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Cytotoxicity, DNA strand breakage and DNA–protein crosslinking by a novel transplatinum compound in human A2780 ovarian and MCF-7 breast carcinoma cells

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Abstract

Cisplatin, *cis*-[$PtCl_2(NH_3)_2$], is commonly utilized in various combination chemotherapy protocols for the treatment of both ovarian and breast cancer while the corresponding *trans* isomer is therapeutically inactive. This work describes efforts to elucidate the cellular mechanism of action of a novel *trans*-platinum compound, *trans*-(dichloroamminethiazole)platinum(II) (ATZ), which demonstrates antiproliferative and cytotoxic effects against both MCF-7 human breast and A2780 human ovarian carcinoma cells in culture. A2780 cells were approximately twofold more sensitive to ATZ than MCF-7 cells in both cell growth and clonogenic survival assays. Dye exclusion studies revealed a 50–70% loss in cell viability within the first 12 h of drug treatment in both cell lines. This initial wave of cell death was succeeded by a prolonged interval of growth arrest during which a small fraction of apoptotic cells was detected. Binding of ATZ to DNA, as estimated by atomic absorption spectroscopy, was similar for the two cell lines and was almost completely reversed 24 h after drug removal. ATZ also induced DNA strand breakage as well as DNA–protein crosslinking during the initial 12 h period when the bulk of cell death was evident. However, neither the extent of DNA strand breakage nor that of DNA protein crosslinking was sufficient to explain the different drug sensitivity in the two cell lines. At 24 and 48 h after exposure of MCF-7 cells to high concentrations of ATZ, the formation of DNA-topoisomerase I complexes is detected, coincident with a high degree of apoptosis. These studies suggest that ATZ has the capacity to interfere with topoisomerase I in the tumor cell, a function not evident in *cis*-platinum-based drugs.

Keywords: DNA strand breakage; DNA–protein crosslinking; Novel transplatinum compound; Apoptosis; MCF-7; A2780

1. Introduction

Platinum-based cancer chemotherapeutic compounds are effective against a variety of malignancies, including testicular cancer and, in combination with other drugs, cancers of the head, neck, bladder, cervix, ovary and lung [1,2] and as second line therapy in the treatment of breast cancer [3].

A feature common to all platinum compounds currently in clinical use is that the exchangeable ligands, e.g., the two chlorides in *cis*-(diamminedichloro)platinum(II) (cisplatin), are in the *cis* configuration. This requirement is generally regarded as being attributable to the ability of the *cis* compounds to form highly toxic bifunctional intrastrand crosslinks between the N7 atoms of adjacent guanines in double-stranded DNA [4,5]. The geometry of *trans*-(diamminedichloro)platinum(II) completely precludes formation of such lesions, and these isomers are much less cytotoxic and not clinically useful [6]. Substitution of an NH_3 ligand by a planar heterocyclic ligand in *trans*-[$PtCl_2(L)(L')$] (L = L′ = pyridine, thiazole or L= NH_3, L′ = pyridine, thiazole, quinoline etc.) affords compounds with cytotoxicity equivalent to cisplatin and significantly greater than that of *trans*-[$PtCl_2(NH_3)_2$], the ''parent'' *trans* isomer [7–9]. Further these new transplanaramine (TPA) compounds maintain cytotoxicity in several cisplatin-resistant cell lines. The spectrum of activity of the TPA compounds against the NIH panel was

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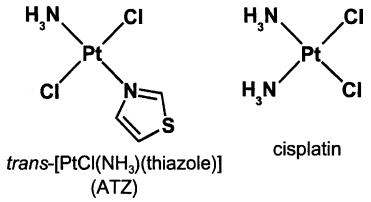

Fig. 1. Structure of *trans*-[$PtCl_2(NH_3)$(thiazole)] (*trans*-amminedichloro(thiazole)platinum(II[TV1]) (ATZ) and cisplatin.

distinctly different—COMPARE analysis indicated little correlation with cisplatin or carboplatin [9,10]. More recent analysis of these data showed that the TPA compounds as a class were relatively more cytotoxic than the conventional cisplatin class toward breast and colon tumor lines, but much less cytotoxic toward central nervous system tumor lines (T. Fojo, N. Farrell, et al., unpublished data). These results raise the possibility that the TPA compounds may have a fundamentally different mechanism of action than the currently approved platinum-based antitumor compounds.

DNA-binding studies show that TPA compounds form significantly higher levels of DNA–DNA interstrand crosslinking than cisplatin [11,12]. Further, while the TPA-induced intrastrand crosslink is formed between the adjacent guanines, similar to that of cisplatin, the interstrand crosslink is distinctly different, involving the guanine N7 and cytosine N3 of the same base pair [13]. Expansion of these mechanistic studies to cellular systems revealed that TPA compounds caused protein-associated DNA strand breaks in murine leukemia L1210 cells [7,9] (N. Farrell, Pommier et al., unpublished data). To further elucidate the nature of these protein-associated DNA strand breaks and the possible differentiation between tissue types, we have examined the cellular and biochemical pharmacology of one specific TPA compound *trans*-[$PtCl_2(NH_3)$(thiazole)] (*trans*-amminedichloro(thiazole)platinum(II) ATZ), (Fig. 1). This compound was chosen because use of the thiazole ligand gave the best combination of aqueous solubility and in vivo antitumor activity in the general structural class found to this point [10]. In this study we confirm the production of DNA strand breaks in human tumor cells and report on the novel formation of DNA-topoisomerase I complexes upon treatment with ATZ. This is the first example of DNA-topoisomerase I complexes induced by platinum-based drugs.

2. Materials and methods

2.1. Materials

ATZ was synthesized as described [14]. Solutions were prepared by dissolving at 100 mM in dimethylformamide (DMF) followed by serial dilution with distilled water to a final concentration of 2.5 mM in 5% dimethylformamide. Cisplatin, a gift of Bristol–Myers Squibb, was similarly dissolved in DMF. Camptothecin (CPT) was provided by the National Cancer Institute Drug Synthesis and Chemistry Branch (Rockville, MD) and dissolved in dimethylsulfoxide (DMSO). Stock solutions of all compounds were stored at −20 °C. A2780 cells were obtained from Dr. Tom Hamilton at the Fox Chase Cancer Center. MCF-7 cells were obtained from the National Cancer Institute, Frederick, MD. Both lines were maintained as monolayer cultures in RPMI 1640 (Gibco) plus 5% fetal bovine serum and 5% bovine calf serum and penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. Cells were routinely subcultured by trypsinization (0.25% trypsin, 0.03% EDTA, Gibco) upon reaching confluence.

2.2. MTT dye assay for drug sensitivity

Cells were plated at a density of 7500 cells per well in 96-well plates and incubated with various drug concentrations for 96 h. Drug was removed, cells were washed twice with cold PBS and incubated with MTT (2 mg/ml in PBS) for 3 h. After removal of the MTT solution, cells were exposed to 100 μl DMSO and the microtitre plate was read at 540 nm in a Molecular Devices Vmax kinetic microplate reader.

2.3. Clonogenic survival

One day before treatment, cells were subcultured in 24-well plates at a density of 3×10^4 cells per well. Cultures were treated with ATZ or cisplatin (or equivalent concentrations of DMF) for 48 h and then trypsinized and plated in duplicate at 800 cells per 100-mm dish. After growth for 8 days, colonies (>50 cells) were stained with crystal violet and counted.

2.4. DNA binding

Overnight monolayer cultures were treated for 5, 12 or 24 h with 25 μM ATZ (or equivalent DMF). One culture was treated for 24 h and then incubated in drug-free medium for 24 h. Cells were harvested by trypsinization, washed, pelleted, and lysed with SDS. Lysates were treated with proteinase K and RNAse, and DNA was isolated by phenol/chloroform-extraction [15]. DNA was precipitated and dissolved in 1 ml of 10 mM Tris–HCl pH 8 and 0.1 mM EDTA (DNA concentration was determined at A260), and bound platinum was determined by atomic absorption spectrometry as described previously [16] using ATZ alone as a reference.

2.5. Alkaline unwinding

Bulk damage to DNA was assessed by alkaline unwinding after 12 h of drug exposure [17] as described in detail previously [18]. To quantify the damage, cultures of each

cell line were exposed to ionizing radiation (1–20 Gy) and a standardization curve for the extent of unwinding versus radiation dose was determined. Drug-induced DNA damage was then expressed in terms of the equivalent radiation dose required to give the same extent of unwinding.

2.6. Detection of apoptotic cells by TUNEL assay

MCF-7 and A2780 cells were grown in chamber slides (100,000 cells/chamber) for 24 h. Cells were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS, pH 7.4 for 1 h at 24 °C. Slides were rinsed with PBS and cells were incubated in freshly prepared permeabilisation solution (0.1 g sodium citrate, 0.1 ml triton X-100 in 100 ml of molecular grade H_2O) for 2 min on ice. Slides were rinsed twice with PBS and 50 μl of TUNEL reaction mixture (In Situ Cell Death Detection Kit, AP. Roche Molecular Biochemicals) was added on the samples. Cells were incubated for 60 min at 37 °C in a humidified atmosphere in the dark. Slides were rinsed three times with PBS and the samples were examined by fluorescence microscopy.

2.7. β-Galactosidase histochemical staining

At the appropriate times after treatment, cells were washed twice with PBS and fixed with 2% formaldehyde, 0.2% glutaradehyde for 5 min. The cells were then washed again with PBS and stained with a solution of 1 mg/ml 5-bromo-4-chloro-3-inolyl-β-galactosidase (X-gal, Gold Biotechnology, St. Louis, MO) in dimethylformamide (20 mg/ml stock), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 40 mM citric acid/sodium phosphate, pH 6.0, and 2 mM $MgCl_2$ [19,20]. Following overnight incubation at 37 °C, the cells were washed twice with PBS, and photographed with a light microscope.

2.8. DNA–protein crosslinks

Cells in log phase of growth were labeled with [^{3}H]thy- μCi/ml, 1 μM) for 24 h, [^{3}H]thymidine was removed and the cells were grown in nonradioactive medium for 4 h prior to addition of drug. After 12 h of drug exposure, cells were washed with PBS and lysed in 1.25% SDS/5 mM EDTA/0.4 mg/ml denatured salmon sperm DNA at 65 °C. Cell lysates were solubilized by four consecutive passages through a 22-gauge needle, and protein was precipitated with KCl at a final concentration of 80 mM [21]. ^{3}H radioactivity in the supernatant was determined and the precipitates were vortexed vigorously, washed and centrifuged to eliminate nonspecifically associated ^{3}H. The precipitates were dissolved in water at 65 °C; DNA–protein crosslink formation was calculated as the fraction of total cell-associated ^{3}H found in the SDS/K precipitates.

2.9. Detection of topoisomerase I-DNA complexes

Topoisomerase I-DNA cleavage complexes were isolated and detected using the immunocomplex of enzyme (ICE) bioassay [22,23]. Briefly, $\sim 1 \times 10^3$ drug-treated or untreated cells were pelleted and immediately lysed in 1% sarkosyl followed by 30 strokes of Dounce homogenizer. Cell lysates were gently layered on CsCl density gradient [22]. After centrifugation (165,000 × *g* in a Beckman SW40 rotor for 24 h at 20 °C), 20 fractions (0.5 ml each) were collected from the bottom of the tubes. For topoisomerase detection, aliquots from each fraction (100 μl) were diluted with an equal volume of 25 mM sodium phosphate buffer, pH 6.5, and applied to Immobilon-P membrane (Millipore, Bedford, MA) by using a slot-blot vacuum manifold. Immunoblotting was performed according to standard procedures using the C21 topoisomerase I monoclonal antibody obtained from Dr. Y.C. Cheng (Yale University, New Haven, CT). Immobilon-P membranes were saturated for 1 h in phosphate buffered saline-Tween 20 (PBS-T) containing 5% non-fat dried milk, probed overnight with the C21 antibody at 4 °C, then probed with the horseradish peroxidase-labeled anti-mouse IgG secondary antibody for an additional hour. Western blotting was visualized by the enhanced chemiluminescence detection system (New England Nuclear/Life Science Products) according to the manufacturer's protocol.

3. Results

3.1. Growth inhibition, clonogenic survival and apoptosis

To compare sensitivity of human MCF-7 breast tumor cells and A2780 ovarian tumor cells to ATZ, cell growth and clonogenic survival were assessed following drug treatment. As shown in Fig. 2A, the IC_{50} values for growth inhibition of MCF-7 and A2780 cells, as determined by MTT tetrazolium dye assay, were approximately 12.5 and 4 μM, respectively. Fig. 2C indicates that the IC_{50} values for clonogenic survival were approximately 2.7 μM (95% confidence limits of 2.25–3.23 μM) and 1.49 μM (95% confidence limits of 1.12–1.98 μM), respectively for MCF-7 and A2780 cells. In both cases, the A2780 cells were approximately two- to threefold more sensitive to the drug. Clonogenic survival assays closely paralleled the MTT assay, indicating that ATZ induced efficient cell killing, rather than merely growth arrest. The cells demonstrated a generally similar pattern of sensitivity to cisplatin using the MTT assay (Fig. 2B), with A2780 cells appearing approximately twofold more sensitive than MCF-7 cells (IC_{50} values of 1.5 and 2.8 μM, respectively).

To determine in more detail the time frame of growth arrest and cell death, viability of MCF-7 and A2780 cells was assessed by trypan blue dye exclusion over the course

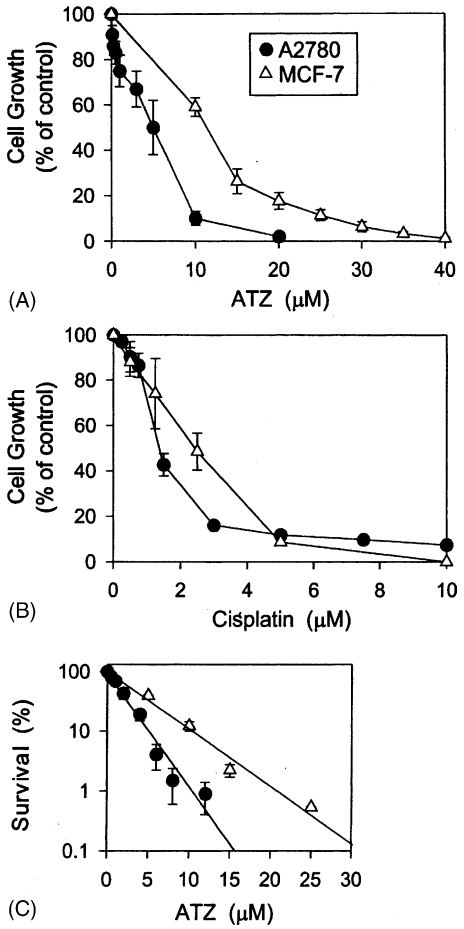

Fig. 2. Sensitivity of A2780 ovarian (●) and MCF-7 breast (△) tumor cells to ATZ and cisplatin. Cells were treated continuously for 3 days with ATZ (A) or cisplatin (B) and cell growth was assessed by MTT conversion. (C) Exponentially growing MCF-7 and A2780 cells were treated with ATZ for 12 h and clonogenic survival was assessed. Each point represents the mean ± S.E.M. for three to four determinations from at least two independent experiments.

of a 3-day treatment with 10 μM ATZ. In addition, apoptosis was assessed by the TUNEL assay. Fig. 3 indicates that the profiles of response to ATZ were virtually identical in the two cell lines; this consisted of a pronounced reduction in viable cell number in the first 12 h after drug exposure followed by a prolonged period of growth arrest (with a further, very gradual decline in viable cell number).

To determine whether the initial cell death was attributable to ATZ-induced apoptosis, TUNEL assays were performed over the course of 3-day treatment with 10 μM ATZ. Quite unexpectedly, few apoptotic cells were evident during the initial interval after drug treatment when cell killing was most pronounced (Fig. 4, 12 h). A small but detectable level of apoptosis was evidcnt in both A2780 and MCF-7 cells at 1, 2 and 3 days after initiation of drug exposure (Fig. 4). A high degree of apoptosis was evident after 24–48 h of treatment of MCF-7 cells with ATZ at 50 μM (data not shown). In contrast, no apoptosis was observed in MCF-7 cells after treatment with 50 μM cisplatin (not shown). Thus, it appears that while apoptosis

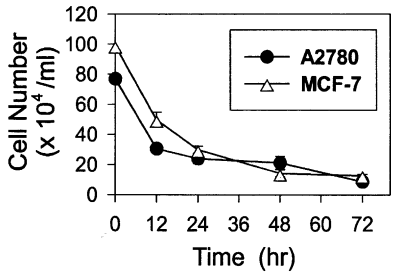

Fig. 3. Cell death and growth arrest in A2780 (□) and MCF-7 (■) cells. Cells were continuously exposed to 10 μM ATZ and cell viability was monitored by trypan blue exclusion over a period of 72 h. Each point represents the mean ± S.E.M. for three independent experiments.

may contribute to loss of clonogenicity, it cannot account either for the initial rapid loss of cell viability, or for the inhibition of overall growth.

3.2. Senescence arrest

In addition to the well-established responses to antitumor drugs such as conventional growth arrest and cell death, there is accumulating evidence that cells exposed to a variety of agents from different drug classes can undergo premature or accelerated senescence [24,25], which is characterized by enlarged flattened cells expressing the senescence marker, β-galactosidase [19]. In view of the prolonged growth arrest response observed after exposure to ATZ, we evaluated the induction of senescence by both ATZ and cisplatin in the MCF-7 cell line, where we have previously shown adriamycin-induced senescence [20]. Fig. 5 indicates that after 96 h, β-galactosidase staining is detectable, but only in a small cohort of the ATZ-treated cell population. This is in contrast to the almost complete senescence that is evident in MCF-7 cells exposed to adriamycin and the large fraction of senescent cells observed after exposure to cisplatin.

3.3. DNA damage

In order to determine whether differences in sensitivity between the cell lines might be attributable to differences in drug uptake, DNA adduction or repair, MCF-7 and A2780 cells were exposed to 25 μM ATZ, and stable (presumably covalent) binding to DNA was quantitated using atomic absorption spectroscopy. (Attempts to measure DNA binding at lower ATZ concentrations did not yield reproducible data.) Fig. 6 indicates that DNA binding and its subsequent reversal were similar in the two cell lines. DNA adducts accumulated over the course of the 24 h of drug treatment and declined to near baseline levels within 24 h after removal of ATZ from the culture medium, presumably reflecting repair of the adducts.

Although the A2780 cells were somewhat more sensitive to ATZ than the MCF-7 cells, there did not appear to be differences in DNA binding, apoptosis or the time course of growth arrest that could account for the differences in

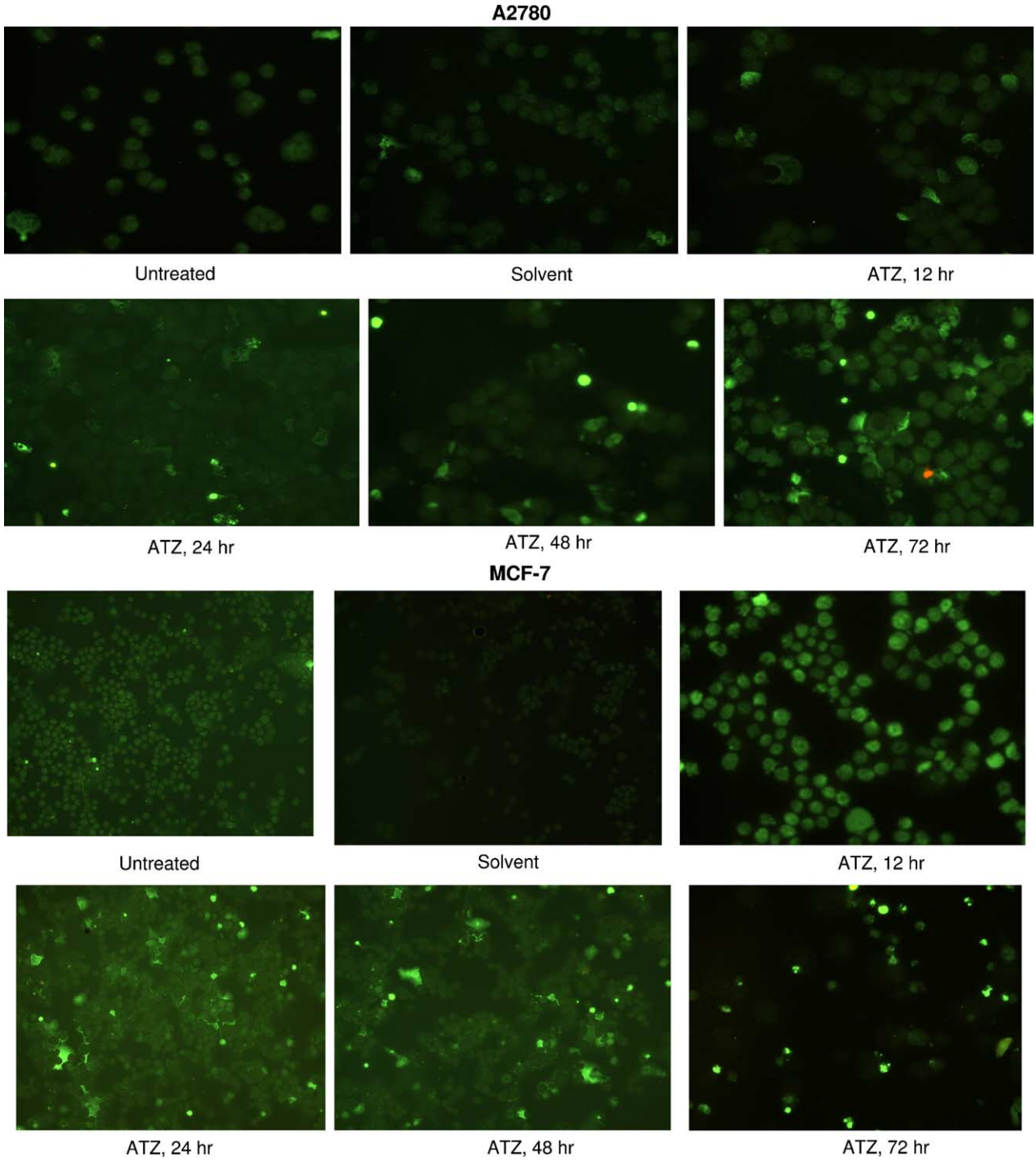

Fig. 4. Detection of apoptosis in MCF-7 and A2780 cells by the TUNEL assay. A2780 and MCF-7 cells were treated with 10 μM ATZ for the indicated times and processed for the TUNEL assay. Cells were examined by fluorescence microscopy. All photomicrographs were taken at equal magnification and exposure times.

sensitivity. As previous work in a leukemic cell line suggested the possibility that topoisomerase I might be a target for the transplatinum compounds [9],[2] we evaluated the induction of DNA strand breaks by ATZ.

Following a 12-h exposure to ATZ, DNA strand breakage in the MCF-7 and A2780 cell lines was assessed by alkaline unwinding, and quantitated in terms of radiation-equivalent damage. As indicated in Table 1, at concentrations of 10 and 25 μM ATZ (corresponding to the IC_{90} and a concentration $\gg IC_{90}$), ATZ produced 280 and 480 rad-equivalents of DNA damage in A2780 cells. At the same concentrations (which correspond to the IC_{30} and the IC_{90} in MCF-7 cells), ATZ produced 275 and 350 rad equivalents of DNA damage in MCF-7 cells. These results are consistent with trapping of topoisomerase I cleavable complexes by ATZ, similar to the action of camptothecin

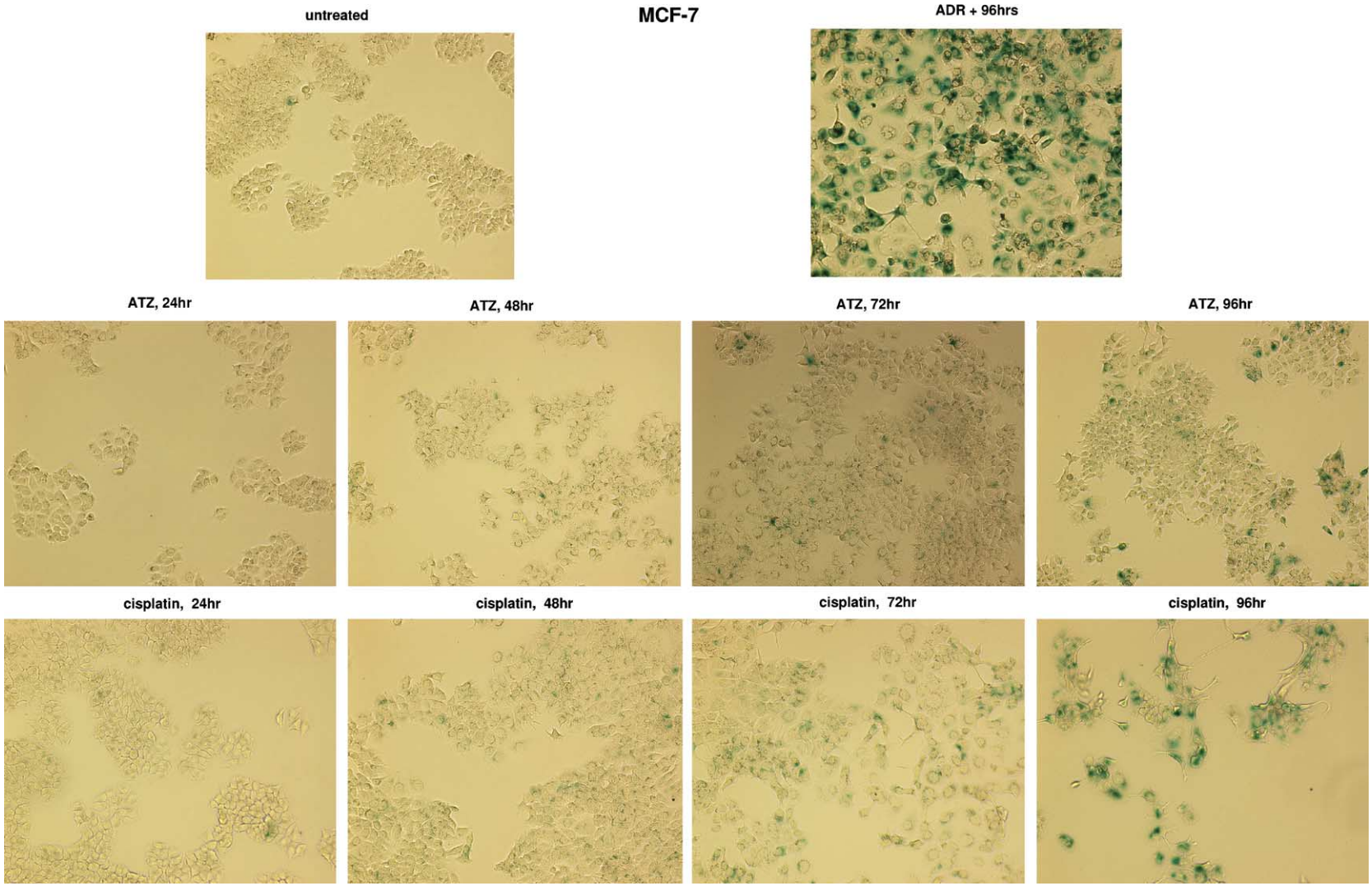

Fig. 5. Detection of senescence in MCF-7 breast tumor cells by β-galactosidase staining. MCF-7 cells were continuously exposed to 10 μM ATZ, 10 μM cisplatin or to 1 μM adriamycin (ADR) for 2 h. β-Galactosidase staining was assessed at the indicated times after initiation of drug exposure.

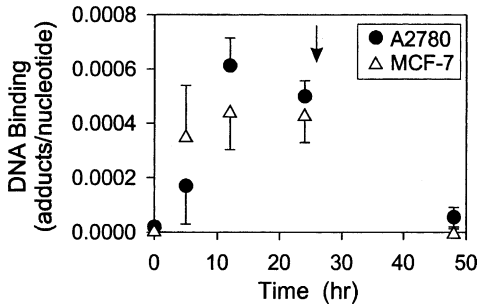

Fig. 6. ATZ binding to DNA. A2780 (●) or MCF-7 (△) cells were treated with ATZ for 5–24 h. One culture was treated for 24 h and then drug was removed (arrow) and the culture incubated in the absence of ATZ for an additional 24 h. Points represent the mean ± S.E.M. of three independent experiments.

[26,27], but could also reflect either inhibition of topoisomerase II, or incision of DNA by repair enzymes as a first step in repair of adducted DNA.

3.4. DNA–protein crosslinking

In order to further explore the possibility that topoisomerases might be a target of ATZ, we assessed DNA protein crosslinking by SDS/K coprecipitation, as described by Hasinoff et al [28]. Fig. 7 (upper panel) indicates that ATZ was capable of inducing DNA–protein crosslinks in A2780 ovarian tumor cells. At concentrations of 25 and 50 μM, ATZ produced increases over baseline of approximately twofold and fourfold, respectively. Cisplatin, even though it was fourfold more cytotoxic than ATZ, failed to produce a detectable increase in DNA–protein crosslinking at 50 μM. Camptothecin, utilized as a positive control at 20 μM, produced an approximately ninefold increase over control values (not shown). Fig. 7 (lower panel) indicates that in MCF-7 cells, 50 μM ATZ produced a 10-fold increase in DNA protein crosslinking compared to controls. Interestingly, in the case of MCF-7 cells, cisplatin produced a small but significant increase in DNA-crosslinks above background. (Camptothecin, utilized as a positive control, produced a 35-fold increase, not shown).

3.5. Formation of topoisomerase I-DNA complexes

Studies in CEM human leukemia cells suggested that ATZ has the capacity to induce the formation of topoisomerase I-DNA (top1-DNA) complexes [9]; in contrast,

Table 1
Rad-equivalent DNA damage induced by ATZ in MCF-7 and A2780 tumor cell lines

Cell line	ATZ 10 μM	ATZ 25 μM
A2780	284 ± 8	484 ± 35
MCF-7	275 ± 7	347 ± 5

Cells were exposed to drug for 12 h and strand breaks were determined using the alkaline unwinding assay, as described in Section 2. Data represent mean and standard error for three to four separate experiments.

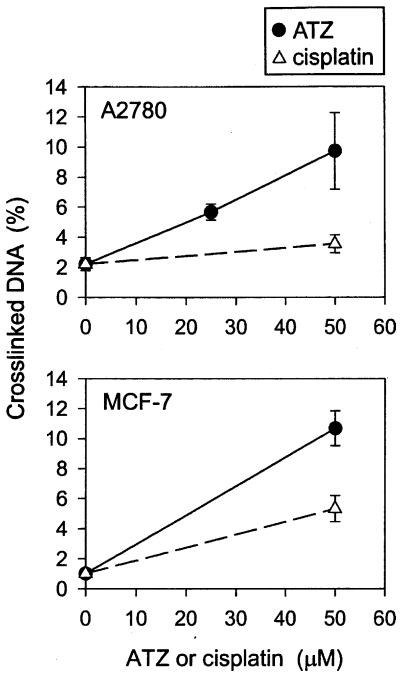

Fig. 7. DNA–protein crosslink induction. The formation of crosslinks between DNA and cell protein was assessed as the percentage of cellular [^{3}H]thymidine associated with protein precipitated by SDS/K. Values are presented as the percentage of total ^{3}H in the precipitate for samples exposed to drug for 12 h, and represent the means and range for two replicate experiments.

no trapping of Topoisomerase II-DNA complexes was observed.[2] Fig. 8 demonstrates the induction of top1-DNA complexes following 24 and 48 h drug treatment in MCF-7 breast carcinoma cells. Although only trace levels of top1-DNA complexes were observed at 10 μM concentration of ATZ, there was substantial increase in the level of these complexes at 50 μM concentration of the drug. As expected, similar top1-DNA complex formation was induced by the known topoisomerase I poison, camptothecin, at 1 μM. Treatment with cisplatin, however, at the same concentrations and under similar conditions to those used for ATZ, did not result in detectable top1-DNA complexes. These results, as well as the strand breakage data, indicate that in contrast to cisplatin, ATZ induces the formation of top1-DNA complexes.

4. Discussion

Cisplatin has found extensive utility in the treatment of testicular and ovarian cancers, as well as in combination chemotherapy for the treatment of breast cancer [1–3]. Although *trans*-platinum-based compounds have generally been considered inactive, a number of modified *trans*-platinum derivatives have shown promise, based on their ability to interfere with the growth of experimental tumor cell lines in culture [7,9]. Furthermore, preliminary work with TPA compounds including ATZ in human leukemic

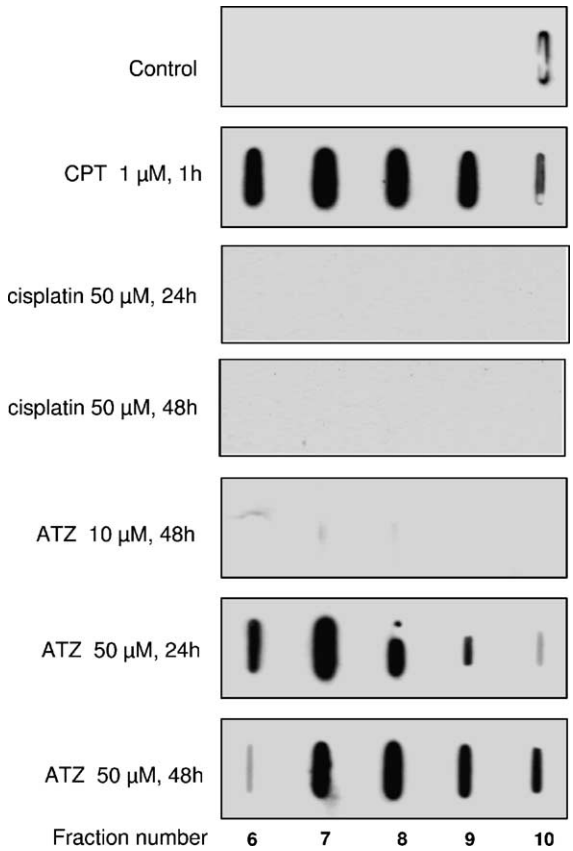

Fig. 8. Detection of topoisomerase I-DNA complexes in MCF-7 cells by ICE bioassay. MCF-7 cells were treated with the indicated concentrations of ATZ, cisplatin, or camptothecin for the times indicated. Cesium chloride fractions were collected from the bottom of the gradients and subjected to topoisomerase I immunoblotting using C21 topoisomerase I monoclonal antibody. Fractions 6–10, encompassing the peak of cellular DNA, are shown. (Previous studies have established that fractions one to five contain neither DNA nor DNA-topoisomerase complexes.)

cells [9] (K. Jones, D. Gewirtz, et al., unpublished data) has suggested the possibility that topoisomerase I may be a molecular target of these agents. Topoisomerase I has been implicated in the cytotoxic effects of camptothecin [26,27] and related compounds, but not those of platinum-based agents.

In view of preliminary evidence from screening against a panel of human tumor cell lines that drugs such as ATZ may have efficacy against breast tumor cells, we studied its biological effects in the well-characterized MCF-7 breast tumor cell line, and an ovarian A2780 tumor cell line. One rationale for this approach was to provide insight into the tumor specificity of platinum-based agents. In general, there is limited understanding of the basis for the action of chemotherapeutic drugs against select malignancies. For instance, it is unclear why drugs such as adriamycin are effective primarily against solid tumors, while cytosine arabinoside is used almost exclusively against hematological malignancies. Similarly, the fundamental basis for activity of cisplatin and carboplatin in testicular and ovarian tumors and, most recently, oxaliplatin in colon cancer, is not fully understood.

The present studies indicate that A2780 cells are approximately two- to threefold more sensitive than MCF-7 cells to both ATZ and cisplatin, but otherwise the response of the two cell lines to ATZ was very similar. DNA binding studies indicated efficient uptake and adduct formation, but equally efficient removal of putative adducts by cellular DNA repair systems. Both lines exhibited a wave of cell death succeeded by a prolonged period of growth arrest. Both cell lines also demonstrated a delayed apoptosis which appeared to involve only a small fraction of the cell population and which was most pronounced only after the bulk of cell death had occurred. In this context, although evidence exists implicating apoptosis in drug sensitivity [29–31], it is becoming increasingly clear that, for many drugs and/or tumor cell types, nonapoptotic modes of cell death account for most of the loss of reproductive capacity [25,32–34].

In the particular case of MCF-7 cells treated with radiation or adriamycin, premature senescence appears to be more important than apoptosis in eliminating the reproductive potential of the cells [20] (K. Jones, D. Gewirtz, et al., unpublished data). Surprisingly, however, ATZ failed to produce a significant degree of senescence even after 96 h. This is in contrast to the senescence produced by cisplatin, which further highlights differences in the mechanisms of action of these two agents.

It is well established that MCF-7 cells lack functional caspase-3 [35] and studies by Blanc et al. [36] have clearly shown that caspase-3 facilitates induction of apoptosis by cisplatin. Although the absence of caspase-3 in MCF-7 cells could in theory explain their greater resistance to ATZ and cisplatin, we detected no difference in apoptotic response between A2780 cells, which have normal caspase-3 and the MCF-7 cell line upon exposure to ATZ. Furthermore, our findings are quite consistent with a number of other reports. Faivre et al. [37] did detect apoptosis at 10 μM concentrations of cisplatin or oxaliplatin after 24 h, similar to our findings; however, far more extensive apoptosis was evident with these agents at concentrations of 50 μM. It is further noteworthy that, as with our own studies, the extent of apoptosis was generally more pronounced at drug concentrations in excess of the IC_{50} values, when examined at 24 h after drug exposure. Similarly, Kolfschoten et al. [38] failed to detect apoptosis in A2780 cells at the IC_{50} value of cisplatin, although these studies were performed with a 1-h incubation rather than continuous drug exposure. Hankel and Turchi [39] also reported low amounts of DNA fragmentation in A2780 cells treated with cisplatin. Taken together, our studies suggest that while apoptosis is clearly one component of the response to ATZ in both MCF-7 and A2780 cells, apoptosis may play a relatively limited role in ATZ cytotoxicity.

Further studies will be required to distinguish whether the covalent binding of topoisomerase I to DNA represents trapping of cleavable complexes [22,23] or bifunctional

binding of ATZ to DNA and topoisomerase I. In preliminary attempts to identify the nature of this association we have thus far been unable to detect ATZ-stimulated DNA strand cleavage by topoisomerase I in vitro (data not shown). However, this failure may reflect the very slow kinetics of ATZ-DNA interactions and/or the fact that direct bifunctional DNA–protein or DNA interstrand crosslinking by ATZ may interfere with detection of the strand breaks. Moreover, although DNA strand cleavage and DNA–protein crosslinking are consistent with trapping of cleavable complexes by ATZ, both strand breaks and crosslinks appeared earlier than covalent topoisomerase-DNA binding, suggesting that some of these lesions may result from other mechanisms and may involve proteins other than topoisomerase I. In particular, strand cleavage could reflect excision repair of ATZ adducts.

Whether topoisomerase I binding is involved in cytotoxicity of ATZ remains to be determined, but the fact that such binding is only detected at doses in excess of the IC_{50} does not necessarily exclude that possibility. As with many DNA damage assays, the ICE assay may not be sensitive enough to detect a level of damage that is nevertheless sufficient to kill a cell. Even with camptothecin, for which topoisomerase I is clearly the primary target [22,27] detection of topoisomerase crosslinking by ICE requires treatment at ~1 μM, which is about 100-fold greater than the IC50 in MCF-7 cells [40]. It is noteworthy that formation of topoisomerase I-DNA complexes in MCF-7 cells at 24 and 48 h after exposure to 50 μM ATZ coincided with the promotion of apoptosis while exposure to cisplatin produced neither topoisomerase I-DNA complexes nor apoptosis. These observations suggest that these complexes could have a close association with the apoptotic response. Regardless of the precise mechanism of top1-DNA complex formation, the finding that these complexes are induced by ATZ but not by cisplatin, suggests that subtle differences in platinum-DNA adduct structure can lead to significantly different downstream effects such as protein recognition and apoptotic pathways.

The greater cytotoxicity of both ATZ and cisplatin toward A2780 cells than toward MCF-7 cells was not associated with greater DNA binding, strand cleavage or DNA–protein crosslinking. Although no differences in gross adduct removal were detected, it remains possible that repair of specific cytotoxic lesions, whether interstrand crosslinks, DNA–protein crosslinks, or protein-associated DNA breaks, is either less efficient or less accurate in A2780 cells. Alternatively, differences in signaling and other events downstream of DNA damage could account for the greater sensitivity.

While relatively few studies have been performed on the cellular effects of ATZ, biochemical studies suggest that its cellular pharmacology may be quite complex, and may involve three distinct types of DNA lesions– monofunctional adducts and bifunctional DNA–DNA crosslinks as well as ternary DNA–protein crosslinks [41–43]. Ternary DNA-Pt-protein adducts for a variety of platinum complexes have been reported [42]. Although cisplatin-induced DNA–protein crosslinks appear not to contribute significantly to cytotoxicity [43], the altered stereochemistry of ATZ could increase its propensity to directly form stable links between DNA and histones or other chromatin proteins, or could make repair more difficult. Model studies on the comparative kinetics of binding of methionine (model for protein) and 5'-GMP (model for DNA) to *trans*-[PtCl(9-EtGuanine)(NH_3)(quinoline)] support the concept that protein binding is kinetically favored [44].

These mechanisms are not necessarily mutually exclusive and if monoadducts, interstrand crosslinks and DNA–protein crosslinks all contribute substantially to ATZ cytotoxicity, acquisition of drug resistance may be a relatively rare event, since it would have to entail resistance to or tolerance of multiple, structurally dissimilar DNA lesions.

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