Nucleic Acids Research, 1997, Vol. 25, No. 3 **491–495**

Selective recognition of a cisplatin-DNA adduct by human mismatch repair proteins

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Received November 4, 1996; Revised and Accepted December 11, 1996

ABSTRACT

The antitumor agent *cis*-diamminedichloroplatinum(II) (cisplatin) introduces cytotoxic DNA damage predominantly in the form of intrastrand crosslinks between adjacent purines. Binding assays using a series of duplex oligonucleotides containing a single 1,2 diguanyl intrastrand crosslink indicate that human cell extracts contain factors that preferentially recognise this type of damage when the complementary strand contains T opposite the 3′, and C opposite the 5′ guanine in the crosslink. Under the conditions of the band-shift assay used, little binding is observed if the positions of the T and C are reversed in the complementary strand. Similarly, duplexes containing CC or TT opposite the crosslink are recognised relatively poorly. The binding activity is absent from extracts of the colorectal carcinoma cell lines LoVo and DLD-1 in which the hMutSα mismatch recognition complex is inactivated by mutation. Extensively purified human hMutSα exhibits the same substrate preference and binds to the mismatched platinated DNA at least as well as to an identical unplatinated duplex containing a single G·T mismatch. It is likely, therefore, that human mismatch repair may be triggered by 1,2 diguanyl intrastrand crosslinks that have undergone replicative bypass.

INTRODUCTION

Cisplatin is used in the treatment of several types of cancer and is particularly effective against testicular tumors (1). In common with many chemotherapeutic agents, the clinical effectiveness of cisplatin is limited by the emergence of drug-resistance (2). Cisplatin adducts in DNA are repaired by nucleotide excision repair (NER) (3,4) and cells deficient in this repair pathway, such as those derived from xeroderma pigmentosum patients, are hypersensitive to cisplatin. Increased excision of cisplatin adducts has been observed in some resistant cell lines derived in the laboratory (5). Other resistant variants can tolerate higher levels of cisplatin-induced DNA damage without detrimental effects on survival (6). 1,2 dipurinyl intrastrand crosslinks comprise ≥80% of cisplatin adducts (7). In comparison to the less abundant 1,3 diguanyl crosslinks, the abundant 1,2 adducts, are rather poor substrates for removal by NER (4,8). Their relatively long persistence in DNA suggests that many 1,2 diguanyl crosslinks may undergo replicative bypass.

In addition to the protection conferred by NER, cells can sometimes acquire cisplatin resistance through an increased ability to tolerate unexcised cisplatin lesions in their DNA. This tolerance arises through loss of a DNA mismatch repair pathway. Cisplatin-resistant variants of a human ovarian carcinoma cell line exhibit microsatellite instability (9) and are mismatch repair deficient owing to defective expression of the hMLH1 mismatch repair protein (10). In addition, some human colorectal carcinoma cell lines deficient in hMLH1 or hMSH2 are more resistant to cisplatin than sublines in which the mismatch repair defect has been complemented by chromosome transfer (11). Thus, in contrast to NER which promotes cell survival, a functional DNA mismatch repair pathway appears to contribute to cisplatin cytotoxicity.

Mismatch repair corrects single base mispairs and the looped intermediates, typically one to three bases, that arise by slippage during replication of repeated DNA sequences (for review see 12). Reversal of these replication errors prevents the increased spontaneous mutation rates and microsatellite instability that characterise mismatch repair defective cells. In normal human cells, DNA mismatches are recognised by one of two known mismatch recognition complexes. The best characterised of these, hMutSα, is a heterodimer of hMSH2 and hMSH6 (also known as GTBP) (13,14). hMutSα binds to single base mispairs, loops of one base and, to a lesser extent, loops of two bases. A second heterodimer, hMutSβ, although able to recognise two adjacent unpaired bases, prefers larger looped structures and binds more effectively to loops of three or four bases. hMutSβ is a heterodimer in which hMSH2 is partnered by hMSH3 (15).

Acquired drug resistance by loss of mismatch repair is a feature of N-methyl-N-nitrosourea (MNU) and N-methyl-N′-nitro-N-nitrosoguanidine tolerant cells. These compounds are analogs of the methylating agents used in clinical practice. Mismatch repair interacts with one particular methylated base, O^6-methylguanine (O^6-meGua) among the numerous DNA methylation products (for review see 16). In particular, binding to O^6-meGua-containing base pairs by a mismatch recognition complex in cell extracts has been demonstrated (17). These data indicated that O^6-meGua:T base pairs are recognised quite well by a mismatch recognition activity [which is now known to be hMutSα (18)] and somewhat better than O^6-meGua:C base pairs. This recognition is thought to provoke incomplete, and therefore lethal, repair attempts at the incorrigible O^6-meGua-containing base pairs. To investigate whether mismatch binding activities can recognise a common cisplatin DNA adduct, we have used synthetic oligonucleotides

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containing a single 1,2 diguanyl cisplatin intrastrand crosslink. By annealing this oligonucleotide to different complementary strands, we have investigated binding to sequences that might arise during replicative bypass of this type of DNA damage. DNA in which the 1,2 diguanyl crosslink is paired to two complementary cytosines is recognised poorly by hMutSα. In contrast, hMutSα binds preferentially to these intrastrand crosslinks if thymine is positioned opposite the 3′ guanine and cytosine opposite the 5′ guanine of the crosslink. Thus, crosslinks that have undergone promutagenic replication are likely to be recognised by this mismatch binding complex.

MATERIALS AND METHODS

Materials

Biochemicals were obtained from Sigma except where stated otherwise. Unmodified oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Oligonucleotides containing a single 1,2 diguanyl cisplatin crosslink (top strand shown below), prepared as described in Szymkowski *et al.* (7) were a kind gift from Dr John Essigmann, MIT. Platinated strands were end-labeled with T4 DNA polynucleotide kinase (New England BioLabs) and annealed to 5-fold excess of non-labeled bottom strands. The sequence of the duplex substrates is as follows:

	Abbreviation
5′-TCTTCTTCTAGGCCTTCTTCTTCT-3′	Pt-GG
3′-AGAAGAAGATCCGGAAGAAGAAGA-5′	
5′-TCTTCTTCTAGGCCTTCTTCTTCT-3′	Pt-GG.TC
3′-AGAAGAAGATTCGGAAGAAGAAGA-5′	
5′-TCTTCTTCTAGGCCTTCTTCTTCT-3′	Pt-GG.CT
3′-AGAAGAAGATCTGGAAGAAGAAGA-5′	
5′-TCTTCTTCTAGGCCTTCTTCTTCT-3′	Pt-GG.TT
3′-AGAAGAAGATTTGGAAGAAGAAGA-5′	

The cross linked guanines are shown underlined.

Binding assay

Band shift assays were performed as previously reported (19). Briefly, cell extract (15–20 µg) was precincubated at room temperature with 40 fmol of matched non-radioactive 34mer in 20 µl reaction buffer comprising 25 mM HEPES·KOH, pH 8.0, 0.5 mM EDTA, 0.1 mM $ZnCl_2$, 10% glycerol, 50 µg poly(dI·dC)poly(dI·dC). After 5 min, the reactions were supplemented with ^{32}P labelled substrate (20 fmol), and incubation continued for a further 20 min. Aliquots of 10 µl, supplemented with bromophenol blue, were analyzed by electrophoresis on 6% polyacrylamide gels. Reaction products were detected by autoradiography. When non-radioactive competitor oligonucleotides were included, they were present during the preincubation and subsequent incubation. In experiments to assess efficiency of binding to different radioactive substrates, equal amounts of radioactivity were used.

Cell culture and extract preparation

The thymidine kinase-deficient subline of the Raji Burkitt's lymphoma was maintained in spinner culture in RPMI medium containing 5% fetal calf serum (Life Technologies, Inc.). Exponentially growing cells were harvested by centrifugation. The human colorectal adenocarcinoma lines LoVo and DLD-1 were grown respectively in Ham's F12 medium or RPMI supplemented by 10% fetal calf serum. LoVo and DLD-1 cells were detached from the flasks by trypsin-free cell dissociation solution (Sigma) and harvested by centrifugation. Cell extracts for binding were prepared from fresh or frozen (–80°C) cells as described previously (19).

Purification of hMutSα

All steps were performed at 0–4°C. The purification was carried out essentially as reported by Drummond *et al.* (13) omitting the final MonoQ step. Extracts were prepared by homogenizing ~5×10^9 cells in Buffer A (25 mM HEPES·KOH, pH 8.0, 1 mM EDTA, 2 mM β-mercaptoethanol, 0.5 mM spermidine, 0.1 mM spermine). The material which precipitated between 5 and 65% saturated $(NH_4)_2SO_4$ was collected by centrifugation, dissolved in Buffer A and dialysed against Buffer A for 5 h. After centrifugation (3000 *g*) for 10 min to remove precipitated material, the sample was applied to a single-stranded DNA cellulose column (1.8 $cm^2 \times 5$ cm) equilibrated with Buffer A containing 0.1 M NaCl. The material that passed through the column was reloaded, and the column was washed at a rate of 1.5 ml/min with Buffer A containing 0.2 M NaCl and 2.5 mM $MgCl_2$. Protein eluting in a subsequent wash with Buffer A containing 0.2 M NaCl, 2.5 mM $MgCl_2$, and 1 mM ATP was retained and loaded onto a Q Sepharose column (0.7 $cm^2 \times 1$ cm, Pharmacia) which was prepared according to the manufacturer's instructions and equilibrated in Buffer A containing 0.2 M NaCl. After washing with 2 ml of the same buffer, hMutSα was obtained by elution with 2 ml of Buffer A containing 0.65 M NaCl. The fraction was concentrated 10-fold by Microcon 30 (Amicon, Inc., MA, USA) and the concentration of NaCl reduced to 0.2 M. Small aliquots were snap frozen and stored at –70°C.

RESULTS

Binding to platinated duplexes by wild-type cell extracts

Extracts of the Burkitt's lymphoma cell line Raji selectively recognise duplex oligonucleotides containing a single G·T mispair. The mismatch-specific complex formed with the standard 34mer heteroduplex is shown arrowed in Figure 1a. When 24mer duplexes containing the 1,2 diguanyl cisplatin crosslink were used as substrates, a band at the position of the G·T complex was seen with the Pt-GG.CT substrate in which the complementary strand contained CT opposite the cross-link (lane 5 from left). This band was not observed with any of the other three platinated substrates, Pt-GG, Pt-GG.TC and Pt-GG.TT or with the non-platinated matched DNA. A non-specific complex was formed with all the substrates tested, including the G·T mismatch and the perfectly matched 24mer duplex (lane 2). A minor band, which migrated between the G·T complex and the non-specific complex, was observed. This minor band was formed with the Pt-GG substrate (lane 3). It was also present with the Pt-GG.CT duplex (lane 5) and binding to this platinated substrate characteristically produced two bands (see below). Thus, under the conditions of these experiments, platinated DNA with CT positioned opposite a 1,2 diguanyl cross-link is recognised more efficiently than similar molecules with other combinations of pyrimidines in the complementary strand. A faint complex that migrated about half-way down the gel was also observed with Pt-GG (lane 3). This is a minor activity under our experimental conditions and may reflect binding by other recognition factors that interact with

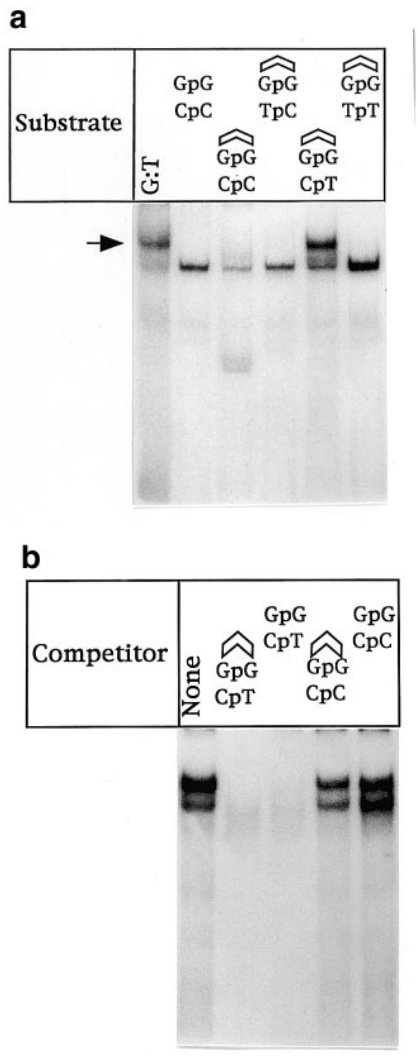

Figure 1. (**a**) Binding to platinated duplex oligonucleotides by Raji cell extracts. Raji cell extracts (15 µg) prepared as described (19) were preincubated with poly(dI:dC) and a non-radioactive matched competitor duplex as described in Materials and Methods. Radioactively labelled G·T mismatched 34mer (lane 1), perfectly matched 24mer (lane 2) or platinated duplex as indicated (lanes 3–6) was then added. Protein–oligonucleotide complexes were separated on a non-denaturing polyacrylamide gel and detected by autoradiography. The free oligonucleotides were allowed to migrate out of the gel in order to improve resolution of the bound complexes. The arrow marks the position of the G·T mismatched oligonucleotide:hMutsα complex. (**b**) Competitive inhibition of binding. Binding to the a platinated duplex (Pt-GG.CT) containing CT in the position complementary to the diguanyl crosslink was carried out as described above except that the preincubation contained non-radioactive competitors (5 pmole, 250-fold excess) as indicated. Bound duplex was analysed as described above.

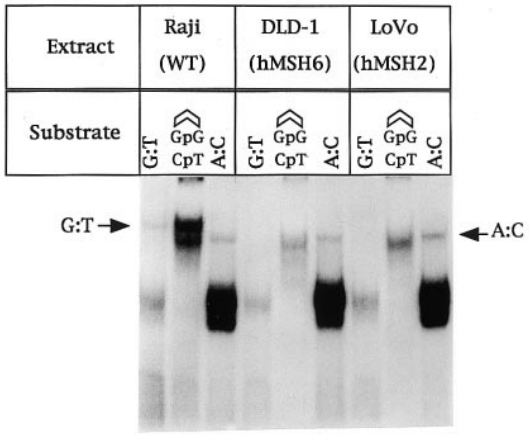

Figure 2. Binding to platinated duplex oligonucleotides by extracts of mismatch binding-defective cells. Binding by extracts (15 µg) of Raji, DLD-1, or LoVo cells to a G·T mismatched 34mer (lane 1 of each series), a platinated duplex Pt-GG.CT (lane 2 of each series) or an A·C mismatched 34mer (lane 3 of each series) was analysed as described in the legend to Figure 1a. The positions of the G·T mismatched oligonucleotide:hMutsα complex and the A·C mismatched oligonucleotide:A·C mismatch binding activity are shown arrowed.

cisplatin modified DNA such as RPA (20) or one of the previously identified proteins containing the HMG box motif (21).

Confirmation of the substrate preference of binding was obtained by including an excess of non-radioactive competitor molecules in the binding reaction (Fig. 1b). A 250-fold excess of the non-radioactive duplexes comprising a platinated or unplatinated top strand with a CT-containing bottom strand were effective competitors and abolished the formation of both complexes on the platinated substrate. In contrast, a matched unplatinated 24mer duplex at the same concentration was an ineffective competitor (lane 5). In agreement with its limited binding, non-radioactive Pt-GG competed to some extent but was much less efficient than the other platinated competitor. Effective competition therefore required either a G·T mismatch (unplatinated substrate) or CT in the complementary strand of the platinated substrate. These data confirm that wild-type cell extracts preferentially recognise platinated oligonucleotides in which the complementary strand contains CT opposite a 1,2 diguanyl cross-link and suggest that this binding is by the complex that recognises G·T mispairs.

Binding to platinated duplexes by mismatch repair-defective cell extracts

In contrast to wild-type Raji cell extracts, neither extracts of hMSH2-defective LoVo cells nor hMSH6(GTBP)-defective DLD-1 cells bound detectably to Pt-GG.CT. Figure 2 shows that whereas Raij cell extracts recognised G·T mispairs, Pt-GG.CT and A·C mispairs, LoVo and DLD-1 extracts recognised only A·C mispairs. The faint band that is apparent with LoVo and DLD-1 extracts may represent some residual non-specific binding to the platinated oligonucleotide and is unrelated to G·T mismatch binding. The absence of G·T mismatch binding by LoVo and DLD-1 extracts confirms previous observations (14,22,23). The poor recognition of the platinated Pt-GG.CT duplex by these extracts is consistent with the involvement of the hMutSα mismatch recognition complex in binding DNA duplexes containing 1,2 diguanyl crosslinks of this type. The A·C mismatch binding activity is known to be independent of both hMSH2 and hMSH6(GTBP) (24) and serves as an internal control for the cell extracts.

Binding to platinated duplexes by purified hMutSα

hMutSα was purified extensively from Raji cell extracts by adsorption to single-stranded DNA cellulose and selective elution with ATP (13). Two prominent proteins of approximate M_r = 100 000 and 160 000 together with a number of minor products were detected by Coomassie staining of an SDS polyacrylamide gel of the purified material (data not shown). The sizes of the major

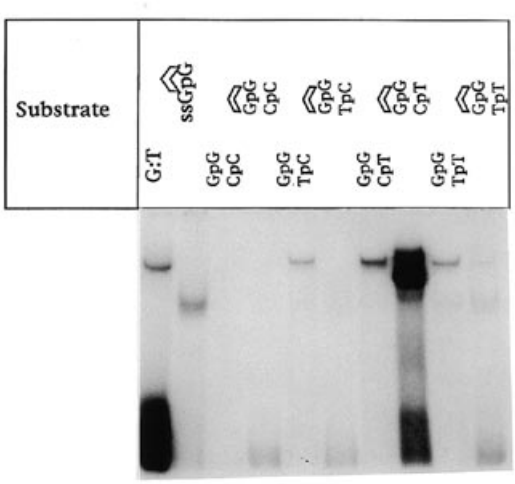

Figure 3. hMutsα binding to platinated and unplatinated duplexes. hMutsα, purified as described in Materials and Methods was incubated with G·T mismatched 34mer (lane 1), unplatinated 24mer duplexes with different complementary strand sequences as indicated (lanes 3, 5, 7 and 9) and their platinated counterparts (lanes 4, 6, 8 and 10). Binding was analysed as described in the legend to Figure 1a.

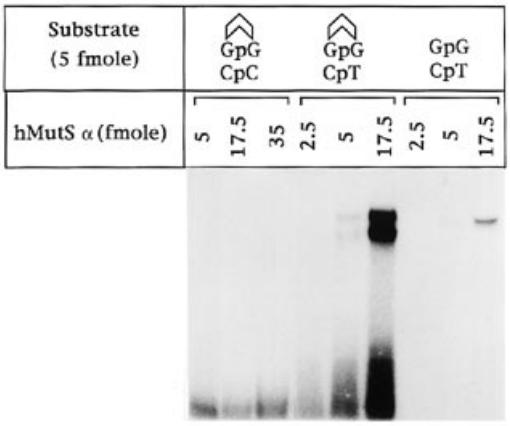

Figure 4. Comparative efficiency of binding by hMutsα. The concentration of purified hMutsα was estimated from a Coomassie stained SDS polyacrylamide gel. The purified material was incubated with the 24mer duplexes shown at the approximate ratios indicated. Binding was analysed as described in the legend to Figure 1a.

components are compatible with hMSH2 and hMSH6(GTBP) and we estimate that the hMutSα preparation was >50% pure. We compared G·T mispair and cisplatin adduct recognition by this purified preparation. Non-platinated duplexes containing different complementary strands which introduced G·T mispairs were compared to their, otherwise identical, platinated counterparts. One or two G·T mispairs in the non-platinated duplexes stimulated binding to approximately similar extents (Fig. 3). In contrast, with platinated substrates which were identical except for the single 1,2 diguanyl crosslink, only Pt-GG.CT was recognised to a detectable extent. This selectivity reflects the preference exhibited by unfractionated cell extracts and confirms that only one of the platinated substrates is recognised to any significant degree. This recognition is most probably by the hMutSα mismatch binding complex.

Comparative binding to G·T mismatches and platinated duplexes

The relative affinity of the purified hMutSα complex for a G·T mispair and 1,2 diguanyl cisplatin cross-link was investigated (Fig. 4). Substrates that were radioactively labelled to comparable specific activities were mixed with increasing amounts of purified hMutSα. The partially purified hMutSα mismatch binding complex recognises this particular platinated substrate at least as well as a single G·T mismatch. Binding to the platinated Pt-GG.CT duplex was detectable at an estimated hMutSα:DNA ratio of ~1. Binding to the G·T mispair in an otherwise identical substrate which did not contain a crosslink was easily detectable at a hMutSα:DNA ratio of ~3.5. The Pt-GG substrate in which the complementary strand contained CC opposite the crosslink, was not detectably bound by ratios of hMutSα:DNA up to 7. Recognition of the platinated substrate always generated two resolvable bands in contrast to the single complex observed with a G·T mispair. The reason for this behaviour is not clear at present although it may reflect different extents of hMutSα loading onto the DNA.

DISCUSSION

Cells can acquire resistance to drugs, such as the methylating agents MNU by becoming tolerant to the presence of O^6-meGua in DNA (16). It is generally considered that O^6-meGua-containing base pairs provoke unsuccessful attempts at mismatch repair that result in cell death. Methylation tolerance arises as a direct consequence of the loss of the mismatch repair pathway (16). Defects in the mismatch repair proteins hMLH1, hMSH2 and hMSH6(GTBP) have been identified among tolerant cell lines (for review see 25) and base pairs involving O^6-meGua are recognised by mismatch binding factors in cell extracts (17) and by a purified hMutSα complex (18).

Cell lines selected for resistance to cisplatin have similar mismatch repair defects (9,10). This observation implies that one of the products of DNA platination might provoke mismatch repair attempts analogous to those at O^6-meGua. Recognition by mismatch binding complexes is a prerequisite for such attempts. The experiments reported here indicate that, among four potential substrates for recognition by mismatch binding activities, a duplex molecule containing a single 1,2 diguanyl cisplatin cross-link in which the complementary strand contains T opposite the 3′, and C opposite the 5′ crosslinked guanine is highly preferred. A 1,2 diguanyl crosslink paired to two cytosines was recognised less favorably in our experiments. This is in agreement with other studies which indicate that purified hMutSα binds to this substrate with about an order of magnitude lower affinity than to a single G·T mispair (18). The same substrate is recognised, although rather poorly, by hMSH2 acting alone (26). Platinated DNA is not *per se* a good substrate for this mismatch recognition complex. This is consistent with previous approaches in which the use of platinated DNA as a probe for possible recognition factors of cisplatin DNA damage identified a member of the HMG group of proteins (21) and RPA (20) but not mismatch binding proteins.

Mismatch repair is a post-replicative correction pathway and a current model for the emergence of methylation tolerance, and the related cross-tolerance to 6-thioguanine (27), invokes replication of adducted bases as a key step (16). Our observation of a more

favorable interaction of hMutSα with duplex DNA containing a single 1,2 diguanyl cisplatin crosslink paired to CT in the complementary strand implies that mismatch correction attempts might be more likely following replication of cisplatin-adducted DNA. This replication would be potentially mutagenic in that insertion of thymine opposite the first guanine of the crosslink would be followed by extension of the daughter strand by incorporation of cytosine. Replication bypass of cisplatin-DNA lesions has been observed in cell extracts (28) and has been inferred in intact cells (29). Some cisplatin-resistant cell lines are apparently more able to perform replicative bypass of platinum-DNA adducts than their sensitive parent cells (30). The mechanism of this type of trans-lesion DNA replication is unknown. In general, however, bypass of 1,2 diguanyl cisplatin crosslinks has been considered inefficient and only DNA polymerase β appeared capable of significant DNA synthesis opposite adducts of this type (31). While the presence of active mismatch repair might explain the poor translesion synthesis in cells or by replication extracts, the apparent failure of purified replicative DNA polymerases δ and/or ε to bypass 1,2 diguanyl adducts is more problematical. Recent evidence indicates, however, that the replicative polymerases are indeed able to bypass these adducts in structures that resemble replication forks (32). Analysis of the products of this type of bypass should define the true probability of the type of miscoding we postulate.

In addition to potentially lethal intervention by mismatch correction, 1,2 diguanyl adducts that have undergone replication bypass may be recognised by proteins of the NER pathway. Positioning a mispaired thymine opposite either, or both platinated guanines of a 1,2 crosslink markedly increases the susceptibility of the crosslink to recognition and removal by NER (33). Post-replicative crosslink removal by NER might therefore exert a protective effect which opposes the lethal processing by mismatch repair. This is consistent with the relative sensitivity of xeroderma pigmentosum cells to cisplatin. It seems likely that a cell's sensitivity to cisplatin treatment will be, at least partly, determined by the relative efficiencies of the mismatch correction and NER pathways.

The data presented here and in the accompanying paper (33) also have implications for the mechanisms of damage recognition by mismatch repair and NER factors. All three unplatinated oligonucleotides which contained one or two GT mispairs were efficiently recognised by hMutSα. The structural alteration introduced by the addition of a 1,2 diguanyl crosslink reduced recognition by the mismatch binding complex in two of the three substrates, but apparently stimulated it in the third. A mispaired thymine opposite either or both crosslinked guanines improves the probability of recognition by NER factors (33). hMutSα and the NER damage recognition factors, such as XPA, in conjunction with oligonucleotides containing defined 1,2 diguanyl cisplatin crosslinks might provide a useful approach to defining the precise structural requirements for effective DNA recognition by mismatch repair and NER proteins.

ACKNOWLEDGEMENTS

We are particularly grateful to John Essigmann for making available the platinated oligonucleotides and we would like to thank him, Rick Wood and Jonathan Moggs for their comments on the manuscript. We also thank Jean-Sebastien Hoffmann for providing a preprint of his paper. The skilled assistance of the Oligonucleotide Synthesis and Cell Production laboratories of the ICRF, Clare Hall is also acknowledged. Y.M. was the recipient of an STA Fellowship and E. O'R. was supported by a Human Capital and Mobility Fellowship from the European Union.

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