GCATTGGTAACTGTCAGACC-3′, identified as SRM, binds to the sequence 3303−3284 and was used to examine the top strand. Linear amplification of DNA was carried out in a total volume of 100 μL containing 0.5 μg of template DNA, 5 pmol of labeled primer, 125 μM each dNTP, 1 U of *Taq* polymerase, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl, pH 9.0, 0.01% Tween, 2.5 mM $MgCl_2$, and 0.05% gelatin. After an initial denaturation at 94 °C for 4 min, the cycling conditions were as follows: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, for a total of 30 cycles. After being amplified, the samples were ethanol precipitated and washed with 70% ethanol.

Sequence Selectivity of Guanine-N7 Alkylation. 5′-Singly end-labeled fragments were obtained by amplification of the template of the AT-rich region with the primers listed above. Amplification with a labeled SCA primer produced the fragment with the top strand labeled, and amplification with a labeled SRM primer produced the fragment with the bottom strand labeled. After amplification, the PCR mixture was precipitated with ethanol, and the labeled fragment was purified by agarose gel electrophoresis. Following the drug incubation, the DNA pellet was resuspended in freshly diluted 10% piperidine and incubated at 90 °C for 15 min to quantitatively convert sites of guanine-N7 alkylation into strand breaks (Mattes et al., 1986). Samples were lyophilized with two additional aliquots of water to remove all piperidine and washed with 70% ethanol.

Sequence Selectivity of Purine-N3 Alkylation. The singly end-labeled fragment was prepared as described above. Following drug incubation, the DNA pellet was resuspended in sodium citrate buffer (pH 7.2) and heated to 90 °C for 30 min to thermally cleave at sites of adenine- or guanine-N3 lesions, as described (Reynolds et al., 1985). Samples were then chilled, precipitated, and dried.

Acrylamide Gel Electrophoresis. Samples were dissolved in formamide loading dye, heated for 2 min at 90 °C, cooled on ice, and electrophoresed at 2500−3000 V for roughly 3 h on a 80 cm × 20 cm × 0.4 mm, 6% acrylamide denaturing sequencing gel (Sequagel, National Diagnostics). The gels were dried, and X-ray film was exposed to the gels (Hyperfilm, Amersham). Densitometry was carried out on a Bio-Rad GS-670 imaging densitometer.

RESULTS

Initially, the drugs were examined on a 265 base pair GC-rich region of pBR322 DNA (375−640) using the *Taq* polymerase stop assay. BAM, at a concentration of 500 μM (its IC_{50} value), produced a pattern of alkylation consistent with that seen for other conventional nitrogen mustards (Ponti et al., 1991). The predominant sites of alkylation were guanines within runs of guanines and in the sequence 5′-TGG (Figure 2, lane b). The monopyrrole BAM conjugate, at a concentration of 10 μM (5-fold lower than its IC_{50} value), also produced a similar pattern in this region, but the intensities of several of the bands differed (Figure 2, lane c). The dose that produced a similar amount of damage to that produced by BAM on the fragment was 50-fold lower. The dipyrrole and tripyrrole conjugates did not produce any significant covalent lesions in this region under identical conditions (Figure 2, lanes d and e), in spite of their higher non-covalent affinity for DNA, as measured by ethidium displacement (Wyatt et al., 1994).

The 213 base pair AT-rich region of pBR322 DNA defined from 3090 to 3303 was examined to further elucidate the specificity of alkylation of the lexitropsin conjugates. This region was chosen because of its high incidence of AT runs, which are known sites of non-covalent binding for pyrrole-containing lexitropsins. Examination of the bottom (Figure 3a) and top (Figure 3b) strand was carried out utilizing the *Taq* polymerase stop assay. The pyrrole conjugates (lanes c−e) showed a markedly altered alkylation specificity from that of BAM (lane b) in this AT-rich region. Damage to the bottom strand, shown in Figure 3a, revealed alkylations at adenines in AT-rich sequences. These were concentration dependent within a dose range that ensured single hit kinetics (data not shown). For the monopyrrole conjugate, several covalent lesions were observed, including strong alkylations at adenines in the sequences 5′-TTTTGA (3205) and 5′-TTTAAA (3250). Sites alkylated to a lesser extent included 5′-ATTTAA (3232) and 5′-ATTGA (3256). The dipyrrole conjugate alkylated fewer sites overall on the strand, while retaining strong alkylation at the adenine in the sequences 5′-TTTTGA (3205) and 5′-TTTAAA (3250), with weaker alkylation observed at 5′-ATTGA (3256) and two occurrences of 5′-TTTAA (3232 and 3237). The tripyrrole conjugate only strongly alkylated at one sequence on the bottom strand, 5′-TTTTGA (3205). It is clear, therefore, that for each increase in the number of pyrroles there is alkylation at fewer sites. For example, while the adenine in the sequence 5′-TTTTGA (3205) is strongly alkylated by each of the conjugates, the adenine in the sequence 5′-TTTAAA (3250) shows a relative decrease in alkylation with the increase in pyrrole units.

Examination of the top strand, shown in Figure 3b, revealed results consistent with those seen for the bottom strand. The monopyrrole conjugate showed alkylation at several sites, including strong alkylation at the sequences

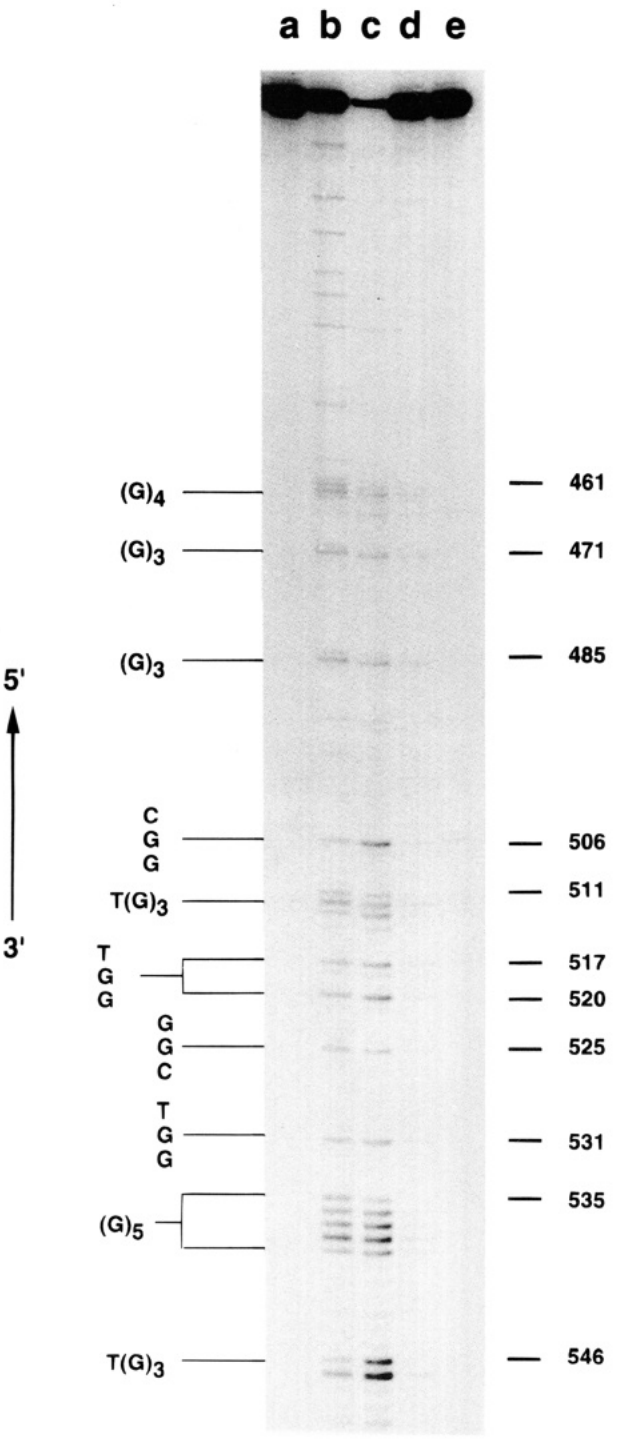

FIGURE 2: *Taq* polymerase gel examining damage on the top strand of the GC-rich region (defined in Materials and Methods). Lane a, control; lane b, BAM, 500 μM; lane c, BAM-Py_1, 10 μM; lane d, BAM-Py_2, 10 μM; lane e, BAM-Py_3, 10 μM.

5′-TTTTAA (3254 and 3235), 5′-TTAAA (3241), and 5′-TTTTGG (3193), while some of the bases alkylated to a weaker extent corresponded to the guanines alkylated by BAM. The dipyrrole conjugate showed alkylation at fewer sites and strong alkylation at the sequences 5′-TTTTGG (3193), 5′-TTTTAA (3254), and 5′-ATATGA (not shown). Weaker alkylation was observed at the sites 5′-TTAAA (3241), 5′-TTTTAA (3235), and 5′-AATGA (3246). The tripyrrole conjugate only strongly alkylated at the sequence 5′-TTTTGG (3193). In agreement with the results from the bottom strand, for each increase in the number of pyrroles there is strong alkylation at fewer sites. Two occurrences of the sequence 5′-TTTTAA (3254 and 3235), in particular,

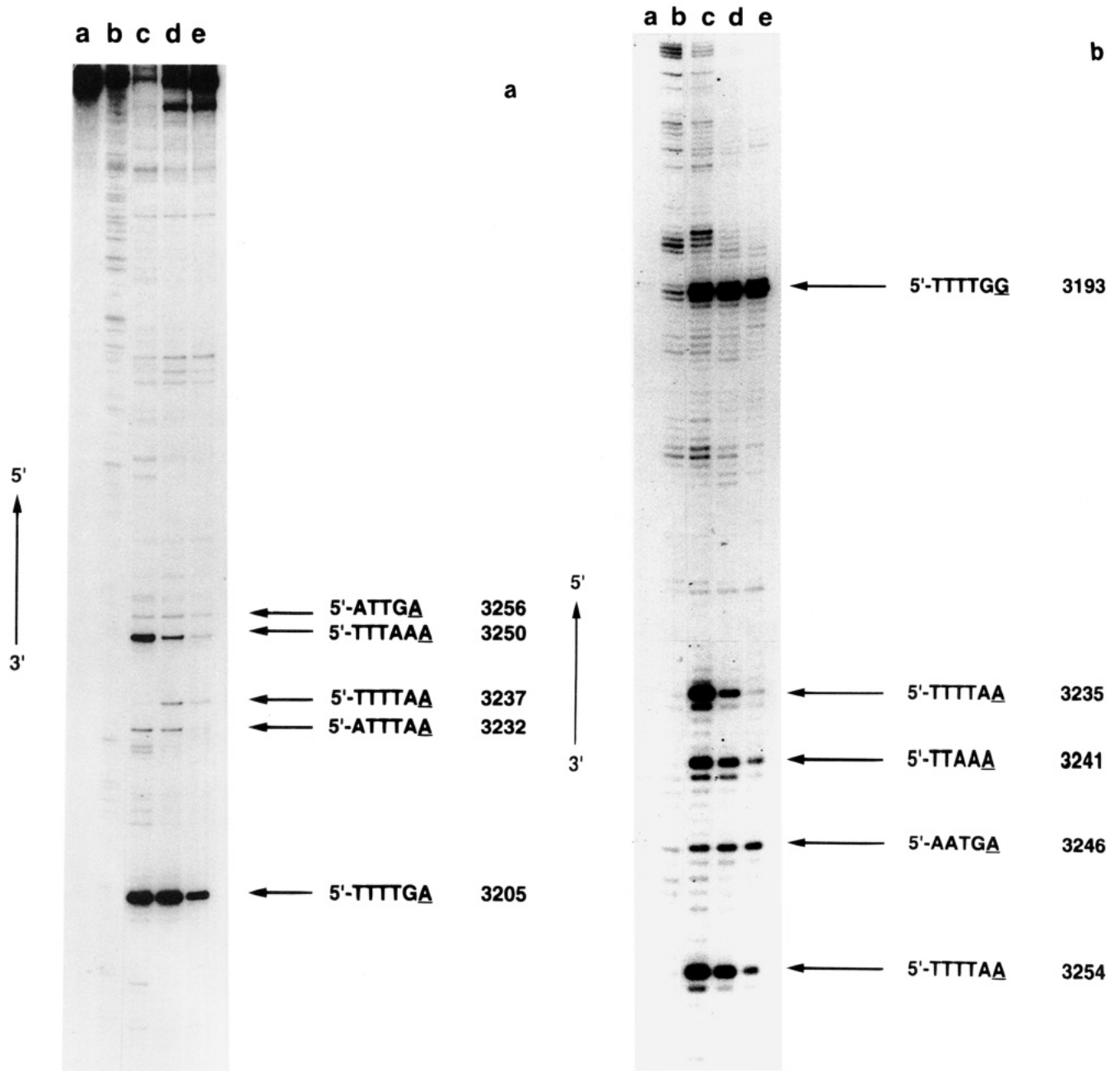

FIGURE 3: (a) *Taq* polymerase gel examining damage on the bottom strand of the AT-rich region (defined in Materials and Methods). Lane a, control; lane b, BAM, 500 μM; lane c, BAM-Py_1, 5 μM; lane d, BAM-Py_2, 5 μM; lane e, BAM-Py_3, 5 μM. (b) Resolvable region of *Taq* polymerase gel examining damage on the top strand of the AT-rich region. Lane a, control; lane b, BAM, 500 μM; lane c, BAM-Py_1, 5 μM; lane d, BAM-Py_2, 5 μM; lane e, BAM-Py_3, 5 μM.

are strongly alkylated by the monopyrrole conjugate, alkylated to a lesser extent by the dipyrrole conjugate, but only alkylated weakly, if at all, by the tripyrrole conjugate. Interestingly, an occurrence of the 5′-TTTTAA sequence on the other strand (3237, Figure 3a) is only weakly alkylated by the dipyrrole conjugate.

The adenine lesions seen on the bottom strand were qualitatively confirmed as minor groove adenine-N3 lesions for the conjugates (Figure 4, lanes d−f), using the thermal cleavage assay. These included the alkylation seen in Figure 3a by all three conjugates at 5′-TTTTGA (3205) and the site in the sequence 5′-TTTAAA (3250) by the monopyrrole conjugate. For the top strand, the adenine lesions and the guanine lesion (3193) were confirmed as adenine-N3 and guanine-N3 lesions, respectively, using the thermal cleavage assay (Figure 5a, lanes c−e). These included the strongest site of alkylation seen in Figure 3b for the conjugates, 5′-TTTTGG (3193), and 5′-TTTTAA (3235 and 3254) for the monopyrrole conjugate.

BAM reacted weakly with guanines on both strands of the AT-rich region. These lesions were confirmed as guanine-N7 lesions by piperidine treatment of a 5′-singly end-labeled fragment of the same region (Figure 5b, top strand, lane c). Furthermore, including equimolar amounts of distamycin in the incubation did not alter the alkylation specificity seen for BAM in either the AT- or GC-rich region (data not shown). As noted on the gel in Figure 5b, BAM alkylated both guanines equally strongly at the sequence 5′-TTGG (3192 and 3193, Figure 5b, lane c). The monopyrrole conjugate showed alkylation of both guanine-N7 positions and a 3-fold preference for the 3′-guanine (lane d). In Figure 5a, however, the thermal depurination assay clearly demonstrates that the alkylation in the minor groove occurs only at the 3′-guanine.

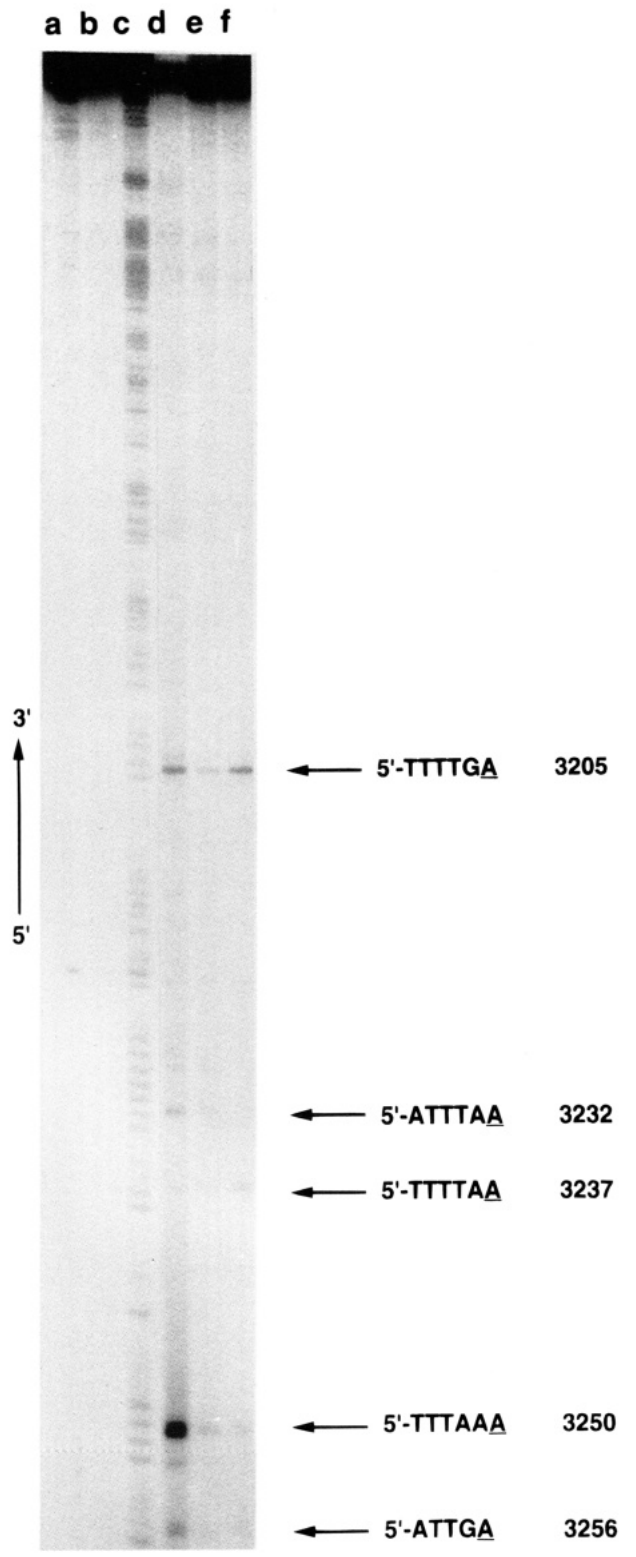

FIGURE 4: Thermal cleavage gel showing purine-N3 lesions on the bottom strand of the AT-rich region. Lane a, control heat treated; lane b, control no heat; lane c, G+A marker lane (formic acid); lane d, BAM-Py_1, 10 μM; lane e, BAM-Py_2, 10 μM; lane f, BAM-Py_3, 10 μM.

Quantitation of the gels in Figure 3a,b by densitometry shows the alkylation pattern for each of the conjugates (Figure 6). Comparison of the alkylation on both strands, shown in Figure 6a, revealed that the preferred sites of alkylation for the monopyrrole conjugate were the sequences 5′-TTGPu, 5′-TTAA, and 5′-TAAA. The preferred sites of alkylation for the dipyrrole conjugate were the sequences 5′-TTTGPu, 5′-TTTAA, 5′-TATGA, and 5′-TTAAA (Figure 6b), while for the tripyrrole conjugate the consensus sequence was 5′-TTTTGPu (Figure 6c). Examination of the strand opposite the two preferred sites of alkylation, 5′-TTTTGPu, did not reveal a corresponding lesion, providing strong evidence that the non-covalent binding and alkylation that occur in the minor groove do not lead to interstrand cross-link formation with these conjugates, even though they possess a nitrogen mustard functionality. Footprinting studies of distamycin and the tripyrrole conjugate in this region revealed identical patterns of non-covalent binding (unpublished results). The tripyrrole conjugate non-covalently recognizes several AT tracts, including sites where there was only weak or no alkylation observed using the above techniques.

DISCUSSION

Conventional nitrogen mustards have been shown to alkylate guanines preferentially in runs of guanines (Mattes et al., 1986). These agents can thus cross-link DNA by alkylating guanines on opposite strands, and cross-link formation is generally assumed to be the mechanism of action of the nitrogen mustards (Hartley, 1993). There have been reports of adenine-N3 alkylation by the aromatic nitrogen mustards melphalan and chlorambucil (Pieper & Erickson, 1990; Wang et al., 1991). Comparison of the results from the *Taq* stop assay and the guanine-N7 assay, however, suggests that guanine-N7 lesions are the significant lesions formed by BAM at the dose used in both regions. It has been shown with a series of conventional nitrogen mustards that the more efficient cross-linker is the more cytotoxic agent (Sunters et al., 1992). The BAM lexitropsin conjugates, however, cross-link DNA poorly (Wyatt et al., 1994). Furthermore, for each increase in the number of pyrrole units there was a corresponding increase in cytotoxicity, with the tripyrrole conjugate being greater than 50-fold more cytotoxic than BAM itself and greater than 5-fold more cytotoxic than the monopyrrole BAM conjugate. It has now been demonstrated that the tripyrrole BAM conjugate is also the most sequence specific of the series. There are several possibilities to consider, with regard to the trend in the cytotoxicity. The first is the beneficial effect of tethering the nitrogen mustard to the DNA binding vector, which targets it to the DNA. Secondly, the vector is targeting the nitrogen mustard away from the major groove and into the minor groove, implying that alkylations in the minor groove are more toxic than major groove alkylation. Lastly, it appears that certain specific sequences in cellular DNA may be more critical as targets, because the most cytotoxic tripyrrole conjugate alkylates DNA at fewer sites compared to the mono- and dipyrrole conjugates, preferentially alkylating at the sequence 5′-TTTTGPu.

In a GC-rich stretch of DNA, the monopyrrole BAM conjugate clearly shows a pattern of alkylation qualititatively similar to BAM itself, albeit at 50-fold lower doses. This difference may be due simply to the cationic C-terminus of the lexitropsin portion and its affinity for DNA. BAM alkylated guanines in the AT-rich region in a consistent manner with conventional nitrogen mustards. In the same AT-rich fragment, however, the monopyrrole conjugate preferentially alkylated sites in the minor groove, as detected by the *Taq* polymerase stop assay. The minor groove lesions were confirmed as adenine-N3 and guanine-N3 lesions using a thermal cleavage assay. The same technique confirmed the presence of a guanine-N3 lesion, originally detected by

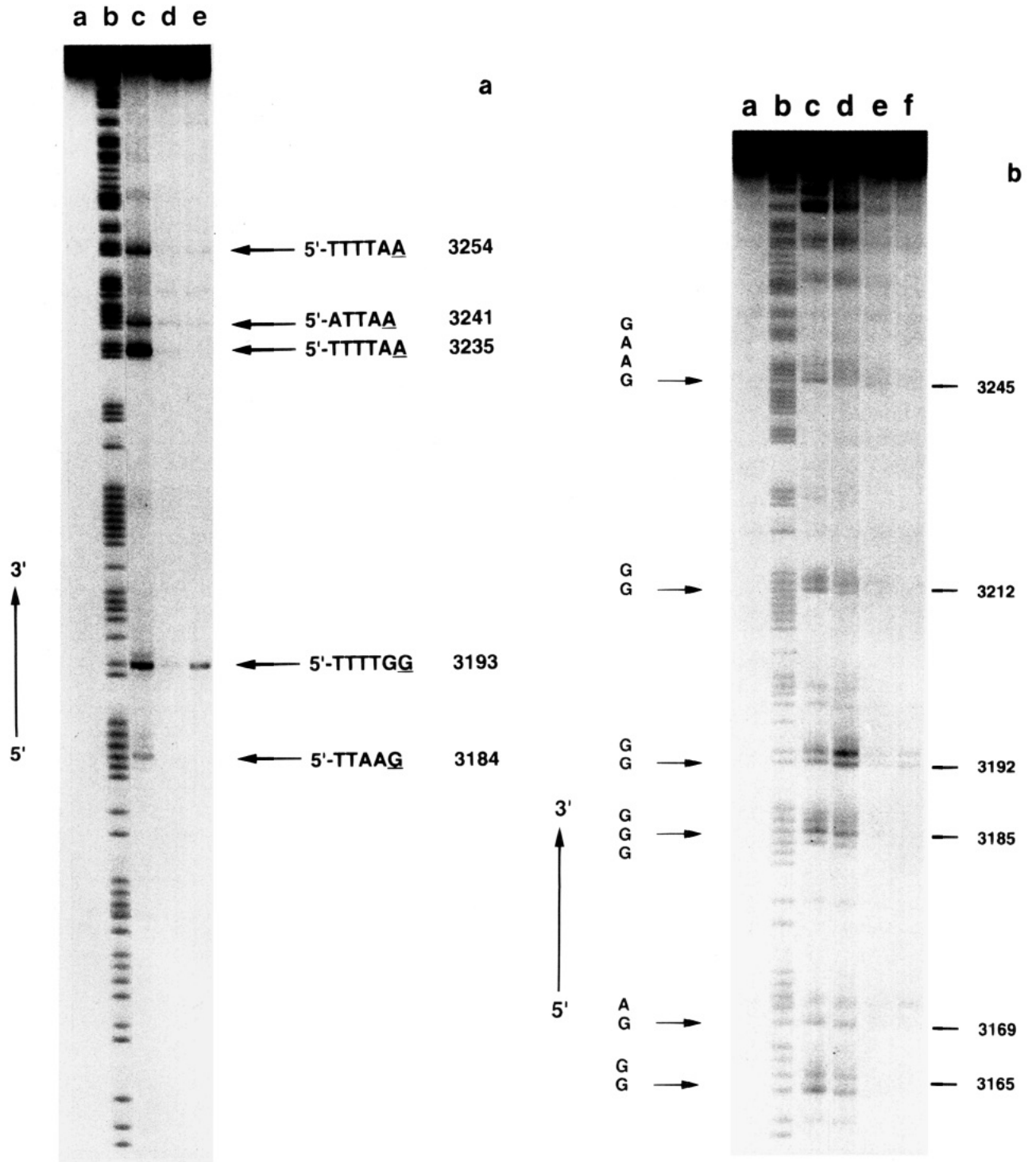

FIGURE 5: (a) Thermal cleavage gel showing purine-N3 lesions on the top strand of the AT-rich region. Lane a, control heat treated; lane b, G+A marker lane (formic acid); lane c, BAM-Py_1, 10 μM; lane d, BAM-Py_2, 10 μM; lane e, BAM-Py_3, 10 μM. (b) Piperidine cleavage gel showing guanine-N7 lesions on the top strand of the AT-rich region. Lane a, control piperidine treated; lane b, G+A marker lane (formic acid); lane c, BAM, 500 μM; lane d, BAM-Py_1, 10 μM; lane e, BAM-Py_2, 10 μM; lane f, BAM-Py_3, 10 μM.

DNA polymerase stop assay, formed by duocarmycin A (Mitchell et al., 1993), and the guanine-N3 lesions by duocarymcin have been reported elsewhere (Asai et al., 1994). Interestingly, the sequence 5′-TTTTAA is not alkylated to the same extent in all three occurrences in this region, implying that the sequence conformation of the neighboring regions must also play a role in the alkylation event.

The dipyrrole and tripyrrole BAM conjugates possess a higher non-covalent affinity for DNA, as measured by an ethidium displacement assay, and show little, if any, retention of the alkylation preference of BAM. Alkylation is strictly limited to labile sites in the minor groove where the lexitropsin preferentially binds. The increase in the reading frame portion of the molecule with a third pyrrole-amide group offers a corresponding increase in the sequence specificity of alkylation. The tripyrrole conjugate binds non-covalently to AT tracts similarly to distamycin, and binding to single-stranded DNA is not seen. The alkylation pattern is similar to the dipyrrole conjugate, but strong alkylation only occurs at two very similar sites, inferring that the third pyrrolamide group places an additional requirement on the alkylation event. This is only met by the consensus sequence 5′-TTTTGPu. This result is in agreement with the work on

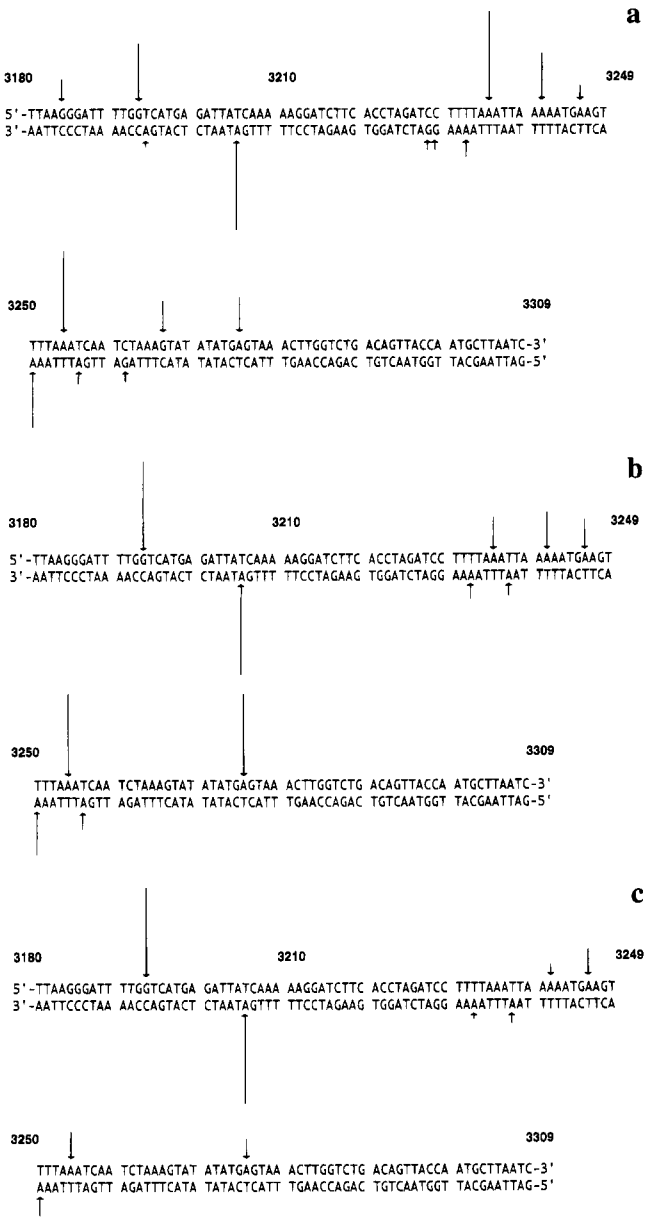

FIGURE 6: Quantitation of the alkylation patterns of the pyrrole conjugates on the top and bottom strands of the AT-rich region taken from the gels in Figure 3a,b. Alkylation on each strand is normalized to the site of strongest alkylation: (a) BAM-Py_1; (b) BAM-Py_2; (c) BAM-Py_3.

N-bromoacetyl distamycin, which showed non-covalent binding at several AT sites identical to distamycin but alkylation at only one of the sites (Baker & Dervan, 1989). The local sequence conformation of the DNA must be crucial in determining whether alkylation is favorable.

Work carried out by Dervan and co-workers has examined many aspects of non-covalent recognition by distamycin analogues. A tripyrrole analogue of distamycin (P3) in which the C-terminal amidinium group has been replaced with a dimethylaminopropyl group was shown to bind identically to distamycin, and the binding site preference on the fragment studied was assigned as 5′-TTTTT > 5′-AATAA > 5′-TTAAT (Wade et al., 1992). More interestingly, one analogue synthesized, pyridine-2-carboxamide netropsin (2-PyN), bound two very different sites, 5′-TTTTT and 5′-TGTCA (Wade et al., 1992). It was found that 2-PyN bound the 5′-TTTTT site in a 1:1 complex, while the wider 5′-TGTCA site could favorably accommodate a 2:1 complex (Wade et al., 1992). Groove width proved to be the key factor in both this example and reports of dimeric binding by distamycin, where the minor groove of T tracts are much more narrow than those of mixed sequence (Pelton & Wemmer, 1989; Fagan & Wemmer, 1992; Mrksich et al., 1992).

The possibility of a dimeric binding motif for the BAM pyrrole conjugates is unlikely in view of the composition of the preferred sequence, 5′-TTTTGPu. Examination of the results from both strands do not indicate that the tripyrrole conjugate is binding in a dimeric motif. The two preferred sites of alkylation, 5′-TTTTGG and 5′-TTTTGA, do not produce a corresponding alkylation event on the opposite strand. Two palindromic sequences on the fragment, 5′-TTTAAA, are bound by the dipyrrole conjugate, and alkylation does occur on both strands. However, these sites are not alkylated to a greater degree than the other sites, discounting the possibility that a cooperative dimeric recognition in non-covalent binding leads to a preferential increase in alkylation efficiency in this case.

The alkylation of the 3′-purine in the sequence 5′-TTTTGPu by the tripyrrole conjugate suggests that the N-terminus of this drug is oriented in the 3′-direction. The covalent modification of the 3′-purine in this consensus sequence suggests that the sequence has unique structural and electrostatic features that facilitate not only non-covalent binding but also the alkylation step. Since the most nucleophilic groups in the minor groove are the adenine-N3, guanine-N3, and guanine-2-NH_2 positions, the alkylation seen by the BAM portion, presumably through an aziridinium intermediate, is not surprising. However, in good agreement with the results found for tallimustine (Broggini et al., 1995), the entire composition of the T_4 tract followed by two purines appears crucial.

The structure and conformation of a number of synthetic oligonucleotides containing 5′-T_4G, 5′-T_4GG, and 5′-T_4GA sequences have been investigated by high field NMR and X-ray crystallography studies. NMR [5′-dCCGTTTTGCC-3′ (Nadeau & Crothers, 1989), 5′-dGGCCCTTTTTGG-3′ (Chen et al., 1992), 5′-dCTTTTGCAAAAG-3′ (Searle & Embrey, 1990), 5′-dGCGTTTTG-3′ (Walker et al., 1994), and 5′-dGCATTTTGAAACC-3′ (Katahira et al., 1990)] and X-ray studies [5′-dCGCTTTTTTGCG-3′ (Nelson et al., 1987)] on oligonucleotides containing the 5′-TTTTG-3′ sequence indicate significant narrowing (3.8−3.9 Å) and propeller twisting (17−25°) in the minor groove of the T-tract from the 5′ to 3′ terminus. In addition, at the T-G junction the minor groove width expands to >4.5 Å, indicating an abrupt disturbance of compression. These results are consistent with junction-induced bending observed for the decamer 5′-CGTTTTGGCC-3′ as indicated by its anomalous gel electrophoretic mobilities (Koo et al., 1986). In view of the unusual features of the 5′-T_4GPu sequence, it is thought that the ability of the DNA to adopt a bent-type structure is an important factor in the specificity of alkylation for the tripyrrole conjugate.

CC-1065 and related analogues specifically alkylate in the minor groove and are quite toxic. The initial studies on CC-1065 found an alkylation pattern that assigned the consensus sequences as 5′-AAAAA and 5′-PuNTTA (Reynolds et al., 1985). A subsequent study to determine the basis of the sequence specificity found the cyclopropyl-containing A pyrroloindole unit to be the determinant of the specificity while the B and C pyrrolindole units facilitate non-covalent interactions preceding covalent bond formation (Hurley et

al., 1988). More recent work by another group found that analogues with one, two, and three additional pyrroloindole units recognized only a subset of those alkylated by the cyclopropylpyrroloindole unit alone, indicating that the increased reading frame portion of the molecule plays a role in the alkylation specificity (Boger et al., 1991).

Confirmation of the covalent sequence specificity of drugs in cells is critical in the rational design of novel DNA binding agents. Those agents designed using models that are predicted from studies with isolated DNA must be shown to bind to cellular DNA with the same specificity, or else the model will have no biological significance. Recently, the sequence specificity of adozelesin and bizelesin was examined in human cells (Lee et al., 1994). It was found that the patterns of alkylation induced by the agents in cellular DNA were similar but not identical to that observed in isolated DNA (Lee et al., 1994). Examination of the nucleotide preference of cisplatin in the N-*ras* gene in human cells revealed a binding site not seen in isolated DNA (Grimaldi et al., 1994). Each of these examples lend further emphasis to the need to determine the sequence specificity of agents in living cells. In addition, the repair of these lesions by the cellular machinery will be important in determining the biologically important and unimportant lesions formed by a sequence-specific drug. A highly specific lesion that is easily recognized and removed by the DNA repair systems may not have the same impact as those lesions that are not easily recognized. Studies are underway to determine whether the sequence specificity noted for the BAM pyrrole conjugates in the present study is retained in cells. In addition, the repair of these lesions at the gene and nucleotide level is being investigated and will be reported in due course.

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