

# Sequence- and Region-Specificity of Oxaliplatin Adducts in Naked and Cellular DNA

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## ABSTRACT

Oxaliplatin is a clinical anticancer drug with a pharmacological profile distinct from that of cisplatin. Our studies compared site- and region-specificity of lesions induced by oxaliplatin and cisplatin in naked and intracellular DNA, respectively. Oxaliplatin adducts in naked Simian virus 40 (SV40 DNA) were mapped by repetitive primer extension. The sites of oxaliplatin adducts were nearly identical to the sites of cisplatin adducts and were focused in G clusters and GNG motifs probably reflecting intrastrand cross-links. Although alkaline agarose electrophoresis of specific SV40 fragments showed that oxaliplatin formed interstrand cross-links, the levels of this lesion type were low. Drug-induced lesions in discrete loci of cellular DNA were assessed by the polymerase chain reaction stop assay in human tumor A2780 cells. Oxaliplatin at 200  $\mu$ M induced  $\sim$ 1300,

$\sim$ 1500,  $\sim$ 800, and  $\sim$ 300 lesions/ $10^6$  bp in the human  $\beta$ -globin, *c-myc*, and *HPRT* genes and in mitochondrial DNA, respectively. Cisplatin formed two to six times more lesions in the same regions. For both drugs, lesion frequencies seem to parallel the density of drug-binding motifs in the nuclear regions, whereas mitochondrial DNA was disproportionately less affected. Despite less potent induction of DNA lesions, oxaliplatin was more cytotoxic than cisplatin against A2780 cells. Because our findings clearly demonstrate that oxaliplatin forms covalent adducts with a similar sequence- and region-specificity to that of cisplatin, other properties of oxaliplatin adducts, factors other than DNA binding, or both determine the unique features of the mechanism of action of oxaliplatin.

DACH [oxaliplatin; oxalato; (1*R*,2*R*)-DACH] (Fig. 1) is a member of the family of DACH platinum complexes and is a third-generation platinum antitumor drug (reviewed by Chaney, 1995). The advantages of oxaliplatin over cisplatin, the classic platinum drug, include a less severe clinical toxicity and a retained activity against cisplatin-refractory tumors (Mathe *et al.*, 1989; Tashiro *et al.*, 1989; Christian, 1992; Kelland, 1993; Weiss and Christian, 1993; Kelland and McKeage, 1994). *In vitro* and *in vivo* studies confirmed that oxaliplatin exhibits different spectra of activity and toxicity than cisplatin, often with a lack of cross-resistance with cisplatin (Tashiro *et al.*, 1989; Pendyala and Creaven, 1993; Chaney, 1995). COMPARE analysis of the sensitivity pattern in a panel of 60 tumor cell lines suggested that oxaliplatin and related DACH-containing platinum analogs differ mechanistically from cisplatin and other platinum drugs (Rixe *et al.*, 1996).

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**ABBREVIATIONS:** DACH, (*trans*-1,2-diaminocyclohexane)platinum(II); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PNK, polynucleotide kinase; mtDNA, mitochondrial DNA; HPRT, hypoxanthine phosphoribosyltransferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RPE, repetitive primer extension; SV40, Simian virus 40; TE, Tris-HCl/EDTA; ORI, origin of replication; bp, base pair(s); kbp, kilobase pair(s).

Studies with various DACH platinum complexes indicate that the *trans*-(*R,R*) configuration of the DACH moiety is critical for the properties of oxaliplatin, such as superior cytotoxicity and drug binding to cellular DNA (Inagaki and Sawaki, 1995; Pendyala *et al.*, 1995). The compounds with DACH isomeric forms, *trans*-(*S,S*) and *cis*-(*R,S*), are less potent. The leaving group (oxalate) also may affect drug pharmacological properties. Oxaliplatin undergoes a transformation under physiological conditions to DACH PtCl<sub>2</sub> (Fig. 1) and other species that seem to be primarily responsible for the reaction with DNA (Chaney, 1995; Luo *et al.*, 1997).

The formation of adducts with cellular DNA generally is regarded as the major determinant of the antitumor activity of platinum drugs. In contrast to the extensively studied cisplatin, however, relatively little is known about the DNA binding of oxaliplatin and related DACH agents. Adducts that involved binding motifs such as d(GpG), d(ApG), and (dG)<sub>2</sub> were identified in drug-treated isolated DNA or short synthetic oligonucleotides using DACH-Pt-SO<sub>4</sub> and DACH-Pt-(NO<sub>2</sub>)<sub>2</sub> (Jennerwein *et al.*, 1989; Boudny *et al.*, 1992). Also, the different Pt-GG and Pt-AG adducts of oxaliplatin were

detected recently by immunochemistry in DNA from oxaliplatin-treated cells (Saris *et al.*, 1996). The sites of oxaliplatin adducts at the nucleotide level, however, have not been characterized.

DACH-Pt adducts, once formed, should be identical to adducts by other DACH compounds with different leaving groups, but it cannot be ruled out that the oxalate DACH-Pt can behave differently than the nitrate form. The precedence of carboplatin, which forms chemically identical adducts as cisplatin but with apparently different nucleotide sequence preferences (Blommaert *et al.*, 1995), demonstrates that the leaving group may affect drug-binding sites. Thus, it remains unclear whether oxaliplatin and cisplatin affect the same or different subsets of potential binding motifs in DNA sequences and consequently target the same or different regions in cellular DNA.

The purpose of our study was to delineate differences and similarities between oxaliplatin and cisplatin in targeting naked and intracellular DNA. Adduct sites were mapped at the nucleotide level in naked SV40 DNA. Drug potential to form adducts in several discrete loci of cellular DNA in intact cells also was examined. The results demonstrate that oxaliplatin shares sequence- and region-specificity with cisplatin. Paradoxically, despite less potent induction of DNA lesions, oxaliplatin was more cytotoxic than cisplatin.

## Experimental Procedures

**Drugs.** Oxaliplatin was obtained from Sanofi Research (Malvern, PA). Cisplatin was from Sigma Chemical (St. Louis, MO). Stock solutions of cisplatin were made in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM  $\text{Na}_2\text{HPO}_4$ , and 1.4 mM  $\text{KH}_2\text{PO}_4$ ) and stock solutions of oxaliplatin in water or in dimethylsulfoxide and stored at  $-20^\circ$ .

**Cell lines and cytotoxic activity.** Green monkey kidney BSC-1 cells were grown as described previously (Zsido *et al.*, 1991). The human ovarian carcinoma A2780 line was obtained from Dr. Kevin J. Scanlon and grown as a monolayer culture in RPMI 1640 medium supplemented with 10% serum. Growth-inhibitory activity was assayed using a standard MTT assay (Arnould *et al.*, 1990). Exponentially growing cells in a 96-well microtiter plate were incubated with the drug for three or four doubling times (3–4 days) and subjected to colorimetric reaction with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide. The results are expressed as drug concentrations that inhibit cell growth by 50% (the  $\text{IC}_{50}$  values).

**Sites of drug adducts: RPE.** Localization of oxaliplatin and cisplatin adducts was determined based on premature termination of primer extension on a drug-modified DNA template. The use of thermally stable *Taq* DNA polymerase and repetitive cycling in a PCR machine enables linear amplification of the signal from the damaged template (Jennerwein and Eastman, 1991; Bubley *et al.*, 1994).

Drugs were reacted in the dark with 0.3–0.4  $\mu\text{g}$  of SV40 DNA (containing ~85% form I, GIBCO Life Technologies, Grand Island, NY) in 25  $\mu\text{l}$  of TE (10 mM Tris-HCl/1 mM EDTA) buffer at  $37^\circ$  for the

indicated times. After the addition of NaCl (0.5 M final concentration), drug-treated DNA was ethanol-precipitated and redissolved in TE buffer.

Primers to be used in RPE were 5'-end-labeled using T4 PNK (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. The labeling reactions consisted of 240 pmol of primer,  $1\times$  reaction buffer (NEB), 25 units of T4 PNK, and 10  $\mu\text{l}$  of [ $\gamma\text{-}^{32}\text{P}$ ]dATP (3000 Ci/mmol, 10.0 mCi/ml; New England Nuclear, Boston, MA) in a total volume of 50  $\mu\text{l}$ . After a 1-hr incubation at  $37^\circ$ , the unincorporated radiolabel was removed using Sephadex G25 Quick Spin Columns (Boehringer-Mannheim, Indianapolis, IN) equilibrated with TE buffer. The primer used in this study was ORI-U, a 21-mer (5'-TTTCTTCTTCATCTCCTCTT-3') complementary to SV40 bottom strand at positions 5010–5030.

RPE reactions for thermal cycling were set up using PCR Core Reagents (Perkin Elmer, Norwalk, CT) and typically consisted of  $1\times$  buffer II, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM concentration of each dNTP, 1 unit of *Taq* polymerase/20  $\mu\text{l}$ , 1.25  $\mu\text{M}$  concentration of end-labeled primer (estimated assuming 100% recovery from the Sephadex G25 column), and 0.1  $\mu\text{g}$  of template DNA. Thermal cycling was performed in a model 9600 Thermal Cycler (Perkin-Elmer). The following conditions were used:  $95^\circ$  for 30 sec followed by 30 cycles of  $35^\circ$  for 20 sec,  $72^\circ$  for 25 sec, and  $94^\circ$  for 15 sec. The final cycle was followed by an extra 6 min of extension at  $72^\circ$ . Equal volume aliquots of each reaction (usually 2  $\mu\text{l}$ ) were analyzed on 8% polyacrylamide/urea sequencing gels along with sequencing reactions, performed with the same primer as in the RPE reactions, using Sequenase v.2 (USA Biochemicals, Cleveland, OH) and [ $^{35}\text{S}$ ]dATP (1000 Ci/mmol, 12.5 mCi/ml; New England Nuclear). After electrophoresis, the gels were dried and subjected to autoradiography. Autoradiographs were scanned on a Molecular Dynamics (Sunnyvale, CA) laser densitometer. Alternatively, dried gels were exposed to phosphor screens and analyzed on a Molecular Dynamics PhosphorImager. The images were processed using Image Quant software (Molecular Dynamics). For representative oxaliplatin and cisplatin lanes, the intensity of each drug-induced band in the position range of 5083–5174 was quantified using Image Quant software. Weak sites (<1.5% total signal intensity) were filtered out, and the intensity of each remaining band was expressed as a percentage of the sum of intensities of all the bands scored for the entire region (Fig. 2C).

**Interstrand DNA cross-links.** A singly end-radiolabeled fragment of SV40 DNA (MAR, 508 bp from positions 3943–4451) was synthesized by PCR as described previously (Woynarowski *et al.*, 1995). Another fragment, ORI (positions 5010–1116), was synthesized in a similar way except that different primers (high performance liquid chromatography purified; Genosys, The Woodlands, TX) were used:  $^{32}\text{P}$ -end-labeled ORI-U (see RPE assay) and unlabeled ORI-L (21-mer, 5'-TCAGCAACCATAGTCCCGCCC-3'). The following thermal cycling conditions were used:  $95^\circ$  for 30 sec followed by 30 cycles of  $45^\circ$  for 20 sec,  $72^\circ$  for 25 sec, and  $94^\circ$  for 15 sec. The final cycle was followed by an extra 6 min of extension at  $72^\circ$ .  $^{32}\text{P}$ -End-radiolabeled PCR product was purified on a Sepharose CL-6B Quick Spin column (Boehringer-Mannheim).

Aliquots of ~2  $\mu\text{g}$  of end-labeled fragment DNA ( $6 \times 10^4$  cpm) were treated with oxaliplatin or cisplatin in 40  $\mu\text{l}$  for 4 hr at  $37^\circ$ . DNA was precipitated with ethanol to remove unreacted drug, redissolved in TE buffer, and analyzed by alkaline agarose electrophoresis (Woynarowski *et al.*, 1995). Cross-link frequencies were estimated based on the fraction of cross-linked DNA in drug-treated samples using a Poisson distribution formula (Woynarowski *et al.*, 1995).

**PCR stop assay.** A2780 cells were prelabeled by overnight incubation in a medium containing 0.05–0.1  $\mu\text{Ci/ml}$  [ $^{14}\text{C}$ ]thymidine (56.5 mCi/mmol, New England Nuclear). The cells were additionally incubated in radiolabel-free medium for 1 hr at  $37^\circ$  and then, with drugs, for 4 hr. After drug treatment, cells were rinsed with PBS, scraped, and resuspended in PBS. Cellular DNA was extracted and partially purified using the PureGene kit (Gentra Systems, Minneapolis, MN)

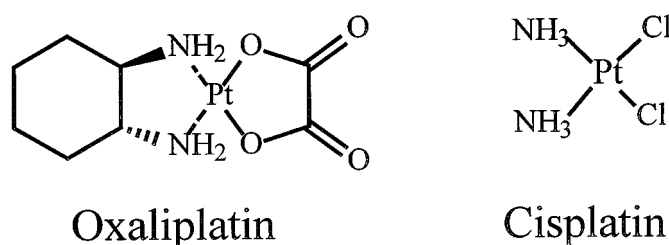


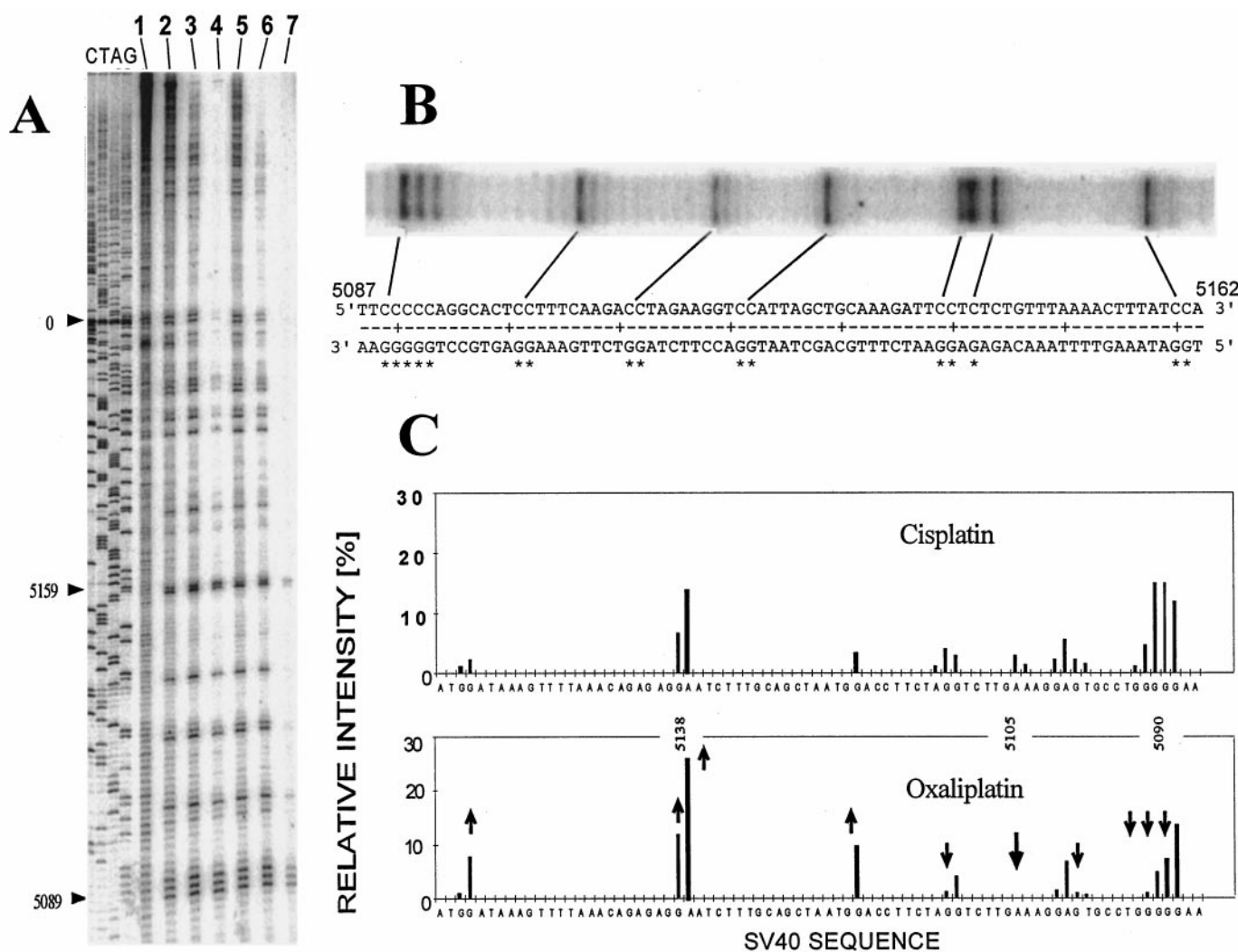
Fig. 1. The structures of oxaliplatin and cisplatin.

according to the manufacturer's protocol. Free drug was assumed to be removed in the course of DNA purification. DNA samples were stored at  $-20^{\circ}$  and used for the PCR determinations in the course of 2–3 weeks. Because the stop assay does not distinguish among different types of lesions as long as they inhibit primer extension, no further attempt was made to prevent the second-arm reaction of platinum monoadducts after DNA isolation.

The PCR stop assay was performed similarly to the previously described procedure (Woynarowski *et al.*, 1997). The concentrations of purified DNA were expressed as "cell equivalents" based on total  $^{14}\text{C}$  radioactivity/ $10^6$  cells determined separately by liquid scintillation counting. PCR reactions (20  $\mu\text{l}$ ) usually were carried out in triplicate at two or three different levels of template DNA (1000–5000 and 100–1000 cell equivalents per reaction for nuclear and mitochondrial regions, respectively). The reactions were set up using PCR Core Reagents (Perkin Elmer, Branchburg, NJ) and typically

consisted of  $1\times$  buffer II, 1.5 mM  $\text{MgCl}_2$ , and 0.2 mM concentration of each dNTP, except for dGTP (0.05 mM), 1 unit of *Taq* polymerase, and 0.04  $\mu\text{Ci}$ /reaction of  $^{32}\text{P}$ dGTP (New England Nuclear). The reactions were subjected to temperature cycling as described below using a Perkin-Elmer model 9600 cycler.

The following primer systems and cycling conditions were used. For a 536-bp segment of the  $\beta$ -globin gene (Daoud *et al.*, 1995), PCR was carried out using 5'-GGTTGGCCAATCTACTCCCAGG-3' and 5'-GCTCACTCAGTGTGGCAAAG-3' as the upper and lower primer, respectively. Initial denaturation at  $95^{\circ}$  for 30 sec was followed by 30 cycles of  $95^{\circ}$  for 20 sec,  $55^{\circ}$  for 20 sec, and  $72^{\circ}$  for 30 sec. For a 2.7-kbp region in the *HPRT* locus (Oshita and Saijo, 1994; Oshita *et al.*, 1995), PCR was carried out using 5'-TGGGATTACACGTGTGAAC-CAACC-3' and 5'-TGTGACACAGGCAGACTGTGGATC-3' as the upper and lower primer, respectively, with initial denaturation at  $95^{\circ}$  for 30 sec followed by 27 cycles of  $95^{\circ}$  for 15 sec,  $60^{\circ}$  for 60 sec, and



**Fig. 2.** Sites of oxaliplatin and cisplatin adducts in purified SV40 DNA determined by RPE using a primer (ORI-U, 21-mer, 5'-TTTTTCTTCATCTC-CTCCTT-3'), complementary to SV40 bottom strand at positions 5010–5030. **A**, A representative sequencing gel showing the products of RPE using SV40 treated for 4 hr with no drug, control DNA (lane 1), 2  $\mu\text{M}$  oxaliplatin (lane 2), 8  $\mu\text{M}$  oxaliplatin (lane 3), 32  $\mu\text{M}$  oxaliplatin (lane 4), 0.5  $\mu\text{M}$  cisplatin (lane 5), 2  $\mu\text{M}$  cisplatin (lane 6), and 8  $\mu\text{M}$  cisplatin (lane 7). The bands reflect primer extensions prematurely terminated opposite the adducts in the template strand. These extension blocks at drug-adducted sites gradually prevent the formation of long products (top of the gel) and random weak background stop sites seen in control samples and at lower drug levels. CTAG, respective DNA bases of the template strand in sequencing reactions (Sequenase version 2) using the same primer as for the extension reactions. Arrows, positions 0, 5089, and 5159 in SV40 sequence. **B**, Enlarged sequence, positions 5089–5189. Enlargement of a lane for oxaliplatin at 2  $\mu\text{M}$  (from a different RPE reaction than **A**) along with the sequence of the respective section of SV40 DNA. \*, Guanine residues in the bottom (3'→5') template strand that are likely to be adducted. **C**, Normalized relative signal intensity for oxaliplatin and cisplatin adducts (based on densitometric quantification of the gels as that in **A**) versus DNA sequence (positions 5083–5174, bottom strand in the opposite orientation, 5'→3', to that in **B**). Weak sites (below 1.5% total signal intensity) have been filtered out. Up and down arrows, oxaliplatin sites whose relative intensities are increased or decreased compared with cisplatin, respectively.



72° for 60 sec. For a 743-bp region in the *c-myc* ORI domain, 5'-GCCGTTTATAGGGTTTGTG-3' and 5'-CAAAAAACATTCTTCTCATCC-3' were used as the upper and lower primer, respectively. PCR was carried out with initial denaturation at 95° for 30 sec followed by 26 cycles of 94° for 15 sec, 55° for 20 sec, and 72° for 30 sec. For a 1100-bp region (Mito) in the mtDNA (Daoud *et al.*, 1995), oligonucleotides 5'-CCACATCACTCGAGACGTAA-3' and 5'-GCG-GTTGTTGATGGGTGAGT-3' were used as the upper and lower primer, respectively, and initial denaturation at 95° for 30 sec was followed by 18 cycles of 94° for 15 sec, 55° for 30 sec, and 72° for 25 sec. For all the systems, terminal extension was performed at 72° for 3–5 min.

The above conditions resulted in a linear response of signal intensity in terms of both the amount of template DNA and increasing number of cycles (data not shown). After electrophoresis in 1% agarose and autoradiography, signal intensities were quantified in a Molecular Dynamics densitometer. Results are normalized to the signal intensity in control samples and averaged for replicates and the different levels of template DNA.

**Estimation of lesion frequency.** Amplification inhibition data were converted to lesion frequency using a Poisson distribution formula (Kalinowski *et al.*, 1992) on the assumption that one lesion in the target area was sufficient to eliminate the amplification of that template. The frequency data are expressed as lesions/1 kbp:  $f = [-\ln(F_a)]/L$ , where  $f$  is the number of breaks per bp,  $F_a$  represents the fraction of remaining amplification (relative to control) in drug-treated samples, and  $L$  is the length of target DNA region in bp.

**Analysis of drug-binding motifs.** The numbers of binding motifs in each region were obtained by analyzing DNA sequences for these regions with a software tool in Oligo (NBI) program originally designed for restriction site searches. The motif input data included common motifs for platinum drugs such as GG, GNG, GC, and CG. These motifs are consistent with our data for cisplatin- and oxaliplatin-binding sites (see Results; Table 1). The number of binding motifs found in each region analyzed were normalized to 1 kbp to compensate for the differences in the lengths of these regions.

TABLE 1

Sites of oxaliplatin adducts in purified SV40 ORI region mapped by repetitive primer extension with ORI-U primer

SV40 DNA was treated with drugs for 4 hr and analyzed as described in the text and the legend to Fig. 2. The table is a composite of several independent experiments of stop sites induced by oxaliplatin in the SV40 ORI region. The sequences are for the bottom (template) strand of SV40 DNA arranged 5' to 3'. Absolute position in the SV40 sequence (leftmost column) refers to the stop site residue (relative position 0). Although oxaliplatin adducts may be 1–2 bp from the stop site, relative position +1 is most likely to correspond to the adducted base. A qualitative determination of intensity of the individual stop sites; S, strong, I, intermediate, was made. Some weak sites (<~1% intensity) are omitted from the table.

Stop site (absolute position in SV40 sequence)	Position relative to stop site									Intensity
	5'	+3	+2	+1	0	-1	-2	-3	3'	
5066		a	g	G	C	a	t	a		S
5071		g	a	G	A	a	a	g		S
5072		t	g	A	G	a	a	a		I
5089		g	g	G	G	a	a	t		S
5090		g	g	G	G	g	a	a		S
5091		t	g	G	G	g	g	a		S
5100		a	g	G	A	g	t	g		S
5110		a	g	G	T	c	t	t		S
5111		t	a	G	G	t	c	t		I
5120		t	g	G	A	c	c	t		I
5138		a	g	G	A	a	t	c		S
5139		g	a	G	G	a	a	t		S
5141		g	a	G	A	g	g	a		I
5154		a	a	G	T	t	t	t		I
5159		t	g	G	A	t	a	a		I
5160		a	t	G	G	a	t	a		S
5161		g	a	T	G	g	a	t		I

## Results

### Lesions in Naked DNA

**Localization of oxaliplatin adducts in a selected region of purified SV40 DNA.** To map the sites of oxaliplatin versus cisplatin adducts, the technique of RPE was used. In an RPE assay, lesions on the template strand result in the premature termination of nascent chain elongation opposite an adducted site (Jennerwein and Eastman, 1991; Bubley *et al.*, 1994). Cisplatin has been shown previously to induce such premature termination sites in naked and intracellular DNA (Hemminki and Thilly, 1988; Jennerwein and Eastman, 1991; Bubley *et al.*, 1994).

Fig. 2A shows an example of the RPE assay with oxaliplatin- and cisplatin-treated SV40 DNA using a primer for a region of SV40 sequence that includes the SV40 origin of replication. Intense, clustered bands at the top of the gel correspond to long products that prevail in control samples (Fig. 2A, lane 1). Treatment of SV40 DNA with either oxaliplatin (Fig. 2A, lanes 2–4) or cisplatin (Fig. 2A, lanes 5–7) decreases the amount of run-off products while generating new, shorter prematurely terminated products. These new bands reflect nascent strands terminated opposite an adduct in the template strand. Sequencing reactions run as parallel samples enabled the localization of these stop sites with the resolution of 1–2 bp.

Drug-induced bands must be distinguished from some prematurely terminated extensions visible in the absence of drug. For example, the altered secondary structure of SV40 DNA near position 0 (a 27-bp palindrome) produces premature termination in the RPE assay as well as in dideoxysequencing reactions (Fig. 2A, \*). Also, the control lane for RPE shows a background noise of 1-bp ladder that most likely reflects nonspecifically nicked SV40 DNA. Chain extension blocked by drug adducts progressively eliminates these background stop sites except for the shortest products.

Fig. 2B details oxaliplatin stop sites on the enlarged gel fragment along with the DNA sequence. Most of the oxaliplatin stop sites are in the runs of guanines, suggesting GG intrastrand cross-linking as the prevailing lesion. For example, the sequence GGGGG at position 5089 shows three strong bands. Also, oxaliplatin seems to modify five of five GG doublets present in the DNA segment shown. Table 1 lists the main sites consistently identified in several independent experiments. These sites are consistent with the motifs (GG or GAG) found previously for cisplatin (Hemminki and Thilly, 1988; Hoffmann *et al.*, 1989) and for DACH-Pt-SO<sub>4</sub> and DACH-Pt-(NO<sub>2</sub>)<sub>2</sub> (Jennerwein *et al.*, 1989; Boudny *et al.*, 1992). The GAG sites might reflect an intrastrand cross-link between both guanines or between adenine and guanine. Consistent with the induction of intrastrand cross-links, oxaliplatin produced unwinding of supercoiled (form I) SV40 DNA (data not shown). This unwinding effect, although significant, was less profound than the unwinding by equimolar cisplatin.

Side-by-side comparison confirmed that oxaliplatin forms adducts at the same sites as cisplatin. Relative intensities of these sites, however, differed somewhat (Fig. 2C). Some sites seem to be slightly enhanced for oxaliplatin, such as two sites at positions 5120 and 5160–61, which contain a sequence 5'-ATGGA-3', and a doublet at 5138–5139 with an AGGA motif (Table 1 and Fig. 2C). Some cisplatin sites (e.g., posi-

tion 5105) seem to be absent from oxaliplatin-treated samples, whereas others show decreased intensity (e.g., sites at 5090–5092).

Although the RPE experiments were not intended to quantify DNA adducts, the results suggested a lower level of oxaliplatin adducts compared with cisplatin. The longest nascent fragments (Fig. 2A, top) are nearly eliminated by 8  $\mu\text{M}$  cisplatin (Fig. 2A, lane 8) but not by oxaliplatin used at the same concentration (Fig. 2A, lane 3). As much as 32  $\mu\text{M}$  oxaliplatin (Fig. 2A, lane 4) was needed to profoundly reduce this signal.

The RPE experiments are consistent with intrastrand cross-links as the primary lesion, although RPE might not detect less frequent interstrand cross-linking. Interstrand cross-links spanning two Gs in the complementary strands, in addition to intrastrand adducts, cannot be ruled out at some sites (such as the strong GGC site at position 5066, Table 1), although several other GC sites showed no indications of adduction.

**Interstrand cross-links in SV40 fragments.** Interstrand cross-linking by DACH compounds other than oxaliplatin was proposed based on the detection of (dG)<sub>2</sub> products in digests from drug-treated DNA (Jennerwein *et al.*, 1989; Boudny *et al.*, 1992). However, no data were available on interstrand cross-links by oxaliplatin, and no direct detection of cross-linked DNA strands was reported thus far for any DACH compound. Given the potential biological significance of interstrand cross-links, the ability of oxaliplatin to form this lesion was directly examined. The induction of interstrand cross-links was assessed in two distinct fragments of SV40 DNA (MAR, positions 3943–4451, and ORI, positions 5010–1116). These fragments, synthesized by PCR with [<sup>32</sup>P] label on a single 5'-end, were treated with drugs and next analyzed by alkaline agarose gel electrophoresis.

Fig. 3 shows the migration of SV40 MAR DNA under strand-separating (alkaline) conditions. Untreated MAR fragments migrated a distance corresponding to the expected

length of 508 nucleotides. Interstrand cross-links present in these fragments would prevent strand separation. Thus, cross-linked DNA would migrate at a position of approximately twice the size of control strands. Both cisplatin and oxaliplatin treatment resulted in the generation of such an additional band, although the majority of DNA remained un-cross-linked.

The proportion of cross-linked DNA generated by oxaliplatin was significantly lower than that of cisplatin. Oxaliplatin-induced cross-links also were observed in the ORI fragment but with even lower yields than in the MAR fragment (data not shown). Cross-links in MAR and ORI at 15  $\mu\text{M}$  cisplatin corresponded to  $\sim 0.8$  and  $\sim 0.16$  cross-links/kbp, respectively. Oxaliplatin-induced significantly lower levels of interstrand cross-links, although the very low signal did not allow us to estimate their frequencies. In part, this very low cross-linking after a relatively short incubation (4-hr) may reflect generally slower kinetics of adduct formation by oxaliplatin. However, oxaliplatin affected SV40 DNA markedly less than cisplatin even after prolonged incubation (up to 48 hr), as judged by less profound DNA unwinding effect (data not shown).

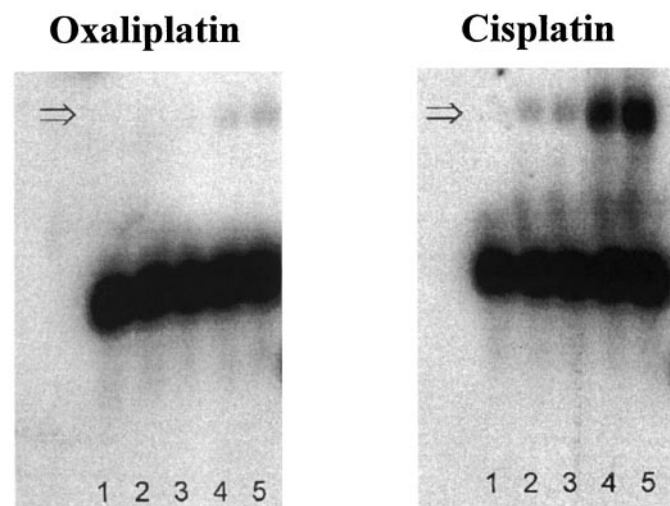
#### Region-Specificity of Lesions in Intracellular DNA in A2780 cells

Despite a similar pattern of lesion sites in naked SV40 DNA, oxaliplatin and cisplatin might differ in the distribution of drug adducts among various regions of cellular DNA (region-specificity). Thus, the PCR stop assay was used to compare the vulnerability of defined regions in cellular DNA to the actions of oxaliplatin and cisplatin. Like the RPE assay, the PCR stop assay capitalizes on the inability of *Taq* polymerase to bypass platinum adducts on DNA, which results in reduced amplification of a target region. The PCR stop assay is expected to reflect mainly intrastrand cross-links, which represent the majority of drug adducts for cisplatin (Plooy *et al.*, 1985; Takahara *et al.*, 1995; Saris *et al.*, 1996) and probably for oxaliplatin as well, as suggested by the RPE experiments.

**Lesions in nuclear versus mtDNA.** Drug effects on cellular DNA were analyzed using PCR systems for three different nuclear regions (a 740-bp ORI domain of the *c-myc* oncogene, a 530-bp domain in the  $\beta$ -globin gene, and a 2.7-kbp region of the *HPRT* locus) and for a system for mtDNA that was used previously for cisplatin (Daoud *et al.*, 1995). The selection of PCR systems was arbitrary and does not imply that any of these regions is a specific target for platinum drugs.

Fig. 4A illustrates a typical setup for the PCR stop assay in the *c-myc*-ORI region with three different amounts of template DNA (expressed as cell equivalents; see Experimental Procedures for details). Both cisplatin and oxaliplatin inhibit the amplification of this region. At 200  $\mu\text{M}$  cisplatin, inhibition of the amplification is nearly complete. For 200  $\mu\text{M}$  oxaliplatin, inhibition of amplification is distinct but incomplete. The differences between cisplatin and oxaliplatin are diminished at 100  $\mu\text{M}$  drug concentrations.

To compensate for significant experimental fluctuations in the PCR stop assay, the results of multiple determinations with different amounts of DNA template were averaged and expressed as relative signal amplification normalized to controls (Fig. 4B). The averaged results show that treatments



**Fig. 3.** Interstrand cross-links in an end-labeled fragment (MAR) of SV40 genome incubated with oxaliplatin (left) and cisplatin (right) for 4 hr at 37°. Arrows, positions of cross-linked DNA species that migrate slower than un-cross-linked molecules in alkaline agarose gel. Drug concentrations were 0  $\mu\text{M}$  (lanes 1), 0.5  $\mu\text{M}$  (lanes 2), 2.5  $\mu\text{M}$  (lanes 3), 7.5  $\mu\text{M}$  (lanes 4), and 15  $\mu\text{M}$  (lanes 5). The figure shows representative gels from at least two separate determinations.

with drugs at 100  $\mu\text{M}$  resulted in marginal-to-modest inhibitions of signal amplification in all four PCR systems. Although the differences between cisplatin and oxaliplatin at 100  $\mu\text{M}$  were small, this drug level seemed to be below the linear range of the assay, at least for oxaliplatin. Therefore, higher drug levels (200  $\mu\text{M}$ ) that showed a progressive inhibition of signal intensity for both drugs may be more suitable for comparing cisplatin and oxaliplatin.

At 200  $\mu\text{M}$ , oxaliplatin consistently caused less inhibition of signal amplification than cisplatin in all four regions examined. The results for cisplatin at 200  $\mu\text{M}$  are consistent with previously published data for  $\beta$ -globin and mtDNA (Daoud *et al.*, 1995) and for HPRT systems (Oshita and Saijo, 1994) in cisplatin-sensitive cell lines, specifically human ovarian carcinoma CaOv-3 ( $\beta$ -globin and mtDNA) and human lung can-

cer PC9 (HPRT). The relative sensitivity of the HPRT system is consistent with its much greater length (2.7 kbp) compared with other regions.

Table 2 shows the estimated frequencies of drug-induced lesions after data conversion to lesions/10<sup>6</sup> bp (Mbp) assuming the Poisson formula. Compared with cisplatin, oxaliplatin induced roughly two to four times fewer lesions in nuclear domains and nearly seven times fewer lesions in mtDNA. Thus, we conclude that oxaliplatin is less reactive than cisplatin with cellular DNA under these conditions. mtDNA seems to be less vulnerable to both oxaliplatin and cisplatin than nuclear DNA. This observation confirms the previously observed preference of cisplatin for nuclear DNA (Daoud *et al.*, 1995).

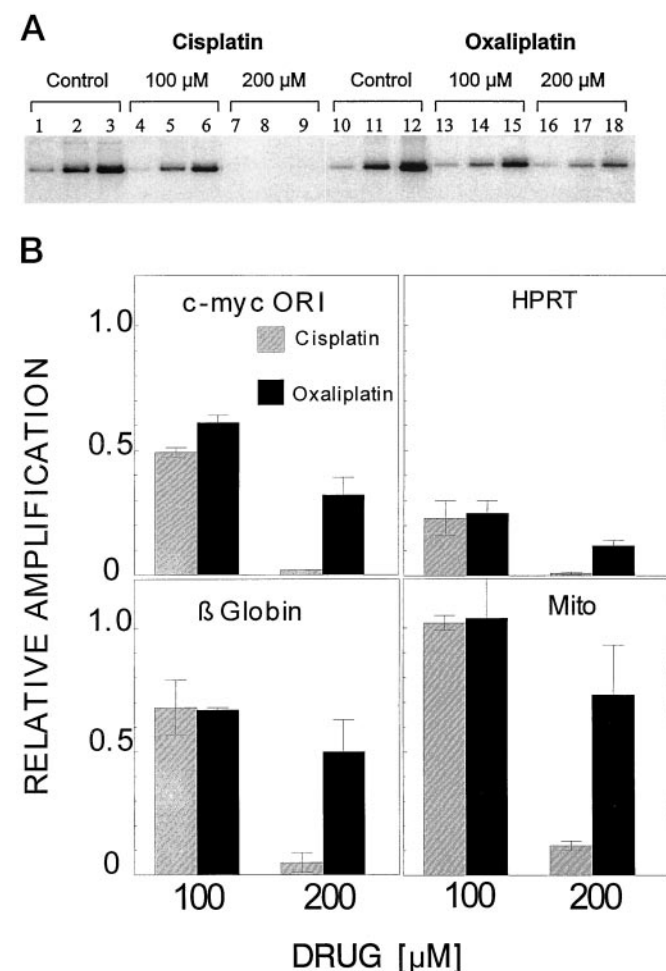
**Region-specific DNA lesions versus drug-binding motifs.** Having estimated oxaliplatin and cisplatin lesions in various regions, we next asked whether the relative vulnerability of these regions is related to the occurrence of known drug-binding sites. The sequences of DNA regions used in the PCR stop assay have been analyzed for the presence of binding motifs consistent with the RPE data (GG, GNG) and interstrand cross-linking (GC). Estimated lesions frequencies were plotted against the density of the drug-binding motifs (Fig. 5). For cisplatin, this analysis showed that more lesions were formed in those regions that are richer in drug-binding motifs. A similar trend was seen for oxaliplatin, although the lower frequency of oxaliplatin lesion makes this trend less pronounced than for cisplatin. For both drugs, mtDNA was clearly the exception from the trend confirming that mtDNA is a disfavored target.

### Cytotoxic Activity of Oxaliplatin and Cisplatin

Drug cytotoxic activity was evaluated using the standard MTT assay. In BSC-1 cells, the resulting drug concentrations that produced 50% growth inhibition (IC<sub>50</sub>, mean  $\pm$  standard error) were  $1.05 \pm 0.06$  and  $4.8 \pm 1.2$   $\mu\text{M}$  for oxaliplatin and cisplatin, respectively. In A2780 cells, respective IC<sub>50</sub> values were 0.56 and 2.3  $\mu\text{M}$ . These results demonstrate that oxaliplatin is approximately four times more cytotoxic than cisplatin in both cell lines.

### Discussion

Our study characterized the sequence- and region-specificity of oxaliplatin adducts with naked and intracellular DNA,



**Fig. 4.** Region-specific lesions in nuclear and mtDNA by PCR stop assay using DNA from drug-treated A2780 cells. A, Representative examples of PCR analysis for a *c-myc* ORI region. DNA preparations isolated from control A2780 cells (lanes 1–3 and 10–12) and cells treated with drugs for 4 hr (lanes 4–9 and 13–18) were subjected to amplification with [<sup>32</sup>P]dGTP, followed by agarose gel electrophoresis and autoradiography. The drug treatments were 100  $\mu\text{M}$  cisplatin (lanes 4–6), 200  $\mu\text{M}$  cisplatin (lanes 7–9), 100  $\mu\text{M}$  oxaliplatin (lanes 13–15), and 200  $\mu\text{M}$  oxaliplatin (lanes 16–18). Three different amounts of template DNA were used per each treatment condition; equivalent to 500 cells (lanes 1, 4, 7, 10, 13, and 16), 2500 cells (lanes 2, 5, 8, 11, 14, and 17), and 5000 cells (lanes 3, 6, 9, 12, 15, and 18). B, Quantification of PCR reactions for nuclear DNA (*c-myc*,  $\beta$ -Globin, HPRT) and mtDNA (Mito). The ordinate shows the relative amplification (control = 1) averaged for all the replicates and different amounts of template DNA using data from two independent experiments (mean  $\pm$  standard error).

TABLE 2

Estimation of region-specific lesions in nuclear and mitochondrial DNA by PCR stop assay after a 4-hr treatment of A2780 cells with cisplatin or oxaliplatin

Signal intensities shown in Fig. 4 were converted to lesion frequencies assuming a Poisson distribution as described in Experimental Procedures.

Drug	Region	Lesion frequency <sup>a</sup>			
		Nuclear DNA			Mt-DNA Mito
		c-myc-ORI	β-Globin	HPRT	
	μM	lesions / 10 <sup>3</sup> bp			
Cisplatin	100	1.0	0.7	0.5	0
	200	5.5	5.4	1.7	1.9
Oxaliplatin	100	0.7	0.7	0.5	0
	200	1.5	1.3	0.8	0.3
Cisplatin/ oxaliplatin ratio		3.7	4.2	2.1	6.7

<sup>a</sup> Values rounded to the nearest 0.1 lesion/kbp.



respectively. The localization of these adducts resembles that of cisplatin/DNA adducts. Analysis at the nucleotide level by RPE technique indicated that oxaliplatin binds mainly to guanines with a preference for guanine clusters. Although the 1–2-bp resolution of the RPE technique does not allow us unequivocally to localize the modified nucleotide residues, our data are consistent with GG and GAG (or GNG) intra-strand cross-links as a prevailing lesion with naked DNA. Despite lower overall reactivity, oxaliplatin forms lesions at similar sequences as does cisplatin. Not only does oxaliplatin share with cisplatin the propensity for adduct formation in G clusters in purified DNA, but also the relative intensities of adduction at the major sites, although not identical, are generally similar. In the context of previous data on types of DNA adducts for other DACH-Pt compounds (Jennerwein *et al.*, 1989; Boudny *et al.*, 1992), oxaliplatin preference for G clusters is not particularly surprising, yet, our results provide the first data on the sequence localization of DACH-Pt DNA adducts. These findings clearly demonstrate that the presence of the bulky and hydrophobic DACH moiety and oxalate leaving group does not significantly influence the sequence specificity of oxaliplatin interaction with DNA.

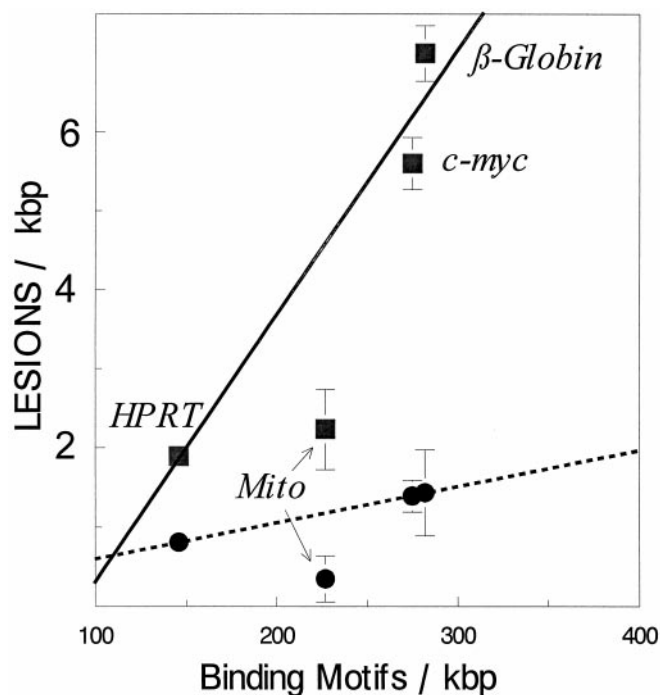
Besides intrastrand cross-links, oxaliplatin forms other types of DNA lesions. The formation of low levels of inter-strand cross-links by oxaliplatin is demonstrated by alkaline electrophoresis of oxaliplatin-treated SV40 fragments. This result is the first direct evidence for cross-linking of complementary strands by oxaliplatin (although indirect evidence has been reported for other DACH compounds; Jennerwein *et al.*,

1989; Boudny *et al.*, 1992). Interstrand cross-links seem too infrequent to significantly contribute to the RPE results. Still, this potentially lethal lesion may contribute to the biological properties of the drug. We recently confirmed inter-strand cross-linking by oxaliplatin in intact cells or isolated nuclei. In these systems, we also found that oxaliplatin can induce DNA-protein cross-links (Woynarowski JM, Faivre S, Chapman WG, unpublished observations).

It could not be ruled out that altered hydrophobic properties might direct oxaliplatin to different cellular compartments than cisplatin. Likewise, similar adduct sites at the nucleotide level did not preclude the possibility that oxaliplatin and cisplatin might target different domains of nuclear DNA. However, the distribution of oxaliplatin adducts among the various regions is similar to that for cisplatin adducts. The lesions in cellular DNA seem to follow the relative density of drug-binding motifs in the regions examined (except for the underprivileged mtDNA; see later). This trend suggests that even low sequence specificity of platinum drugs may determine the relative vulnerability of various genomic loci to drug adduction. Thus, sequence analysis may predict region-specificity for platinum drugs. This novel finding also is potentially significant for the efforts to design drugs targeted at specific domains of genomic DNA.

The significance of targeting mtDNA by platinum drugs remains controversial. Cisplatin has been reported to prefer nuclear DNA over mtDNA (Daoud *et al.*, 1995). However, a preference for mtDNA also has been postulated (Murata *et al.*, 1990; Olivero *et al.*, 1995, 1997), and certain cisplatin lesions were suggested to be inefficiently repaired in mtDNA (LeDoux *et al.*, 1992). Given that no data were available for oxaliplatin, it was important to determine whether the drug can target mtDNA. Our results indicate that oxaliplatin forms lesions in mtDNA. However, unless drug adducts in the examined 1.1-kbp region of mtDNA are not representative for the adducts in the entire ~16-kbp mtDNA, nuclear DNA, not mtDNA, seems to be the main target for both drugs.

Collectively, our findings and other data emphasize the fact that despite the obvious similarities in the pattern of DNA adduction, significant mechanistic differences exist between oxaliplatin and cisplatin. Although the primary focus of this study was on lesion localization with only a semiquantitative estimation of lesion frequencies, the results suggest that oxaliplatin is less reactive toward both naked and intracellular DNA than cisplatin. In contrast, oxaliplatin is more cytotoxic in the A2780 cells used in our studies and in numerous other cell lines (Tashiro *et al.*, 1989; Pendyala and Creaven, 1993; Chaney, 1995). The lower reactivity of oxaliplatin than cisplatin with defined regions of cellular DNA is consistent with the results of a recent study (Saris *et al.*, 1996) that used two other methods to compare cisplatin and oxaliplatin binding to bulk DNA in A2780 cells: <sup>32</sup>P-postlabeling and total platination levels by atomic absorption. This paradoxical trend suggests that although DNA adducts generally are believed to be the main effect of platinum drugs, other effects may contribute to oxaliplatin antiproliferative properties. Likewise, based on differing profiles of activity against selected cell lines in the NCI screen (COMPARE program analysis), Rixe *et al.* (1996) concluded that oxaliplatin and cisplatin differ in their mechanisms of action, resistance, or both.



**Fig. 5.** Region-specific lesions versus drug binding motifs for cisplatin (■) and oxaliplatin (●). The values of lesion frequencies (for drugs at 200  $\mu$ M) are from Table 2. The numbers of binding motifs in each region were obtained by analyzing DNA sequences for these regions with a modified Oligo program. Values were normalized to 1 kbp to compensate for differences in the length of these sequences. Region-specific lesions induced by cisplatin and oxaliplatin seem to reflect the densities of drug-binding motifs in the respective regions of nuclear DNA (HPRT, *c-myc*, and  $\beta$ -Globin). Both drugs affect mtDNA (*Mito*) to a lesser extent than suggested by the number of binding motifs.

Oxaliplatin-induced lesions in cellular DNA could be more difficult to repair or could lead to different biological consequences. Our separate study, which characterized in-depth total platination, interstrand crosslinks, and DNA-protein cross-links, confirmed that oxaliplatin forms fewer primary lesions than equimolar cisplatin. However, at comparable levels of DNA adducts, oxaliplatin was approximately twice as effective as cisplatin in inducing apoptosis (Faivre and Woynarowski, 1998). Studies are under way to further explore the relative significance of DNA lesions and other effects to the proapoptotic and antiproliferative actions of oxaliplatin.

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