Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 225–237
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

DNA strand breaks and apoptosis induced by oxaliplatin in cancer cells☆

Sandrine Faivre[a,1], Denise Chan[a], Richard Salinas[b], Barbara Woynarowska[b], Jan M. Woynarowski[a,b,*]

[a]*Institute for Drug Development, Cancer Therapy and Research Center, San Antonio, TX, USA*
[b]*Department of Radiation Oncology, University of Texas Health Science Center, IDD Bldg., 14960 Omicron Drive, San Antonio, TX 78245-3217, USA*

Received 10 September 2002; accepted 20 December 2002

Abstract

Platinum anticancer drugs, such as cisplatin, are thought to exert their activity by DNA damage. Oxaliplatin, a clinically active diaminocyclohexane platinum compound, however, requires fewer DNA-Pt adducts than cisplatin to achieve cell growth inhibition. Here we investigated whether secondary DNA damage and apoptotic responses to oxaliplatin compensate for the reduced formation of DNA adducts. Oxaliplatin treatment of leukemic CEM and ovarian A2780 cancer cells resulted in early (4 hr) induction of DNA single-strand breaks measured by nucleoid sedimentation. These infrequent early lesions progress with time into massive double-stranded DNA fragmentation (fragments >50 kbp) paralleled by characteristic apoptotic changes revealed by cell morphology and multivariate flow cytometry. Profound oxaliplatin-induced apoptotic DNA fragmentation was detectable following a 24 hr treatment of A2780 and CEM cells with 2 and 10 μM oxaliplatin, respectively. This DNA fragmentation was inhibited completely by the broad-spectrum caspase inhibitor Z-VAD-fmk. Cisplatin, which forms markedly more DNA-Pt adducts in CEM and A2780 cells than equimolar oxaliplatin, was similarly potent as oxaliplatin in terms of early strand breaks and later apoptotic responses. Oxaliplatin was also profoundly apoptotic in several other tumor cell lines of prostate origin but had only a marginal effect in normal prostate PrEC cells. Collectively, the results demonstrate that, relative to the magnitude of the primary DNA-Pt lesions, oxaliplatin is disproportionately more potent than cisplatin in the induction of apoptosis. Apoptosis induction, possibly enhanced by a contribution of targets other than DNA, seems to be an important factor in the mechanism of action of oxaliplatin.

Keywords: Oxaliplatin; Cisplatin; DNA adducts; DNA strand breaks; Apoptosis; Caspases; Cancer cells

1. Introduction

Oxaliplatin, L-OHP, oxalatoplatinum, eloxatin (Fig. 1) is a third generation platinum antitumor analog in which 1,2-diaminocyclohexane (DACH) ligand substitutes for the amine groups of cisplatin (for review see [1,2]). Oxaliplatin has demonstrated a broad spectrum of activity in a wide range of human tumors *in vitro* and *in vivo*. Oxaliplatin also exhibits a safe toxicity profile and in various clinical situations is superior to cisplatin by being less toxic and retaining activity against cisplatin-resistant tumors [3–5]. Oxaliplatin is approved for use in the treatment of advanced colorectal cancer in the United States, Europe, Asia, and Latin America.

Oxaliplatin shares various mechanistic properties with the parent platinum drug, cisplatin. Both drugs react with the same GC-rich sites in naked DNA and similarly prefer

☆ Preliminary account of this study was presented in part at the 89th Annual Meeting of the American Association for Cancer Research, New Orleans, LA, March 28–April 1, 1998, Proceedings, p. 158, and 90th Annual Meeting of the American Association for Cancer Research, Philadelphia, PA, April 10–14, 1999, Proceedings, p. 488.

* Corresponding author. Address for correspondence: University of Texas Health Science Center. Tel.: +1-210-677-3832; fax: +1-210-677-0058.
E-mail address: jmw1@saci.org (J.M. Woynarowski).
[1] Present address: Departement de Medecine, Institut Gustave-Roussy, 39 rue Camille-Desmoulins, 94800 Villejuif, France.

Abbreviations: DACH, diaminocyclohexane; oxaliplatin, [oxalato-(*trans*-L-1,2-diaminocyclohexane)platinum(II)]; DSB, double-strand break; GI_{50}, drug concentration inhibiting net cell growth by 50%; GI_{80}, drug concentration inhibiting net cell growth by 80%; MMR, mismatch repair; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PCA, perchloric acid; SSB, single-strand break.

0006-2952/03/$ – see front matter © 2003 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00260-0

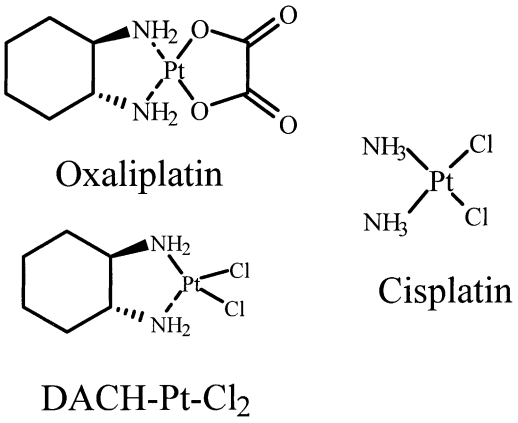

Fig. 1. The structures of oxaliplatin, cisplatin, and the reactive form of oxaliplatin, DACH-$PtCl_2$.

GC-enriched regions of cellular DNA [6]. Like cisplatin, oxaliplatin induces mainly intrastrand crosslinks but forms also interstrand crosslinks (ISC) and DNA-protein crosslinks (DPC) in cellular DNA [6,7]. All these oxaliplatin-induced DNA lesions are likely to play a role in cell growth inhibition. Oxaliplatin tends to be at least as cytotoxic as cisplatin but is often more active in various cisplatin-refractory cell lines [7–12].

Several studies repeatedly show that oxaliplatin is markedly less reactive with naked DNA [6,13] and forms fewer adducts with cellular DNA than equimolar cisplatin [6,7,12]. For example, our investigations demonstrated that total platination, ISC, and DPC induced by oxaliplatin in human leukemic CEM cells and ovarian carcinoma A2780 cells under various treatment schemes amount to approximately one-half of the respective lesions induced by equimolar cisplatin [7]. Yet, oxaliplatin is as potent as cisplatin in growth inhibition of these cell lines. Recent studies by Pendyala and coworkers using several models of cisplatin and oxaliplatin-sensitive and -resistant cell lines concluded that, compared to cisplatin, lower intracellular concentration and fewer DNA-Pt adducts are sufficient for oxaliplatin to exert its cytotoxicity [12].

It is not always apparent whether and how different responses to DNA adducts might compensate for the reduced initial platination levels by oxaliplatin. Although differential adduct repair plays a role in cellular responses to platinum drugs [14–17], oxaliplatin-induced DNA damage appears to be no more difficult to repair than cisplatin-induced damage in cellular systems [7,12] and in cell-free excision repair systems [18]. A potentially important difference between cisplatin and oxaliplatin is that only cisplatin-DNA adducts are recognized by MMR proteins, probably reflecting the different structure of the oxaliplatin adduct, in which the bulky and hydrophobic DACH ring protrudes directly outward into the major groove [19,20]. Cisplatin cytotoxicity is thought to be actually enhanced by the MMR system due to futile cycles of *trans*-lesion synthesis [21,22]. Loss of MMR that desensitizes cells to cisplatin but not to oxaliplatin, might thus explain the higher oxaliplatin cytotoxicity in some MMR-deficient cells, although cisplatin cytotoxicity showed no correlation with the levels of MMR proteins in the NCI panel of 60 cell lines [23]. The differences in MMR-proficient cells must, on the other hand, reflect other factors, possibly including a contribution of targets other than DNA.

For various anticancer drugs, regardless of their specific mechanism of action, diverse initial signals are integrated by apoptotic machinery, giving rise either to cell commitment to death or to cell survival. The higher cytotoxicity of oxaliplatin in various MMR-proficient cell systems might reflect death responses beyond the level anticipated when only the magnitude of DNA adducts is considered. Little is known, however, about the apoptotic potential of oxaliplatin and, especially, how it compares to the apoptotic potential of cisplatin.

In this study, we determined the timing of early cellular responses to oxaliplatin, including secondary DNA lesions, and their progression to massive apoptosis. Oxaliplatin and cisplatin were compared using two human neoplastic cell lines, leukemic CEM and ovarian carcinoma A2780, for which the levels of drug-induced lesions have been characterized previously [7]. The results demonstrate that oxaliplatin is disproportionately more potent than cisplatin, given the lower levels of DNA adducts, in inducing early secondary DNA strand breaks and massive apoptosis. These findings are further expanded by documenting that oxaliplatin promotes apoptosis in other cancer cell lines but not in normal cells.

2. Materials and methods

2.1. Drugs

Oxaliplatin and DACH-$PtCl_2$ were obtained from Sanofi-Synthelabo Research. Cisplatin was purchased from Sigma. Stock solutions of oxaliplatin and cisplatin were made in water and in saline, respectively, and stored at -20°.

2.2. Cell culture

Human cell lines, ovarian carcinoma A2780 (from Dr. S. Chaney, originally provided by Dr. T. Hamilton) and leukemia CEM (from Dr. W.T. Beck), were cultured as described previously [7] using RPMI 1640 (Gibco) and minimal essential medium Eagle, Joklik's modification (Sigma), respectively, each supplemented with 10% fetal bovine serum. Prostate cancer cells LNCaP-Pro5, LNCaP-LN3 (provided by Dr. C.A. Pettaway), PC-3 (from American Type Culture Collection, ATCC), and normal prostate PrEC cells having a finite life span (from Clonetics) were cultured as described elsewhere [24].

Table 1
Summary of cytotoxic activity and apoptotic potential of oxaliplatin in human cancer and normal cell lines

Cell line	Type/properties[a]	GI_{50} (μM)[b]	Apoptosis (%)[c]
CEM	ALL leukemia, mutated p53, MMR: possibly deficient [MLH1(−)]	1.19 ± 0.17	33 ± 4
A2780	Ovarian cancer, w.t. p53, MMR: competent	0.21 ± 0.04	31 ± 5
LNCaP-Pro5	Prostate cancer, androgen responsive nonmetastatic, w.t. p53, Bcl-2(+)	0.16 ± 0.02	25 ± 3
LNCaP-LN3	Prostate cancer, androgen responsive metastatic, w.t. p53, Bcl-2(++)	0.23 ± 0.04	13 ± 4
PC-3	Prostate cancer, androgen-independent p53-negative, Bcl-2(++), MMR: competent	2.6 ± 0.17	2.1 ± 0.6
PrEC	Normal prostate, w.t. p53, MMR: ? (presumably competent)	5.2 ± 0.2	1.2 ± 1.2

[a] Cell line characteristics based on [23,30–33]; w.t.: wild-type.
[b] Mean values (±SEM or 1/2 range for N = 2) determined by MTT assay after 72 hr incubation except for CEM cells in which cell counts after a 48 hr incubation were used (approximately 3× cell generation time). Data for CEM and A2780 cells were reported previously [7].
[c] Percentage of fragmented DNA after 24 hr continuous treatment with 25 μM drug (corrected for the levels of fragmentation in control cells, mean values ± SEM or 1/2 range for N = 2) based on quantitative DNA fragmentation assay shown in Figs. 3 and 7.

2.3. Cytotoxic activity

Growth inhibitory activities of oxaliplatin and cisplatin in CEM and A2780 cells, based on cell counting with an electronic counter following a 48 hr continuous incubation were reported previously [24]. Cytotoxic activities in other cell lines were determined by the standard microplate colorimetric assay that follows the accumulation of a formazan dye generated by the conversion of MTT by mitochondrial dehydrogenases in living cells as described elsewhere [24]. Briefly, exponentially growing cells were incubated with a series of drug concentrations for 72 hr (approximately 2–3× generation times). Following a 4 hr incubation with MTT, absorbance at 595 nm was measured on a BioRad model 3550 microplate reader. The MTT results given in Table 1 represent means ± SE from two independent experiments each carried out with six replicates/condition. All the cytotoxicity data are expressed as drug concentrations that inhibit net cell growth by 50% (GI_{50}) [24].

2.4. Nucleoid sedimentation

Nucleoids are residual nuclear structures, sometimes referred to as "halo" structures, in which partially deproteinized large DNA loops remain attached to the nuclear matrices maintaining their supercoiling status [25,26]. Relaxation of this supercoiling results in slower sedimentation of nucleoids. Since one SSB completely relaxes a given DNA loop, nucleoid sedimentation provides a sensitive method for SSB detection in intact cells [25]. Nucleoid sedimentation was analyzed essentially as described previously [27]. Briefly, cells were incubated with drugs, washed with PBS and loaded on the top of 15–30% linear sucrose gradients (10 mL) overlaid with a lysis layer (0.4 mL) containing 0.7% Triton X-100 (all in 1.9 M NaCl, 50 mM Tris–HCl, 20 mM EDTA, pH 8.0). After 30 min of lysis at 20°, the gradients were centrifuged in an SW 41 rotor (Beckman Instruments) at 17,500 rpm (52,400 *g*) for 120 min (CEM cells) or for 180 min (A2780 cells). Following gradient fractionation, the radioactivity of the collected fractions was determined in a scintillation counter. The results show either the sedimentation profiles or relative sedimentation (RS) normalized to the sedimentation of DNA from control cells.

The RS data are used to coarsely estimate the frequency of drug-induced strand breaks. In this normalization, RS = 1 reflects the original state of nucleoid supercoiling with no drug-induced SSB. Fully relaxed nucleoids migrate at a RS of 0.4–0.5 (RS_{REL}), as found with saturating concentrations of ethidium bromide (data not shown). Any given RS would thus reflect a remaining fraction of original supercoiling FS:

$$FS = \frac{RS - RS_{REL}}{1 - RS_{REL}} \quad (1)$$

Since one SSB per DNA loop is sufficient for its relaxation, the frequency of SSB/bp (f) is estimated using a Poisson formula:

$$f = \frac{-\ln(FS)}{L} \quad (2)$$

where L is the size of DNA loops, assumed typically to be 100 kbp [28].

2.5. Quantitative apoptotic DNA fragmentation assay

Unlike the nonquantitative laddering assay that follows only short oligonucleosomal fragments, the quantitative assay employed in this study measures both short as well as long (up to at least 150 kbp) DNA fragments based on their extractability from nuclear structures from drug-treated cells [27,29]. The assay was performed as described previously [24,27,29] with slight modifications. For suspension cells (CEM), cells were prelabeled overnight with 0.05 μCi/mL [^{14}C]-thymidine and resuspended in a fresh medium at 0.25×10^6 cells/mL for drug treatment as indicated. For monolayer cultures (A2780 and prostate cancer and normal cell lines), cells were plated (0.2×10^6 cells/35 mm dish) 1 day before treatment and prelabeled in medium with [^{14}C]-thymidine, treated with drugs as indicated, and trypsinized.

Further steps were common to suspension and monolayer cells. Aliquots of cell suspension were washed with PBS, resuspended in 300 μL hypotonic buffer (10 mM

Tris, pH 7.5, 10 mM EDTA, 0.1 mM PMSF, 0.2% Triton X-100), and incubated on ice for 1 hr. Permeabilized cells were centrifuged (10 min at 4° at 200 *g*). The supernatants (S1) were collected, the pellets (P1) were suspended in 300 μL of 2 mM EDTA and incubated for 2 hr on ice to extract fragmented DNA. Samples were centrifuged 5 min at 20,000 *g*, and the supernatants (S2) were collected. Remaining pellets (P2) containing unfragmented DNA were hydrolyzed in 300 μL 0.5 M PCA 30 min at 70°. Supernatants, hydrolyzed pellets, and the culture medium combined with PBS washes were counted on a microplate liquid scintillation counter (Top Count, Packard Instruments). The results are expressed as the percentage of the total DNA released (Eq. (3)):

$$\text{released fragments}\,(\%) = \frac{\text{cpm S1} + \text{cpm S2} + \text{cpm (medium)}}{\text{cpm S1} + \text{cpm S2} + \text{cpm P2} + \text{cpm (medium)}} \quad (3)$$

If DNA fragmentation determinations were carried out in the presence of caspase inhibitors Ac-DEVD-CHO and Z-VAD-fmk, the inhibitors were added to cell cultures at 100 μM 2 hr prior to drug addition and were constantly present during the incubation with oxaliplatin.

2.6. Determination of DNA fragment size distribution

Aliquots of the double-stranded DNA fragments generated by drug treatment and extracted as described above for the quantitative fragmentation assay were analyzed by field-inversion gel electrophoresis (FIGE) using a BioRad FIGE apparatus (BioRad Laboratories) and the manufacturer's program 4. The gel was stained with SyBr Green to visualize the high molecular weight markers (CHEF DNA size standards, 8–48 kbp, BioRad) and next dried and autoradiographed to detect ^{14}C-labeled DNA fragments.

2.7. Terminal transferase apoptotic flow cytometry assay, cell morphology and "viability"

Multiparametric flow cytometry assay used terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling and propidium iodide staining as a measure of DNA content. The assay was performed as exactly as described previously [24,27].

Cell morphology was examined under fluorescence microscope after staining with Hoechst 33258 and cell membrane integrity (cell "viability") was measured under visual microscopy based on trypan blue exclusion.

3. Results

3.1. Model cell lines and drug cytotoxic activities

In the main part of this study, we compared oxaliplatin and cisplatin for various effects pertinent to apoptosis induction, with the focus on drug-induced DNA fragmentation. These experiments were carried out in MMR-proficient human leukemic CEM and ovarian carcinoma A2780 cells, for which we have previously determined the levels of total DNA adducts and specific types of DNA lesions and their localization at the genomic level [6,7]. While A2780 cells are more sensitive than CEM cells, in both cell lines, oxaliplatin and cisplatin are similarly cytotoxic with the respective GI_{50} values of 1.19 ± 0.17 μM and 1.11 ± 0.05 μM in CEM cells and 0.21 ± 0.04 μM and 0.38 ± 0.16 μM in A2780 cells (Table 1) [7]. The slopes or the cytotoxicity curves were also similar for both drugs, for instance 80% inhibition of CEM cell growth required approximately 25 μM of either drug (data not shown).

Findings with CEM and A2780 cells were next expanded by establishing the ability of oxaliplatin to induce apoptosis in several human prostate cancer cell lines and in normal prostate cells. These cell lines ranged from highly sensitive, e.g. LNCaP-Pro5, to relatively resistant normal prostate cells PrEC (GI_{50} values of 0.16 ± 0.02 μM and 5.2 ± 0.2 μM, Table 1). These cell lines differed also in their p53 status and other properties (Table 1).

3.2. Early DNA single-strand breaks (SSB) by oxaliplatin and cisplatin

To quantitate the induction of SSB by cisplatin and oxaliplatin, the sensitive technique of nucleoid sedimentation was used. Unlike other methods involving alkaline conditions that would detect also alkali-labile sites, this method uses neutral conditions and assesses the formation of true SSB based on changes in DNA supercoiling status.

The decreased rate of nucleoid sedimentation produced by both oxaliplatin and cisplatin is an unequivocal indication of strand breaks. These changes are illustrated for oxaliplatin by shifts of peak positions of nucleoid sedimentation profiles from fraction 15 to 9 for 0 and 50 μM drug, respectively (Fig. 2A). The normalized sedimentation data (Fig. 2B) summarize the concentration- and time-dependence of these effects for oxaliplatin and cisplatin in CEM and A2780 cells. For instance, treatment of CEM cells for 4 hr with 50 μM oxaliplatin reduced nucleoid sedimentation to 75% of control rate (an estimated five SSB/10^6 bp). After a 24 hr exposure, 50 μM oxaliplatin further reduced nucleoid sedimentation (to ~50%, estimated 18 SSB/10^6 bp). Even oxaliplatin at low 2 μM level produced a noticeable change (Fig. 2B), indicating roughly one SSB every 250–500 kbp. The magnitude of oxaliplatin-induced strand breakage was higher in A2780 than in CEM cells. A 4 hr incubation of A2780 cells with oxaliplatin (Fig. 2B, bottom panel) produced a similar reduction of nucleoid sedimentation as a 24 hr incubation of CEM cells. A 6 hr treatment of A2780 cells produced an even greater effect (an estimated 10 SSB/10^6 bp at 5 μM oxaliplatin). Cisplatin treatment of both cell lines resulted in a similar pattern of decreased nucleoid sedimentation.

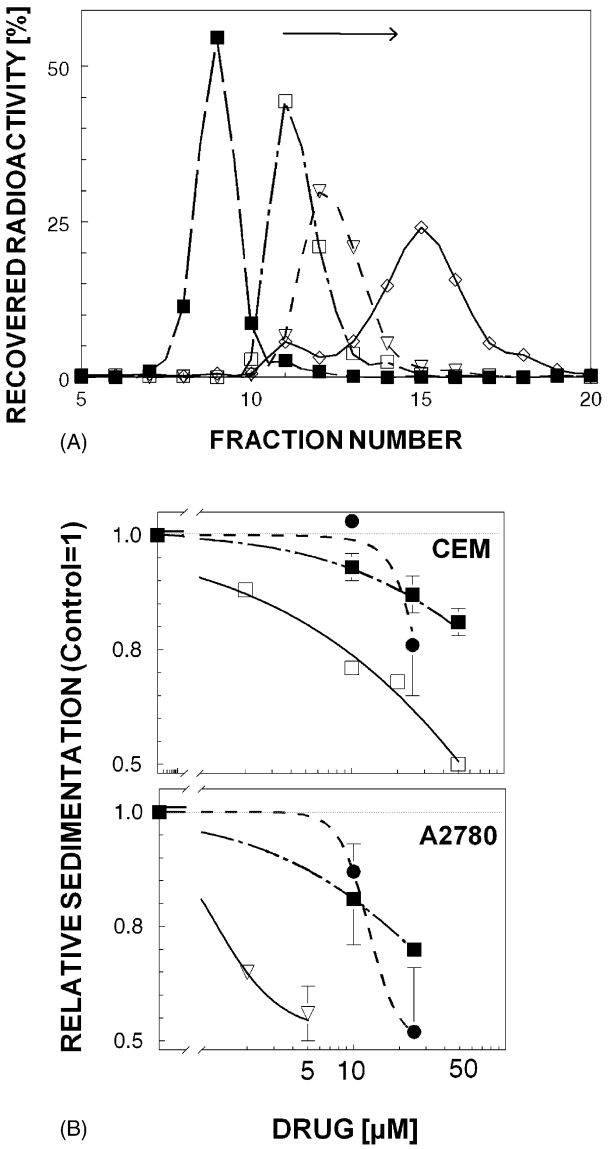

Fig. 2. Sedimentation of nucleoids from oxaliplatin- and cisplatin-treated CEM and A2780 cells. Panel A: Representative sedimentation profiles of nucleoids from CEM cells incubated with oxaliplatin at 0 μM (◇), 10 μM (▽), 25 μM (□), and 50 μM (■) for 4 hr. The arrow indicates the direction of sedimentation. Panel B: Relative nucleoid sedimentation (normalized for sedimentation of nucleoids from untreated cells) in CEM (top) and A2780 (bottom) cells treated with oxaliplatin for 4 hr (■), 6 hr (▽) or 24 hr (□) or cisplatin for 4 hr (●). The points represent mean values from two to four independent determinations (±SEM or 1/2 range for N = 2) except for the data for 24 hr incubation which are from a single experiment. Decrease in nucleoid sedimentation is indicative of DNA single-strand breakage.

Whereas 25 μM cisplatin was slightly more potent and 10 μM cisplatin less potent than equimolar oxaliplatin, these differences are insignificant (Fig. 2B).

3.3. Double-strand breakage of DNA in oxaliplatin- and cisplatin-treated cells

To follow the progression of the initial drug-induced SSB, we examined the ability of both drugs to generate DSB. The size distribution of DNA fragments resulting from DSB induction in CEM cells was analyzed by FIGE (Fig. 3A). The majority of fragments generated by both oxaliplatin and cisplatin are markedly larger than 50 kbp after 24 hr (Fig. 3A, left, lanes 3–6 and 7–10, respectively). After a prolonged incubation (48 hr), the fragments seem to be shorter, approaching 50 kbp at higher drug concentrations (Fig. 3A, right, lanes 6 and 10, respectively). Thus, both drugs cause a high molecular weight fragmentation in CEM cells with no significant amounts of oligonucleosomal "ladder". A similar pattern was seen for oxaliplatin in some other cell lines (data not shown). The induction of DSB after prolonged incubation with platinum drugs is consistent with an advanced apoptotic process.

Further experiments used the quantitative fragmentation assay to corroborate that both platinum drugs induce massive DNA breakage and to characterize time- and concentration-dependence of these effects. In CEM cells treated continuously for 24 hr with 10 μM oxaliplatin, 24 ± 6% of total cellular DNA becomes fragmented, compared to the base level of 9 ± 2% in untreated cells (Fig. 3B, top). The magnitude of the oxaliplatin effect increases to 67 ± 1% at 50 μM drug. A massive progression of fragmentation is also seen for a 48 hr continuous treatment (e.g. to ~52% for 10 μM oxaliplatin, Fig. 3B, open symbols). The elapsed time from the initial insult, rather than just drug presence, seems to be an important factor for such progression as drug removal after a 24 hr treatment and additional 24 hr incubation in drug-free medium resulted in similar levels of DNA fragmentation (data not shown). Also, a 4 hr pulse treatment of CEM cells followed by a 17 hr postincubation in drug-free medium produced a reduced, but still quite high effect (18 and 30% of fragmented DNA for oxaliplatin at 25 and 50 μM, respectively), compared to the continuous treatment by a similar elapsed time.

Importantly, cisplatin induced similar levels of DNA fragmentation as oxaliplatin in both CEM and A2780 cells. In CEM cells, the profiles for both drugs are nearly identical (Fig. 3B, top). A product of oxaliplatin bioconversion DACH-$PtCl_2$ was also as potent as cisplatin in fragmenting DNA in CEM cells (data not shown). In A2780 cells, cisplatin at the higher drug concentrations (25 and 50 μM) appears to be somewhat more potent than equimolar oxaliplatin, although these differences are not dramatic (Fig. 3B, bottom). Like it was the case with the early cellular responses, these data demonstrate again that despite the lower level of platinum adducts, oxaliplatin is as capable as cisplatin of inducing advanced secondary lesions in cellular DNA.

3.4. Apoptotic nature of cellular responses to oxaliplatin

The pattern of cellular responses to oxaliplatin is consistent with a typical progression of apoptosis. Massive

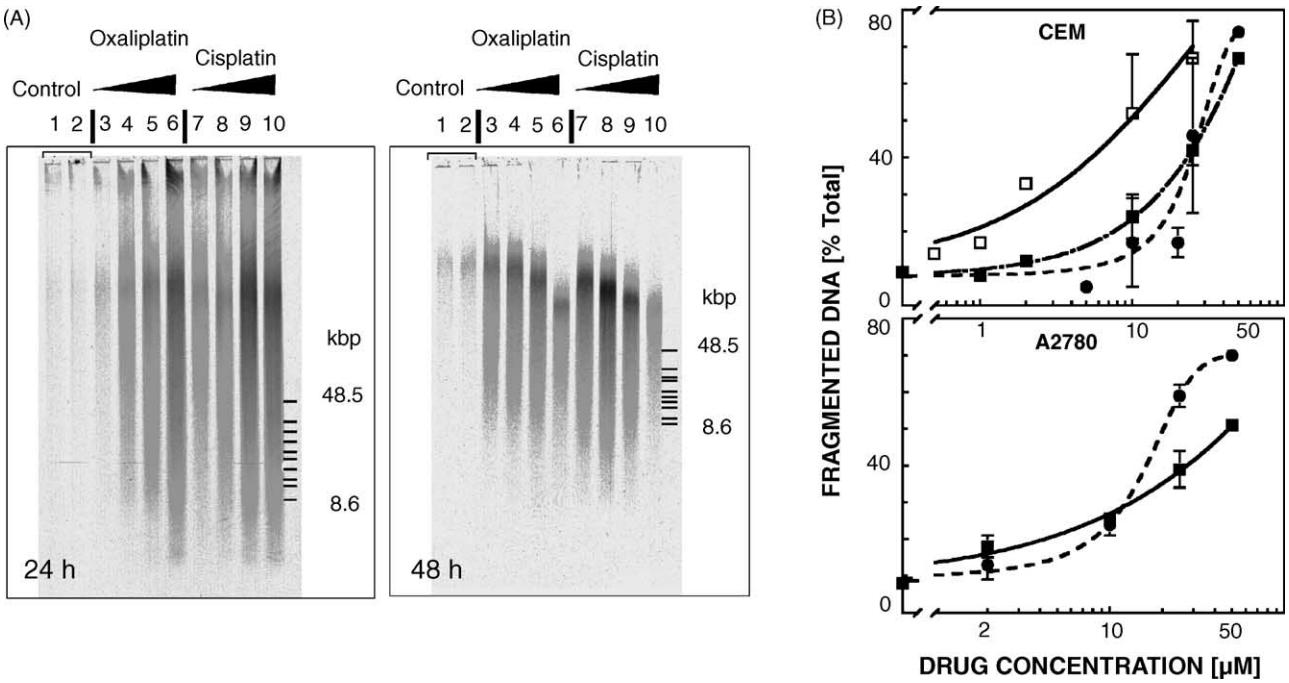

Fig. 3. Double-stranded DNA fragmentation by platinum drugs in oxaliplatin- and cisplatin-treated CEM and A2780 cells. Panel A: Size distribution of drug-induced DNA fragments in CEM cells. Cells with [^{14}C]-thymidine-prelabeled DNA were incubated for 24 or 48 hr with no drugs (lanes 1, 2), with oxaliplatin at 2, 5, 10, and 25 μM (lanes 3–6, respectively) and with cisplatin at 2, 5, 10, and 25 μM (lanes 7–10, respectively). Double-stranded DNA fragments were extracted as described for the quantitative DNA fragmentation assay and analyzed by FIGE electrophoresis and autoradiography. Bars indicating the positions of size markers between the markers for 8.6 and 48.5 kbp correspond to 10.1, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, and 38.4 kbp. Gels shown are representative for two independent experiments. Panel B: Quantitation of fragmented DNA in CEM (top) and A2780 cells (bottom). Cells with [^{14}C]-thymidine-prelabeled DNA were treated with oxaliplatin (■) or cisplatin (●) for 24 hr or with oxaliplatin for 48 hr (□). Double-stranded DNA fragments were extracted as described in Section 2. Each point represents the mean value from two to three determinations (±SEM or 1/2 range for N = 2).

DNA fragmentation coincides with the appearance of apoptotic cell morphology for CEM cells treated with oxaliplatin (Fig. 4A) as well as cisplatin (data not shown). Moreover, drug-induced massive DNA fragmentation is caspase-dependent. The pan-caspase inhibitor Z-VAD-fmk completely abrogates oxaliplatin-induced DNA fragmentation after 24 hr treatment (Fig. 4B). Partial inhibition effected by the caspase-3 inhibitor Ac-DEVD-CHO is also observed. Furthermore, after a 24 hr incubation, CEM cells treated with oxaliplatin up to 25 μM concentration show an essentially unaltered "viability" (Fig. 4C). Thus, the above mentioned massive DNA fragmentation observed at 25 μM drug (Fig. 3) precedes the changes in cell "viability" (Fig. 4C), which is a hallmark of the apoptotic process. Higher drug levels and prolonged incubation (48 hr) result in significant decreases in cell "viability", indicative of severely compromised membrane integrity (Fig. 4C). Such changes that follow apoptosis are usually referred to as "secondary necrosis". The distinction between various modes of cell death, however, is not always clear and some contribution of direct necrosis at high drug levels cannot be ruled out. A similar pattern was observed for cisplatin in CEM cells (Fig. 4C) and for both drugs in A2780 cells (data not shown).

A further comparison of the apoptotic potential of oxaliplatin and cisplatin is provided by multiparametric flow cytometry determinations. This assay simultaneously measured DNA content and cells or cell fragments positive for apoptotic strand breaks with 3′-OH groups that were determined by the terminal transferase reaction (TUNEL). The 2D histograms for drug-treated CEM cells (examples shown in Fig. 5A) show profound increases in cells with a high signal for apoptotic breaks. At the same time, DNA content indicates the appearance of sub-G1 particles independently indicative of apoptotic events (Fig. 5A and data not shown). Both oxaliplatin and cisplatin produced a persistent accumulation of cells in the S phase. For example, the percentage of cells in the S phase increased from ∼41 to 44% in control cells to 70 ± 11% and 85 ± 0.3% in cells treated with oxaliplatin for 24 and 48 hr, respectively. The 2D histograms, like the ones depicted in Fig. 5A, demonstrate that many of the TUNEL-positive cells are recruited from the S phase compartment. Apoptotic events, however, also comprise cells from other phases of the cell cycle (Fig. 5A and data not shown).

As was the case with the other endpoints, the magnitude of the oxaliplatin-induced apoptotic events determined by flow cytometry closely compares to the effects of cisplatin. For example, with a marginal level of spontaneous apoptosis in untreated CEM cells (2.4 ± 0.9%), the apoptotic events produced by 25 μM oxaliplatin and cisplatin after 24 hr incubation amounted, respectively, to 15.0 ± 2.9% and 19.8 ± 4.3% (Fig. 5B). The temporal progression of apoptotic events by flow cytometry (Fig. 5B) also closely

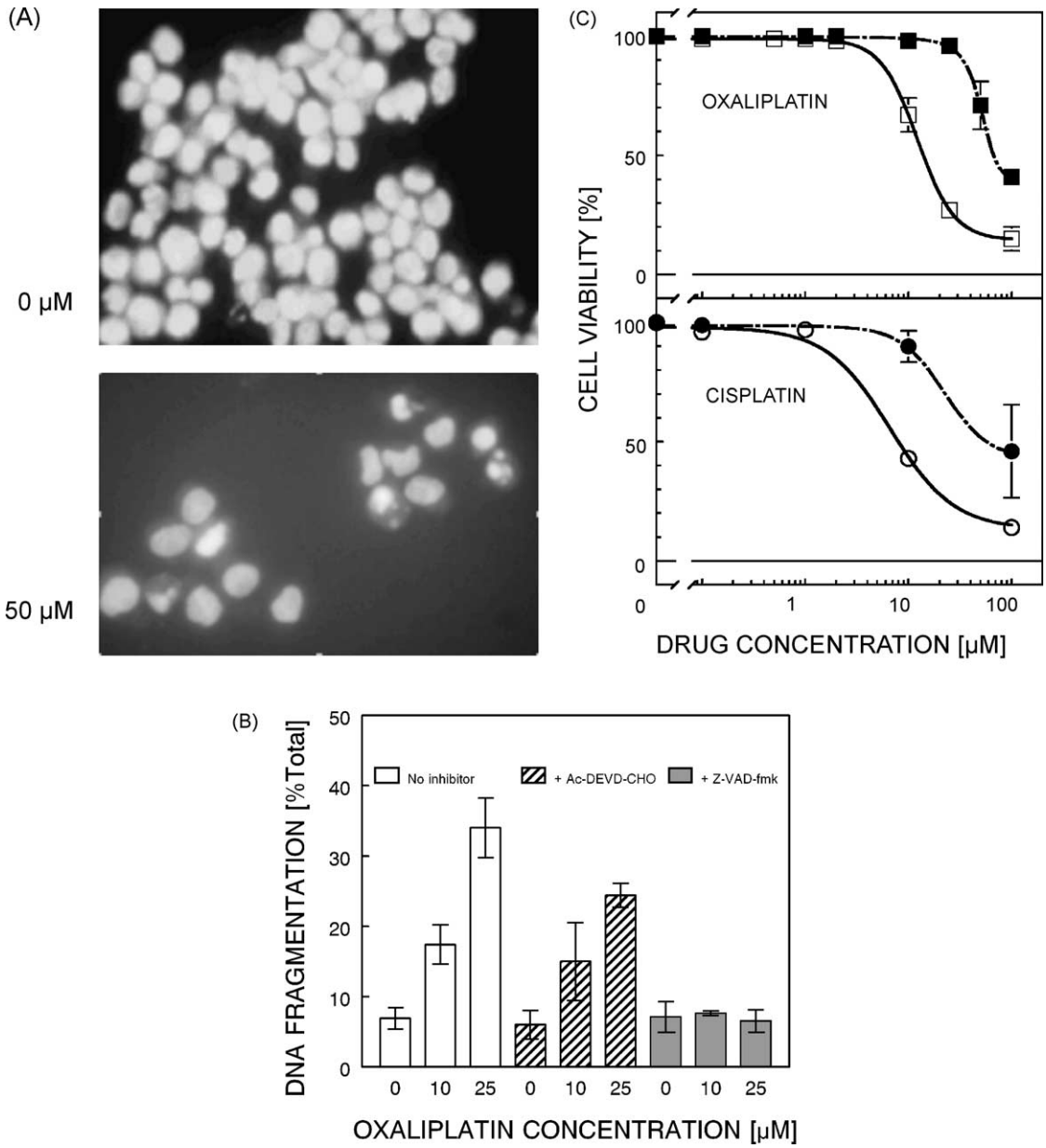

Fig. 4. Apoptotic nature of responses to oxaliplatin in CEM cells. Panel A: Cell morphology by Hoechst 33258 staining of cells after 24 hr incubation with 0 and 50 μM oxaliplatin. Panel B: The effects of a caspase-3 inhibitor, Ac-DEVD-CHO (100 μM), and a pan-caspase inhibitor, Z-VAD-fmk (100 μM) on oxaliplatin-induced DNA fragmentation in CEM cells (24 hr treatment, means ± SE from two independent experiments). Panel C: Changes in the membrane integrity (“cell viability” by trypan blue exclusion) of CEM cells incubated with oxaliplatin and cisplatin for 24 hr (solid symbols) and 48 hr (open symbols). The results represent means from one to three independent experiments (±SEM for N = 3 or 1/2 range for N = 2).

parallels the data from the fragmentation assay (Fig. 3B) in that both indices increase profoundly between 24 and 48 hr. After 48 hr with 25 μM oxaliplatin, nearly one-half of all the recorded flow cytometry events (51.5 ± 2.5%) corresponds to apoptotic events.

3.5. Apoptotic potential of oxaliplatin in other cancer cells

To expand the findings with CEM and A2780 cells, we examined the ability of oxaliplatin to induce apoptosis in several prostate cancer cell lines that differ in their molecular characteristics and apoptotic propensity. Normal prostate cells were also examined. The rationale for selecting prostate cancer cells is that this type of neoplasia typically tends to exhibit a reduced rate of spontaneous cell death reflecting various deficiencies in the apoptotic machinery [34,35]. The selected cell lines included androgen-dependent LNCaP-Pro5 and LNCaP-LN3 and apoptosis-resistant androgen-independent PC-3 (Table 1). These cell lines differ also in the p53 status, the levels of antiapoptotic factors such as Bcl-2, and their metastatic potential, which was reported to correlate with apoptotic resistance [30]. To account for possible

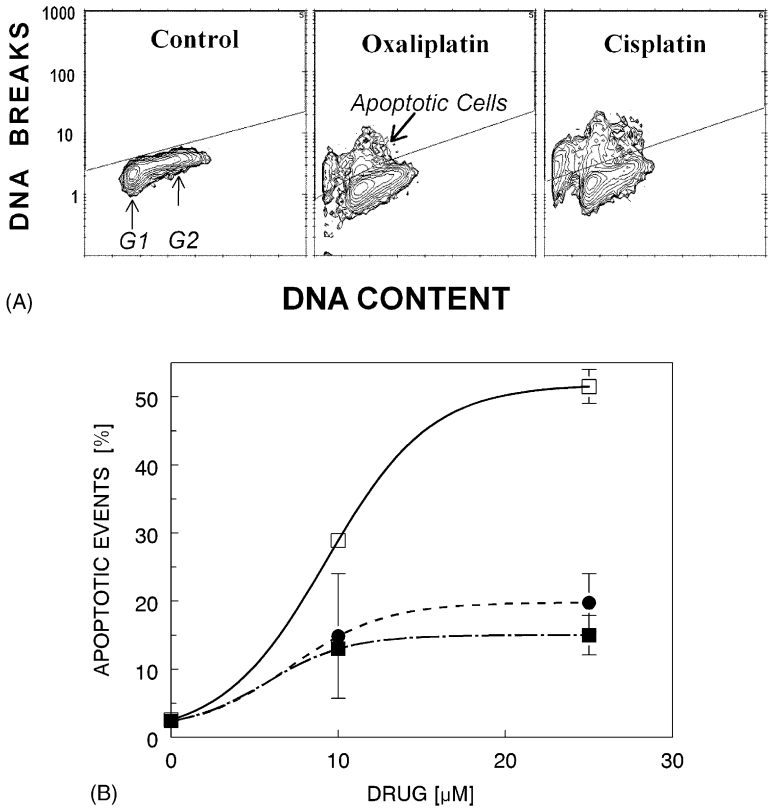

Fig. 5. Apoptosis by oxaliplatin and cisplatin in CEM cells by terminal transferase assay. Panel A: Examples of multiparametric flow cytometry profiles for control cells and cells treated for 24 hr with 25 μM oxaliplatin and cisplatin. Panel B: Quantitation of apoptotic events in cells treated with oxaliplatin (■, □) and cisplatin (●) for 24 hr (solid symbols) and 48 hr (open symbols). The results represent means (1/2 range) from two determinations.

differences in the timing of responses, three different treatment schemes were tested: ranging from a 4 hr pulse drug treatment followed by a 20 hr postincubation in a drug-free medium (4/20 hr), through 24 hr continuous treatment (24/0 hr), to 24 hr treatment and 24 hr postincubation (24/24 hr).

Quantitative DNA fragmentation assay demonstrates that oxaliplatin induces apoptosis in all three tested prostate cancer cell lines, although the magnitude and timing of these effects vary (Fig. 6). LNCaP-Pro5 cells show a profound apoptosis even after a 4/20 hr pulse treatment. Cisplatin, also analyzed in LNCaP-Pro5 cells, induced similar levels of apoptotic DNA fragmentation as oxaliplatin, except for the highest concentration (50 μM) in 24/0 scheme, where cisplatin was somewhat more potent. LNCaP-LN3 cells were as sensitive to oxaliplatin as LNCaP-Pro5 at the 24/0 and 24/24 hr schemes, although the 4/20 hr scheme was less effective. PC-3 cells, known for their resistance to apoptosis by other agents, are also relatively resistant to oxaliplatin. Still, the 24/24 hr scheme results in a small but clear increase in DNA fragmentation for 25 and 50 μM drug. In striking contrast to tumor cells, normal prostate cells (PrEC) and normal fibroblasts WI-38 tend to be remarkably resistant to the induction of apoptosis by oxaliplatin, regardless of treatment schedule (Table 1 and data not shown).

4. Discussion

Cytotoxic effects of platinum compounds, such as cisplatin, are believed to be exerted through drug-induced DNA adducts. Oxaliplatin, a third generation clinical platinum analog, is also definitely a DNA-reactive drug. It is puzzling, however, that oxaliplatin needs to form markedly fewer primary DNA lesions than cisplatin for equitoxic effects, even though the types of Pt-DNA adducts formed by oxaliplatin, their location and their removal closely resemble the respective attributes of cisplatin-DNA adducts [6,7,12]. This investigation demonstrates that, given the reduced level of Pt-DNA adducts, oxaliplatin displays a disproportionately greater ability to induce secondary lesions in DNA that are precursors to massive apoptosis. These results are the first to systematically characterize apoptosis induction by oxaliplatin in comparison to cisplatin and to relate the apoptotic potential to the primary lesions in DNA.

In drug-treated CEM and A2780 cells, both oxaliplatin and cisplatin induce early SSB as unequivocally demonstrated by the nucleoid sedimentation analysis (Fig. 2). For both drugs, the timing (commencing with 4 hr) and concentration-dependence of SSB induction are comparable to the conditions needed for the previously reported induction of various types of DNA-Pt lesions [7]. These early SSB, however, probably reflect a secondary, not primary, DNA

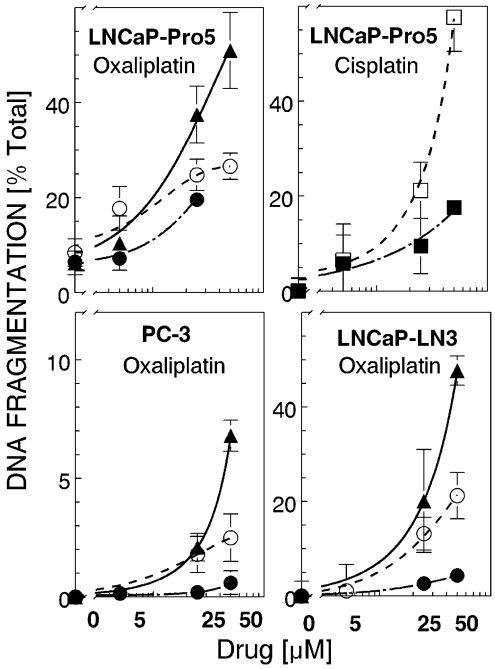

Fig. 6. Apoptosis by oxaliplatin and cisplatin in prostate tumor cells. Cells were treated with oxaliplatin (circles and triangles) and cisplatin (squares) using three different schemes before the determination of apoptotic DNA fragmentation: 4 hr with drug followed by a 20 hr postincubation in drug-free medium (●, ■, 4/20); continuous drug treatment for 24 hr (○, □, 24/0); or 24 hr with drug followed by a 24 hr postincubation in drug-free medium (▲, 24/24). The points are means from one to three independent experiments carried out in duplicate (±SEM for N = 3 or 1/2 range for N = 2).

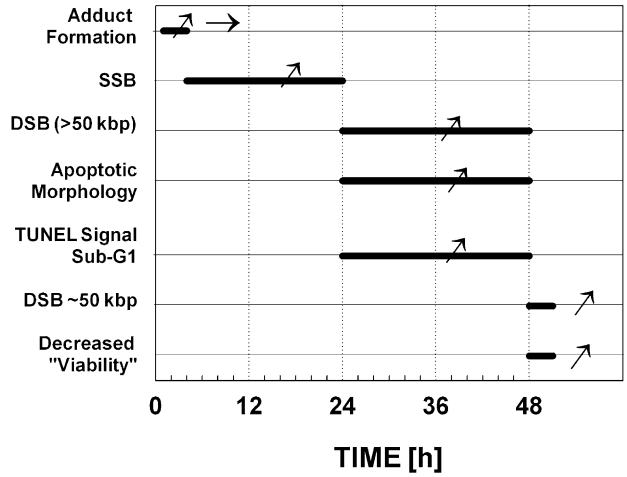

Fig. 7. Summary of cellular responses to oxaliplatin in CEM cells. Horizontal bars indicate the timing when specific responses become prominent. Arrows (→, ↗) indicate whether a specific effect is sustained, or progresses.

damage, as neither cisplatin nor oxaliplatin are capable of direct cleavage of naked DNA [6]. Early SSB induction by cisplatin has been known for a long time [36], although its nature has not been clearly characterized.

Unlike the similar induction of SSB, previous investigations demonstrated that cisplatin consistently produces more primary DNA lesions than equimolar oxaliplatin [6,7]. For example, depending on a treatment schedule, cisplatin formed 1.3–2.7-fold more DNA-Pt adducts than oxaliplatin in both CEM and A2780 cells. After a continuous 4 hr treatment of CEM cells, cisplatin formed 2.6-fold more ISC and 2.3-fold more DPC. Under the same conditions in A2780 cells, cisplatin produced 2.4-fold more ISC and 1.4-fold more DPC than oxaliplatin. Also, the level of platinum adducts induced by oxaliplatin in various regions of DNA from drug-treated A2780 cells was two to six times lower than that of cisplatin [6]. Contrasting with these data, the similar levels of the early DNA fragmentation (SSB) reveal the disparity between the primary and secondary DNA lesions by these two platinum drugs.

The properties of the early oxaliplatin-induced strand breaks and their temporal progression to massive DSB (Figs. 2 and 3, summarized in Fig. 7) are noticeably reminiscent of apoptotic DNA fragmentation, which is, in general, known to progress in discrete steps that are presumably mediated by distinct sets of gradually activated nucleases [37]. Early SSB detected by nucleoid sedimentation (for instance after 4 hr with 25–50 μM oxaliplatin in CEM cells) seem to occur approximately every few hundred kbp, which matches the early long-range apoptotic cleavages to ~300–1000 kbp sizes [37–39]. DSB detected by FIGE and the quantitative fragmentation assay (Fig. 3) produce smaller fragments that approach ~50 kbp after 48 hr in CEM cells. Such fragment sizes are characteristic of advanced apoptosis in various systems [37–39]. DNA fragments of 50–300 kbp were also reported for cisplatin-induced apoptosis in A2780 cells [40]. An analogous high molecular weight but progressing fragmentation was reported for cisplatin in the genomic and extrachromosomal DNA in human epidermoid KB-V1 cells [41]. On the other hand, neither cisplatin nor oxaliplatin produced oligonucleosomal size fragmentation (Fig. 3). While cisplatin was reported to produce the oligonucleosomal ''ladder'' of fragments in some systems, including A2780 cells [42,43], this historical hallmark of apoptosis is now regarded as a late and dispensable step of the cell death process [27,37,44]. As it is common to various apoptotic stimuli, oxaliplatin-induced cell death may have also nonapoptotic components, but rather only at high drug levels and prolonged treatment times.

The connection between the early SSB by oxaliplatin and later double-stranded, presumably apoptotic fragmentation, is further documented by the dependence of both effects on caspase inhibitors. DEVD-type caspase, such as caspase-3, a main apoptosis executioner, is likely to be involved, similar to the execution of apoptosis induced by cisplatin [45–48]. Other characteristic indicators of apoptotic death by oxaliplatin include the appearance of condensed chromatin that parallels massive DSB induction but clearly precedes decreases in membrane integrity (Fig. 4) and sub-G1 events with high TUNEL signal in flow cytometry (Fig. 5). Collectively, these findings

demonstrate that oxaliplatin is an apoptosis inducer as potent as cisplatin at equimolar levels.

Given the close resemblance of the cellular effects of oxaliplatin and cisplatin, the mechanisms of apoptosis induction by both drugs might be qualitatively similar. Consistent with a role of cisplatin-DNA adducts as apoptotic stimulus, various proteins that recognize and process DNA lesions, such as p53, MDM2, DNA-PK, and p21WAF were implicated in cisplatin effects [42,43,46,49–54]. Like other responses to cisplatin, apoptotic propensity is also thought to be dictated by the levels and persistence of Pt adducts in cellular DNA [40,55]. As mentioned in Section 1, oxaliplatin and cisplatin adducts are similarly persistent [7,12].

The lower level of DNA adducts formed by equimolar oxaliplatin would thus suggest that oxaliplatin should be inferior, not equal, to cisplatin in apoptosis induction. Moreover, differential recognition and removal of DNA-Pt adducts, if any, would be expected to manifest itself in rather late responses. In contrast, the early induction of SSB indicates that cellular responses to oxaliplatin match those of cisplatin right from their early onset and oxaliplatin is as potent apoptosis inducer as cisplatin.

It remains unknown what factors might compensate for the reduced levels of DNA-Pt adducts by oxaliplatin but one possibility to consider would be targets other than DNA. The apoptotic process integrates various signal transduction pathways and apoptotic stimuli are not limited to DNA damage. DNA adducts of platinum drugs constitute probably only a minor fraction (1%) of cell-associated drug molecules [56]. Cisplatin adducts to DNA amount to ~10% whereas protein adducts comprise 75–85% of all cellular adducts to macromolecules [57,58]. Using the ratios of DNA/protein adducts reported by Akaboshi *et al*. [57,58], and the frequency of DNA-Pt lesions in CEM cells [7], we estimate that 25 μM cisplatin produces, respectively, $\sim 5 \times 10^4$ vs. $(1–2) \times 10^6$ DNA and protein adducts/cell. Such massive reactivity with protein sulfhydryls is likely to distort the redox homeostasis of the cell, analogous to various protein-reactive agents for which the ability to damage proteins only (no DNA adducts) is sufficient for apoptosis [59–62].

It is likely that targeting critical protein regulators of redox homeostasis contributes to apoptosis by platinum drugs. One such protein, thioredoxin, has been implicated in the resistance of cancer cells to cisplatin [63–65]. A recent investigation demonstrated that cisplatin can directly inactivate thioredoxin and its regenerating enzyme thioredoxin reductase [64]. Our preliminary data show that both enzymes are also inhibited by oxaliplatin.[2] Other potentially important protein targets probably exist, but they remain obscure due to the scarcity of published studies on the protein reactivity of platinum drugs. However, analogies to protein-reactive single-action agents with well defined roles in apoptosis can point to some candidate proteins. For example, given the crosslinking properties of platinum drugs, adenine nucleotide transporter (ANT), a mitochondrial membrane protein, could perhaps be relevant as a target. Crosslinking of critical sulfhydryl groups in ANT by some protein-reactive agents directly results in mitochondrial dysfunction, a "point of no return" in apoptosis [59,66]. While further studies are needed to investigate the ability of oxaliplatin to damage specific proteins, such reactivity might be facilitated, compared to cisplatin, by the interaction of the hydrophobic DACH moiety with hydrophobic protein pockets ([7] and references therein).

In a separate study, we have compared apoptotic potential of drugs that react with both DNA and proteins (dual-action drugs), including cisplatin and oxaliplatin, to single-action drugs that react only with proteins or only with DNA [67]. The results showed that dual-action drugs tend to be more potent apoptosis inducers than single-action agents when the effects are related to equitoxic drug levels. Dual-action drugs induced apoptosis at concentrations comparable to a nearly complete cell growth inhibition ($(1–2) \times GI_{80}$). Agents that react only with proteins, diamide and helenalin, although somewhat less potent, were still apoptotic at $\sim 2 \times GI_{80}$. In contrast, the purely DNA-damaging drugs (bizelesin and adozelesin) produced more than marginal apoptosis only at concentrations equal or greater than 5–10 times the IC_{80}. Also, ionizing radiation, which is believed to kill cells by DNA damage (although it can also affect proteins *via* free radical generation), was consistently observed by us and others to produce only marginal apoptosis at clinically relevant doses. As reviewed recently, based on these and other data, we propose that DNA damage and protein damage may be mutually-potentiating factors in apoptosis induction [68].

Deficiencies in the apoptotic machinery are linked to some cases of resistance to cisplatin [40,69–74]. Cancer cells with the naturally high expression of Bcl-2 may be less susceptible to apoptosis by cisplatin [75]. Cisplatin effects are sometimes but not always affected by p53 status [52,54,74,76–80]. Data in this report indicate that p53-defective cells are not necessarily less sensitive to growth inhibition and apoptosis induction by oxaliplatin (Table 1). Apoptosis definitely plays an important role in the cisplatin's antiproliferative effects in cancer cells [42,43,81–83]. The ability of oxaliplatin to induce apoptosis is not limited to leukemic CEM and ovarian carcinoma A2780 cells, as illustrated by the results with several prostate cancer cell lines (Fig. 6, Table 1). Apart from our presentation of the results covered by this report [84,85], only recently was oxaliplatin-induced apoptosis reported in other systems including myeloma and colon carcinoma cells [86,87]. Interestingly, stable transfection of human ovarian carcinoma cells with antiapoptotic survivin inhibited apoptosis induction by taxanes but not by oxaliplatin and cisplatin [88]. Resistance to oxaliplatin-induced

[2] Woynarowska et al., unpublished results.

apoptosis was implicated in the oxaliplatin-resistant phenotype [87].

Profound apoptotic responses of cancer cells to oxaliplatin are observed after rather brief treatments. In contrast, normal prostate cells seemed refractory even to prolonged incubations with oxaliplatin. The ability to induce differential apoptosis in cancer but not normal cells has been extensively documented for another structurally unrelated dual-action alkylating drug of clinically promising properties, irofulven [24,29,68]. This aspect remains to be investigated for platinum drugs. Cisplatin-induced apoptosis was implicated not only in the context of anticancer effects but also in the renal and neurological toxicity of the drug [46,89,90]. An intriguing possibility is that the superior toxicological profile of oxaliplatin might in part reflect oxaliplatin's potential to differentiate between tumor cells and normal cells in apoptosis induction. Collectively, this report and other studies point to the significance of apoptosis in the antiproliferative effects of oxaliplatin and warrant further investigations to determine its underlying mechanism.

Acknowledgments

This study was supported in part by a grant from Sanofi-Synthelabo Research and an NIH Grant CA78706. Skilful technical assistance of Ms. B. Arnett, A. Hidgon, and B. Leal is acknowledged. We thank also Dr. Paul E. Juniewicz (Sanofi-Synthelabo) for encouragement, numerous helpful discussions, and support of these investigations, and Dr. Maryanne Herzig for the critical reading of the manuscript.

References

[1] Raymond E, Faivre S, Woynarowski J, Chaney S. Oxaliplatin: mechanism of action and antineoplastic activity (review). Semin Oncol 1998;25:4–12.
[2] Raymond E, Faivre S, Chaney S, Woynarowski J, Cvitkovic E. Cellular and molecular pharmacology of oxaliplatin (review). Mol Cancer Therap 2002;1:227–35.
[3] Pelley RJ. Oxaliplatin: a new agent for colorectal cancer. Curr Oncol Rep 2001;3:147–55.
[4] Mani S, Graham MA, Bregman DB, Ivy P, Chaney SG. Oxaliplatin: a review of evolving concepts. Cancer Invest 2002;20:246–63.
[5] Misset JL, Bleiberg H, Sutherland W, Bekradda M, Cvitkovic E. Oxaliplatin clinical activity: a review. Crit Rev Oncol Hematol 2000; 35:75–93.
[6] Woynarowski JM, Chapman WG, Napier C, Herzig MCS, Juniewicz P. Sequence- and region-specificity of oxaliplatin adducts in naked and cellular DNA. Mol Pharmacol 1998;54:770–7.
[7] Woynarowski JM, Faivre S, Herzig MC, Arnett B, Chapman WG, Trevino AV, Raymond E, Chaney SG, Vaisman A, Varchenko M, Juniewicz PE. Oxaliplatin-induced damage of cellular DNA. Mol Pharmacol 2000;58:920–7.
[8] Dunn TA, Schmoll HJ, Grunwald V, Bokemeyer C, Casper J. Comparative cytotoxicity of oxaliplatin and cisplatin in nonseminomatous germ cell cancer cell lines. Invest New Drugs 1997; 15:109–14.
[9] Rixe O, Ortuzar W, Alvarez M, Parker R, Reed E, Paull K, Fojo T. Oxaliplatin, tetraplatin, cisplatin, and carboplatin: spectrum of activity in drug-resistant cell lines and in the cell lines of the National Cancer Institute's Anticancer Drug Screen panel. Biochem Pharmacol 1996; 52:1855–65.
[10] Pendyala L, Creaven PJ. In vitro cytotoxicity, protein binding, red blood cell partitioning, and biotransformation of oxaliplatin. Cancer Res 1993;53:5970–6.
[11] Pendyala L, Kidani Y, Perez R, Wilkes J, Bernacki RJ, Creaven PJ. Cytotoxicity, cellular accumulation and DNA binding of oxaliplatin isomers. Cancer Lett 1995;97:177–84.
[12] Hector S, Bolanowska-Higdon W, Zdanowicz J, Hitt S, Pendyala L. In vitro studies on the mechanisms of oxaliplatin resistance. Cancer Chemother Pharmacol 2001;48:398–406.
[13] Saris CP, Van de Vaart PJM, Rietbroek RC, Blommaert FA. In vitro formation of DNA adducts by cisplatin, lobaplatin and oxaliplatin in calf thymus DNA in solution and in cultured human cells. Carcinogenesis 1996;17:2763–9.
[14] Damia G, Guidi G, D'Incalci M. Expression of genes involved in nucleotide excision repair and sensitivity to cisplatin and melphalan in human cancer cell lines. Eur J Cancer [A] 1998;34:1783–8.
[15] Hibino Y, Hiraoka Y, Kamiuchi S, Kusashio E, Sugano N. Enhancement of excision repair of cisplatin-DNA adducts by cell-free extract from a cisplatin-resistant rat cell line. Biochem Pharmacol 1999;57: 1415–22.
[16] Koeberle B, Masters JRW, Hartley JA, Wood RD. Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours. Curr Biol 1999;9:273–6.
[17] Dunkern TR, Fritz G, Kaina B. Cisplatin-induced apoptosis in 43-3B and 27-1 cells defective in nucleotide excision repair. Mutat Res 2001;486:249–58.
[18] Reardon JT, Vaisman A, Chaney SG, Sancar A. Efficient nucleotide excision repair of cisplatin, oxaliplatin, and bis-aceto-ammine-dichloro-cyclohexylamine-platinum(IV) (JM216) platinum intrastrand DNA diadducts. Cancer Res 1999;59:3968–71.
[19] Zdraveski ZZ, Mello JA, Farinelli CK, Essigmann JM, Marinus MG. MutS preferentially recognizes cisplatin- over oxaliplatin-modified DNA. J Biol Chem 2002;277:1255–60.
[20] Scheeff ED, Briggs JM, Howell SB. Molecular modeling of the intrastrand guanine–guanine DNA adducts produced by cisplatin and oxaliplatin. Mol Pharmacol 1999;56:633–43.
[21] Fink D, Zheng H, Nebel S, Norris PS, Aebi S, Lin T-P, Nehme A, Christen RD, Haas M, MacLeod C, Howell SB. In vitro and in vivo resistance to cisplatin in cells that have lost DNA mismatch repair. Cancer Res 1997;57:1841–5.
[22] Vaisman A, Varchenko M, Umar A, Kunkel TA, Risinger JI, Barrett JC, Hamilton TC, Chaney SG. The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance: correlation with replicative bypass of platinum-DNA adducts. Cancer Res 1998;58: 3579–85.
[23] Taverna P, Liu L, Hanson AJ, Monks A, Gerson SL. Characterization of MLH1 and MSH2 DNA mismatch repair proteins in cell lines of the NCI anticancer drug screen. Cancer Chemother Pharmacol 2000;46: 507–16.
[24] Woynarowska BA, Woynarowski JM, Herzig MCS, Roberts K, Higdon AL, MacDonald JR. Differential cytotoxicity and induction of apoptosis in tumor and normal cells by hydroxymethylacylfulvene (HMAF). Biochem Pharmacol 2000;59:1217–26.
[25] Ahnstrom G. Techniques to measure DNA single-strand breaks in cells: a review. Int J Radiat Biol 1988;54:695–707.
[26] Glazkov MV. Ultrastructure of somatic and meiotic nucleoids. Electron Microsc Rev 1989;2:197–229.
[27] Woynarowski JM, Napier C, Koester S, Chen S-F, Troyer D, Chapman W, MacDonald JR. Effects on DNA integrity and apoptosis induction of a novel antitumor sesquiterpene, 6-hydroxymethyl acylfulvene (HMAF). Biochem Pharmacol 1997;54:1181–93.

[28] Das AT, Luderus ME, Lamers WH. Identification and analysis of a matrix-attachment region 5′ of the rat glutamate-dehydrogenase-encoding gene. Eur J Biochem 1993;215:777–85.
[29] Herzig MC, Liang H, Johnson AE, Woynarowska B, Woynarowski JM. Irofulven induces apoptosis in breast cancer cells regardless of caspase-3 status. Breast Cancer Res Treat 2002;71:133–43.
[30] McConkey DJ, Greene G, Pettaway CA. Apoptosis resistance increases with metastatic potential in cells of the human LNCaP prostate carcinoma line. Cancer Res 1996;56:5594–9.
[31] Borner MM, Myers CE, Sartor O, Sei Y, Toko T, Trepel JB, Schneider E. Drug-induced apoptosis is not necessarily dependent on macromolecular synthesis or proliferation in the p53-negative human prostate cancer cell line PC-3. Cancer Res 1995;55: 2122–8.
[32] Ng AY, Bales W, Veltri RW. Phenylbutyrate-induced apoptosis and differential expression of Bcl-2, Bax, p53 and Fas in human prostate cancer cell lines. Anal Quant Cytol Histol 2000;22:45–54.
[33] Aquilina G, Ceccotti S, Martinelli S, Soddu S, Crescenzi M, Branch P, Karran P, Bignami M. Mismatch repair and p53 independently affect sensitivity to *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea. Clin Cancer Res 2000;6:671–80.
[34] Kyprianou N. Apoptosis: therapeutic significance in the treatment of androgen-dependent and androgen-independent prostate cancer. World J Urol 1994;12:299–303.
[35] Tang DG, Porter AT. Target to apoptosis: a hopeful weapon for prostate cancer. Prostate 1997;32:284–93.
[36] Pera Jr MF, Rawlings CJ, Shackleton J, Roberts JJ. Quantitative aspects of the formation and loss of DNA interstrand crosslinks in Chinese hamster cells following treatment with *cis*-diamminedichloroplatinum(II) (cisplatin). II. Comparison of results from alkaline elution. Biochim Biophys Acta 1981;655:152–66.
[37] Walker PR, Sikorska M. New aspects of the mechanism of DNA fragmentation in apoptosis. Biochem Cell Biol 1997;75:287–99.
[38] Khodarev NN, Sokolova IA, Vaughan ATM. Association between DNA cleavage during apoptosis and regions of chromatin replication. J Cell Biochem 1998;70:604–15.
[39] Rusnak JM, Calmels TP, Hoyt DG, Kondo Y, Yalowich JC, Lazo JS. Genesis of discrete higher order DNA fragments in apoptotic human prostatic carcinoma cells. Mol Pharmacol 1996;49:244–52.
[40] Ercoli A, Battaglia A, Raspaglio G, Fattorossi A, Alimonti A, Petrucci F, Caroli S, Mancuso S, Scambia G. Activity of cisplatin and ICI 182,780 on estrogen receptor negative ovarian cancer cells: cell cycle and cell replication rate perturbation, chromatin texture alteration and apoptosis induction. Int J Cancer 2000;85:98–103.
[41] Schoenlein PV, Barrett JT, Welter D. The degradation profile of extrachromosomal circular DNA during cisplatin-induced apoptosis is consistent with preferential cleavage at matrix attachment regions. Chromosoma 1999;108:121–31.
[42] Fajac A, Da Silva J, Ahomadegbe JC, Rateau JG, Bernaudin JF, Riou G, Benard J. Cisplatin-induced apoptosis and p53 gene status in a cisplatin-resistant human ovarian carcinoma cell line. Int J Cancer 1996;68:67–74.
[43] Henkels KM, Turchi JJ. Induction of apoptosis in cisplatin-sensitive and -resistant human ovarian cancer cell lines. Cancer Res 1997;57: 4488–92.
[44] Watanabe H, Kanbe K, Shinozaki T, Hoshino H, Chigira M. Apoptosis of a fibrosarcoma induced by protein-free culture involves DNA cleavage to large fragments but not internucleosomal fragmentation. Int J Cancer 1995;62:191–8.
[45] Mese H, Sasaki A, Nakayama S, Alcalde RE, Matsumura T. The role of caspase family protease, caspase-3 on cisplatin-induced apoptosis in cisplatin-resistant A431 cell line. Cancer Chemother Pharmacol 2000;46:241–5.
[46] Cummings BS, Schnellmann RG. Cisplatin-induced renal cell apoptosis: caspase-3-dependent and -independent pathways. J Pharmacol Exp Ther 2002;302:8–17.
[47] Kuwahara D, Tsutsumi K, Kobayashi T, Hasunuma T, Nishioka K. Caspase-9 regulates cisplatin-induced apoptosis in human head and neck squamous cell carcinoma cells. Cancer Lett 2000;148:65–71.
[48] Blanc C, Deveraux QL, Krajewski S, Janicke RU, Porter AG, Reed JC, Jaggi R, Marti A. Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. Cancer Res 2000;60:4386–890.
[49] Kondo S, Barnett GH, Hara H, Morimura T, Takeuchi J. MDM2 protein confers the resistance of a human glioblastoma cell line to cisplatin-induced apoptosis. Oncogene 1995;10:2001–6.
[50] Kondo S, Barna BP, Kondo Y, Tanaka Y, Casey G, Liu J, Morimura T, Kaakaji R, Peterson JW, Werbel B, Barnett GH. WAF1/CIP1 increases the susceptibility of p53 nonfunctional malignant glioma cells to cisplatin-induced apoptosis. Oncogene 1996;13:1279–85.
[51] Siemer S, Ornskov D, Guerra B, Boldyreff B, Issinger OG. Determination of mRNA, and protein levels of p53, MDM2 and protein kinase CK2 subunits in F9 cells after treatment with the apoptosis-inducing drugs cisplatin and carboplatin. Int J Biochem Cell Biol 1999;31:661–70.
[52] McKay BC, Becerril C, Ljungman M. P53 plays a protective role against UV- and cisplatin-induced apoptosis in transcription-coupled repair proficient fibroblasts. Oncogene 2001;20:6805–8.
[53] Niedner H, Christen R, Lin X, Kondo A, Howell SB. Identification of genes that mediate sensitivity to cisplatin. Mol Pharmacol 2001;60: 1153–60.
[54] Qin LF, Ng IO. Induction of apoptosis by cisplatin and its effect on cell cycle-related proteins and cell cycle changes in hepatoma cells. Cancer Lett 2002;175:27–38.
[55] Yang Z, Faustino PJ, Andrews PA, Monastra R, Rasmussen AA, Ellison CD, Cullen KJ. Decreased cisplatin/DNA adduct formation is associated with cisplatin resistance in human head and neck cancer cell lines. Cancer Chemother Pharmacol 2000;46:255–62.
[56] Gonzalez VM, Fuertes MA, Alonso C, Perez JM. Is cisplatin-induced cell death always produced by apoptosis? Mol Pharmacol 2001;59: 657–63.
[57] Akaboshi M, Kawai K, Maki H, Akuta K, Ujeno Y, Miyahara T. The number of platinum atoms binding to DNA, RNA and protein molecules of HeLa cells treated with cisplatin at its mean lethal concentration. Jpn J Cancer Res 1992;83:522–6.
[58] Akaboshi M, Kawai K, Ujeno Y, Takada S, Miyahara T. Binding characteristics of (−)-(*R*)-2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)-2-platinum(II) to DNA, RNA and protein molecules in HeLa cells and its lethal effect: comparison with *cis*- and *trans*-diamminedichloroplatinums(II). Jpn J Cancer Res 1994;85:106–11.
[59] Costantini P, Belzacq AS, Vieira HL, Larochette N, de Pablo MA, Zamzami N, Susin SA, Brenner C, Kroemer G. Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. Oncogene 2000;19:307–14.
[60] Zamzami N, Marzo I, Susin SA, Brenner C, Larochette N, Marchetti P, Reed J, Kofler R, Kroemer G. The thiol crosslinking agent diamide overcomes the apoptosis-inhibitory effect of Bcl-2 by enforcing mitochondrial permeability transition. Oncogene 1998;16:1055–63.
[61] Dirsch VM, Stuppner H, Vollmar AM. Helenalin triggers a CD95 death receptor-independent apoptosis that is not affected by overexpression of Bcl-x(L) or Bcl-2. Cancer Res 2001;61:5817–23.
[62] Pallis M, Grundy M, Turzanski J, Kofler R, Russell N. Mitochondrial membrane sensitivity to depolarization in acute myeloblastic leukemia is associated with spontaneous in vitro apoptosis, wild-type TP53, and vicinal thiol/disulfide status. Blood 2001;98:405–13.
[63] Yokomizo A, Ono M, Nanri H, Makino Y, Ohga T, Wada M, Okamoto T, Yodoi J, Kuwano M, Kohno K. Cellular levels of thioredoxin associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide. Cancer Res 1995;55:4293–6.
[64] Arner ESJ, Nakamura H, Sasada T, Yodoi J, Holmgren A, Spyrou G. Analysis of the inhibition of mammalian thioredoxin, thioredoxin reductase, and glutaredoxin by *cis*-diamminedichloroplatinum(II) and

its major metabolite, the glutathione–platinum complex. Free Radic Biol Med 2001;31:1170–8.
[65] Kawahara N, Tanaka T, Yokomizo A, Nanri H, Ono M, Wada M, Kohno K, Takenaka K, Sugimachi K, Kuwano M. Enhanced coexpression of thioredoxin and high mobility group protein 1 genes in human hepatocellular carcinoma and the possible association with decreased sensitivity to cisplatin. Cancer Res 1996;56:5330–3.
[66] Belzacq AS, Vieira HL, Kroemer G, Brenner C. The adenine nucleotide translocator in apoptosis. Biochimie 2002;84:167–76.
[67] Woynarowski JM, Koester S, Woynarowska B, Arnett B, Trevino AV, Chan D, Higdon A, Munoz R, Herzig MCS, Faivre S. Is drug-induced DNA damage sufficient for apoptosis induction? Proc Am Assoc Cancer Res 1999;40:736.
[68] Woynarowska BA, Woynarowski JM. Preferential targeting of apoptosis in tumor vs. normal cells. Biochim Biophys Acta 2002;1587: 309–17.
[69] Sartorius UA, Krammer PH. Upregulation of Bcl-2 is involved in the mediation of chemotherapy resistance in human small cell lung cancer cell lines. Int J Cancer 2002;97:584–92.
[70] Helmbach H, Kern MA, Rossmann E, Renz K, Kissel C, Gschwendt B, Schadendorf D. Drug resistance towards etoposide and cisplatin in human melanoma cells is associated with drug-dependent apoptosis deficiency. J Invest Dermatol 2002;118:923–32.
[71] Alas S, Ng CP, Bonavida B. Rituximab modifies the cisplatin-mitochondrial signaling pathway, resulting in apoptosis in cisplatin-resistant non-Hodgkin's lymphoma. Clin Cancer Res 2002;8:836–45.
[72] Li J, Feng Q, Kim JM, Schneiderman D, Liston P, Li M, Vanderhyden B, Faught W, Fung MF, Senterman M, Korneluk RG, Tsang BK. Human ovarian cancer and cisplatin resistance: possible role of inhibitor of apoptosis proteins. Endocrinology 2001;142:370–80.
[73] Mese H, Sasaki A, Alcalde RE, Nakayama S, Matsumura T. Regulation of apoptosis reduction in the cisplatin-resistant A431 cell line by Bcl-2 and CPP32. Chemotherapy 2000;46:69–76.
[74] Segal-Bendirdjian E, Mannone L, Jacquemin-Sablon A. Alteration in p53 pathway and defect in apoptosis contribute independently to cisplatin-resistance. Cell Death Differ 1998;5:390–400.
[75] Han JY, Chung YJ, Park SW, Kim JS, Rhyu MG, Kim HK, Lee KS. The relationship between cisplatin-induced apoptosis and p53, Bcl-2 and Bax expression in human lung cancer cells. Korean J Int Med 1999;14:42–52.
[76] Burger H, Nooter K, Boersma AW, Kortland CJ, Stoter G. Lack of correlation between cisplatin-induced apoptosis, p53 status and expression of Bcl-2 family proteins in testicular germ cell tumour cell lines. Int J Cancer 1997;73:592–9.
[77] Ikeguchi M, Tatebe S, Kaibara N, Ito H. Changes in levels of expression of p53 and the product of the Bcl-2 in lines of gastric cancer cells during cisplatin-induced apoptosis. Eur Surg Res 1997;29:396–402.
[78] Minagawa Y, Kigawa J, Itamochi H, Kanamori Y, Shimada M, Takahashi M, Terakawa N. Cisplatin-resistant HeLa cells are resistant to apoptosis via p53-dependent and -independent pathways. Jpn J Cancer Res 1999;90:1373–9.
[79] Burger H, Nooter K, Boersma AW, Kortland CJ, Stoter G. Expression of p53, Bcl-2 and Bax in cisplatin-induced apoptosis in testicular germ cell tumour cell lines. Br J Cancer 1998;77:1562–7.
[80] Chang FL, Lai MD. Various forms of mutant p53 confer sensitivity to cisplatin and doxorubicin in bladder cancer cells. J Urol 2001;166: 304–10.
[81] Barry MA, Behnke CA, Eastman A. Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol 1990;40:2353–62.
[82] Evans DL, Tilby M, Dive C. Differential sensitivity to the induction of apoptosis by cisplatin in proliferating and quiescent immature rat thymocytes is independent of the levels of drug accumulation and DNA adduct formation. Cancer Res 1994;54:1596–603.
[83] Henkels KM, Turchi JJ. Cisplatin-induced apoptosis proceeds by caspase-3-dependent and -independent pathways in cisplatin-resistant and -sensitive human ovarian cancer cell lines. Cancer Res 1999;59: 3077–83.
[84] Woynarowska B, Woynarowski JM, Higdon AL, Munoz R. Induction of apoptosis in prostate tumor cells by hydroxymethylacylfulvene (HMAF) and oxaliplatin. Proc Am Assoc Cancer Res 1999;40:488.
[85] Faivre S, Woynarowski JM. Oxaliplatin effects on DNA integrity and apoptosis induction in human tumor cells. Proc Am Assoc Cancer Res 1998;39:158.
[86] Tassone P, Tagliaferri P, Galea E, Palmieri C, Bonelli P, Martelli ML, Tuccillo F, Turco MC, Venuta S. Oxaliplatin (L-OHP) treatment of human myeloma cells induces in vitro growth inhibition and apoptotic cell death. Eur J Cancer 2002;38:1141–7.
[87] Gourdier I, Del Rio M, Crabbe L, Candeil L, Copois V, Ychou M, Auffray C, Martineau P, Mechti N, Pommier Y, Pau B. Drug specific resistance to oxaliplatin is associated with apoptosis defect in a cellular model of colon carcinoma. FEBS Lett 2002;529:232.
[88] Zaffaroni N, Pennati M, Colella G, Perego P, Supino R, Gatti L, Pilotti S, Zunino F, Daidone MG. Expression of the anti-apoptotic gene survivin correlates with taxol resistance in human ovarian cancer. Cell Mol Life Sci 2002;59:1406–12.
[89] Okuda M, Masaki K, Fukatsu S, Hashimoto Y, Inui K. Role of apoptosis in cisplatin-induced toxicity in the renal epithelial cell line LLC-PK1. Implication of the functions of apical membranes. Biochem Pharmacol 2000;59:195–201.
[90] McDonald ES, Windebank AJ. Cisplatin-induced apoptosis of DRG neurons involves Bax redistribution and cytochrome *c* release but not Fas receptor signaling. Neurobiol Dis 2002;9:220–33.