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ORIGINAL ARTICLE

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DNA guanine-guanine crosslinking sequence specificity of isophosphoramide mustard, the alkylating metabolite of the clinical antitumor agent ifosfamide

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Abstract *Purpose*: The purpose of this investigation was to determine the base sequence specificity of isophosphoramide mustard (IPM), the alkylating metabolite of ifosfamide, by crosslinking of designed DNA oligomers in comparison with the clinical alkylating agents mechlorethamine (ME) (nitrogen mustard) and phosphoramide mustard (PM), the alkylating metabolite of cyclophosphamide. *Methods*: IPM, as well as PM and ME were each reacted with three dodecameric duplexes, which were designed to detect interstrand crosslinking between guanines in 5′-GC-3′ (I), 5′-GNC-3′ (II) or 5′-GNNC-3′ (III) sequences (N = A or T). *Results*: All three agents preferentially react with 5′-GNC-3′ target sequences. The 5′-GNNC-3′ target sequence is less reactive by a factor of approximately 2.5- to 10-fold, while 5′-GC-3′ is of even lower reactivity. *Conclusion*: These results indicate that all three agents show approximately equal preference for reaction with a 5′-GNC-3′ target sequence in spite of the fact that IPM yields a 7-atom crosslink, while the other two agents yield 5-atom crosslinks.

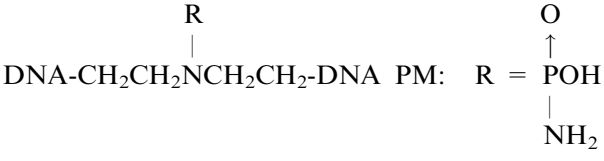
Key words DNA · Crosslinking · Isophosphoramide mustard

Introduction

Cyclophosphamide (CPA) and ifosfamide (Ifos) are important clinical antitumor alkylating agents that require cytochrome P450 oxidation primarily by CYP3A4 to generate corresponding intermediate hydroxylation products, which subsequently yield the ultimate alkylating metabolites phosphoramide mustard (PM) and isophosphoramide mustard (IPM), respectively [10].

Recent studies in the laboratories of Povirk and associates [1] and Friedman and associates [6] have identified the preferred DNA base sequence for PM for DNA interstrand guanine-guanine crosslinking as 5′-G-N-C-3′. This result was not unexpected since previous studies by Loechler and associates [7, 9] and Millard and associates [8] determined that mechlorethamine (ME) preferred the same sequence. Both ME and PM contain the same bis(2-chloroethyl)amine alkylating moiety, which would produce a 5-atom crosslink in duplex DNA:

Mechlorethamine: R = CH_3



We report here analogous studies on the DNA guanine-guanine crosslinking sequence specificity of IPM, the alkylating metabolite of the clinical antitumor agent Ifos, in order to determine whether it is identical to or different from that produced by CPA and ME. Because IPM would produce a 7-atom crosslink in duplex DNA instead of a 5-atom crosslink, it appeared likely that IPM would prefer a longer sequence such as 5′-GNNC-3′.

Material and methods

ME was purchased from Aldrich Chemicals, Milwaukee, Wis., and PM and IPM were obtained from Dr. Ven Narayanan, Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, Md. The DNA twelvemers were purchased from GENOSYS, The Woodlands, Tex., and Cruachem, Dulles, Va., USA.

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Crosslinking of the twelvemer duplexes was accomplished as previously reported [9] as follows:

Labeling of oligonucleotides

Approximately 100 μg of oligonucleotides TZ1 (G-C pair), TZ3 (G-X-C pair), or TZ5 (G-X-X-C pair) were phosphorylated in the presence of 68 μmoles of [γ-^{32}P] ATP (ICN Pharmaceuticals, Irvine, Calif.; approximately 4500 Ci/mmol) using T4 polynucleotide kinase (40 units; New England Biolabs, Beverly, Mass.) for 45 min at 37 °C. Cold ATP (2.5 mmol) and an additional 20 units of T4 polynucleotide kinase (final volume, 140 μl) were added and incubated for an additional 30 min at 37 °C. Unincorporated radiolabel was removed by the use of Bio-Spin 6 chromatography columns (Bio-Rad, Hercules, Calif., USA).

Reaction of oligonucleotides with alkylating agents

One hundred micrograms of labeled oligonucleotides TZ1, TZ3, and TZ5 was annealed to their respective complementary unlabeled oligomers by heating samples at 65 °C for 2 min in 200 m*M* sodium phosphate buffer (total volume 500 μl) followed by slow cooling of samples to 30 °C over a period of 30 min. PM, IPM, or nitrogen mustard (10 μmol) were added to each sample, and reactions were incubated at room temperature for 25 h. Sixty microliters of 1 N NaOH was added to the samples, incubated at 37 °C, and 50 μl of 1 N HCl was added to neutralize the reactions. The samples were desalted with C-18 SEP-PAK cartridges (Millipore, Milford, Mass., USA), and concentrated to a volume of 100 μl in a Speed Vac concentrator.

Quantitation of crosslinked products

Crosslinked oligonucleotides were analyzed on denaturing polyacrylamide gels (20.9% acrylamide/1.1% bis-acrylamide; TBE; pH 8.0). Approximately 5×10^5 cpm of each sample was loaded per lane and electrophoresed at 1600 V for 5 h. Labeled oligonucleotide sizing markers (Pharmacia Biotech, Piscataway, N.J., USA) ranging in size from 8 to 32 bases were used to determine oligonucleotide sizes. The wet gels were autoradiographed at room temperature for 8 h. The autorads were scanned [pdi 420oe scanner using pdi Quantity One (Version 2.6) software] to obtain a digital output, and the relative intensity across each lane was printed out. The relative intensities of the overlapping signals (i.e., distinct bands vs smears) were resolved with a Du Pont Model 310 Curve Resolver by a method that we have described previously [2]. Where values are reported, the number of readily discernible distinct bands was 3 for ME with each oligonucleotide, 3 for IPM with each oligonucleotide, and 2 for PM with each oligonucleotide. Previous work suggests that these different bands represent conformational isomers [9]. The values for the relative intensities reported in Table 1 are in arbitrary units. Relative intensity can only be rigorously compared for a particular lane (e.g., distinct bands, smear, and total for ME reacted with II); however, it is likely that all of the data can be reliably compared, given that the conditions were identical for all of the reactions and equal amounts of total radioactivity were loaded in each lane.

Results and discussion

ME was reacted with the oligonucleotides I (5′-GC-3′ target), II (5′-GNC-3′ target), and III (5′-GNNC-3′ target) under identical conditions. Bands that migrated considerably slower than starting material in a denaturing polyacrylamide gel were observed when ME was reacted with II (Fig. 1a, lane 2). Such bands have been shown to contain crosslinked oligonucleotides by us [7, 9] and others [1, 6, 7, 8] using ME, as well as other crosslinking agents. In this region of the gel there appeared to be two well-resolved bands along with an ill-defined smear of radioactivity. In contrast, the reaction of ME with oligonucleotide III principally gave a smear, although several weak bands were present, and the reaction of ME with I gave only a smear. The relative intensity of the radioactivity was quantitated following autoradiography and image analysis, and Table 1 gives quantitation for both the distinct bands, which were observed most clearly in the reaction of ME with II, as well as radioactivity associated with the smears for I, II, and III.

CGCGTATGCATT	CGCGTGTCGACT	CGCGTTGATCAT
\	\ or \	\
ATACGTAAGATC	ACAGCTGAGATC	AACTAGTAGATC
I	II	III
5′-GC-3′	5′-G-N-C-3′	5′-G-N-N-C-3′

Using identical reaction conditions, PM and IPM were also reacted with I, II, and III. Similar results were obtained in that both PM and IPM gave distinct bands with II, while smears were observed with all three oligonucleotides, I, II, and III (Figs. 1a, 1b, and 1c, respectively). This is also quantitated in Table 1. While we are not certain of the source of the smearing, it seems unlikely to be attributable to the presence of crosslinked oligonucleotides for four reasons:

1. The smearing is unlikely to be due to the breakdown of the major crosslinked oligonucleotides, because the smears (principally associated with the reaction of ME, PM, and IPM with II) migrate more slowly than the distinct bands.

Table 1 Relative amount of radioactivity associated with slowly migrating material in the case of the reaction of ME, PM, and IPM with oligonucleotides I, II, and III based upon an analysis of polyacrylamide gels in Fig. 1. *ME* mechlorethamine, *PM* phosphoramide mustard, *IPM* isophosphoramide mustard

	Distinct Bands			Smears			Total		
	GC	GNC	GNNC	GC	GNC	GNNC	GC	GNC	GNNC
ME	< .0012 (< .013)	.086[a] (1.0)	.011 (.13)	.046	.113	.083	.046	.199	.094
PM	< .0012 (< .018)	.067 (1.0)	.013 (.19)	.090	.130	.079	.090	.197	.092
IPM	.0032 (.03)	.094 (1.0)	.0045 (.048)	.018	.037	.024	.021	.130	.028

[a] Values are the relative amounts of radioactivity (in arbitrary units) associated with the distinct bands, the smears and the total based on the data in Fig. 1. Values in parenthesis are normalized to the highest signal (i.e., 5′-GNC-3′) for each agent. All values in the table can be compared for reasons discussed in Material and methods

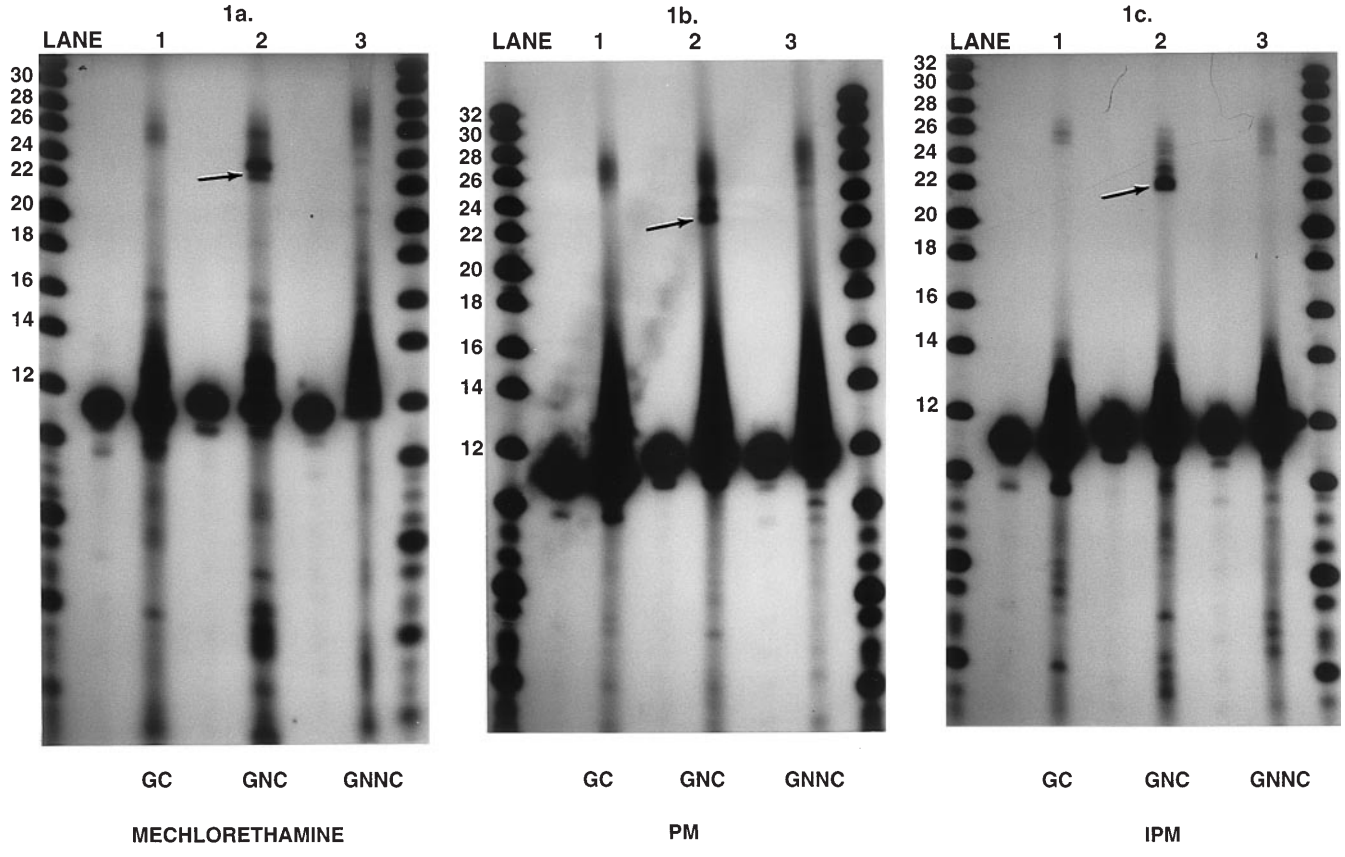

Fig. 1 Denaturing polyacrylamide gels showing reaction products in **1a–1c** in *lanes 1–3* generated from oligonucleotides I, II and III and ME, PM and IPM, resp. Arrows indicate major crosslinked products, and lanes preceding *lanes 1–3* include the appropriate control oligomers

2. Thus, if the smearing represents breakdown of crosslinked oligonucleotides, it must be due to other crosslinked entities. In the case of each agent, the apparent ratio of radioactivity due to the smearing is approximately 0.5:1.0:0.5 (average 0.47:1.0:0.62) for I:II:III. It seems unlikely that this represents the relative yield of some kind of crosslinked entities, given that the ratios for the distinct bands, which are known to be attributable to crosslinked material, are so markedly different.

3. In the past we have tried to isolate the putative crosslinked oligonucleotides associated with the smears that sometimes appear when ME is reacted with II, but have never been able to isolate anything (J. Ojwang, M. Berardini and E. Loechler, unpublished results).

4. In some cases, the reaction of ME with an oligonucleotide containing only a single 5′-GC-3′ target sequence (i.e., analogous to I) gave neither detectable bands, nor smears [9]. While none of these four arguments are rigorous, collectively they suggest that it is unlikely that the smearing is due to crosslinked oligonucleotides. This conclusion is certainly most consistent with previous work that indicated that ME was much more reactive toward a 5′-GNC-3′ sequence than either a 5′-GNNC-3′ or a 5′-GC-3′ sequence [1, 6, 7, 8, 9]. In fact, the analysis for the distinct bands in Table 1 indicates that the 5′-GNC-3′ sequence (oligonucleotide II) is preferred by a factor of approximately 2.5- to 10-fold compared with a 5′-GNNC-3′ sequence (oligonucleotide III) for ME, PM, and IPM, and even greater than that compared to a 5′-GC-3′ sequence (oligonucleotide I). This factor is corrected for the fact that oligonucleotides I and III have a single 5′-GC-3′ and 5′-GNNC-3′ target sequence, respectively, while oligonucleotide II has two 5′-GNC-3′ target sequences. It should also be noted that prior experiments revealed that a duplex lacking the 5′-GNC-3′ sequence but containing the 5′-GC-3′ sequence failed to react with ME [4], which, coupled with the results for duplexes I and III, clearly indicate the base sequence specificity for ME, PM, and IPM.

In order to confirm that the crosslinking sites in II, crosslinked with IPM, were identical to the sites crosslinked in II by ME, the crosslinked product was treated identically to the procedure previously reported for II crosslinked by ME [9], and analysis by the Maxam-Gilbert sequencing method yielded identical results, revealing that the guanines in positions 6 and 9 in both oligomers (5′ → 3′) were the major sites of adduction with some adduction occurring at position 4 in both oligomers, as also observed for ME [9].

Because IPM should yield a 7-atom bridging crosslink [DNA-CH_2CH_2NH-P(O_2H)-$NHCH_2CH_2$-DNA], we considered it possible that IPM might show a different sequence reactivity preference compared with ME and PM, which both yield a 5-atom bridging crosslink.

However, IPM does not show evidence of enhanced reactivity with either I or III compared with II, based upon analysis of either the distinct bands or total radioactivity. If anything, it appears that IPM might in fact show greater specificity for reaction with II than does either ME or PM based upon the relative yield of distinct bands with II compared with I and III (Table 1).

The preference of IPM for crosslinking II rather than III, in spite of its 7-atom crosslink and its likely capability of crosslinking over longer distances such as G-N-N-C, is nonetheless consistent with similar observations on other agents capable of crosslinking G-N-N-C but preferring G-N-C [5]. Because the chemical nature of the crosslinks derived from PM and IPM is different, differences in the rate of crosslink formation and differences in crosslink repair could allow for differences in their overall therapeutic effect, and investigation of these two effects is in progress. In addition, since PM and IPM also yielded products from the 5′-GC-3′ and 5′-GNNC-3′ duplexes, it is possible that one of the minor crosslinks may be the major contributor to cytotoxicity for PM or IPM, possibly also yielding a qualitative difference in the major cytotoxic crosslink for PM and IPM.

Following our reported procedures [4, 7, 9], PM- and IPM-crosslinked duplexes II, which include appropriate overhangs and a restriction enzyme recognition site, will be ligated into a plasmid for subsequent studies in *E. coli* and human 293 cells to determine effects of the crosslinks on the production of progeny plasmids, as determined for ME [3, 4].

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