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Promotion of DNA strand breaks, interstrand cross-links and apoptotic cell death in A2780 human ovarian cancer cells by transplatinum planar amine complexes

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ABSTRACT

Cisplatin is one of the primary drugs utilized in the treatment of ovarian cancer. However, despite the initial effectiveness of chemotherapy in suppressing this disease, drug resistance almost invariably develops and cures are relatively rare. While it is generally thought that only compounds of the *cis* geometry express antitumor activity, a number of transplatinum derivates have shown preclinical promise. The current work investigates the influence of transplanaramine (TPA) compounds of structure *trans*-[Pt $(O_2CR)_2$ (L) (L′)], (L = NH_3, L′ = pyridine, quinoline, isoquinoline; L = L′ = pyridine; R = H, CH_3, CH_2OH, etc.) (with a focus on the contribution of the carboxylate leaving group to drug action) on growth and viability of A2780 human ovarian carcinoma cells as well as their putative mechanism(s) of cytotoxicity. The compounds, as a class, induce cell death through caspase-dependent apoptosis, with activation of both caspase 3 and caspase 9 and concomitant PARP cleavage. The *trans*-platinum compounds tested show induction of p53 as well as time dependent γH2AX induction, consistent with the promotion of DNA lesions. *trans*-[$Pt(O_2CH)_2(NH_3)$(4-pic)] can be shown to promote significant DNA strand breaks and DNA interstrand cross-linking. The enhanced cytotoxicity of *trans*-[$Pt(O_2CH)_2(NH_3)$(4-pic)] compared to its isostructural $-O_2CCH_3$ and $-O_2CCH_2OH$ analogs may be a consequence of its accelerated cellular accumulation, increased hydrolytic activation, interstrand cross-linking and abortive efforts by the cell to repair the cross linked DNA.

1. Introduction

The platinum based anticancer drugs cisplatin and carboplatin (*cis*-[$PtCl_2(NH_3)_2$], c-DDP; [$Pt(CBDCA)(NH_3)_2$], CBDCA = 1,1-cyclobutanedicarboxylate, carboplatin), either alone or in combination with other drugs, have proven effective against a variety of malignancies including ovarian, testicular, head, neck and bladder cancers [1–3]. More recently, oxaliplatin in combination with 5-fluorouracil (5-FU) has found clinical use for treatment of colorectal cancer [4,5]. These platinum compounds all have their exchangeable ligands in the *cis* configuration and form presumably toxic bifunctional 1,

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2-intrastrand cross-links between the N7 atoms of adjacent guanines in double-stranded DNA [6]. The *trans* isomer, *trans*-[$PtCl_2(NH_3)_2$], *trans*-DDP is much less cytotoxic than its *cis* counterparts, due in part to the fact that the *trans* geometry precludes formation of these 1,2-intrastrand cross-links [7].

Substitution of an NH_3 ligand by a planar heterocyclic ligand in *trans*-[$PtCl_2(L)(L')$] (L = L′ = pyridine, thiazole; L = NH_3, L′ = pyridine, quinoline, isoquinoline, thiazole, etc.) affords transplanaramine (TPA) compounds with cytotoxicity significantly greater than that of the ''parent'' *trans*-[$PtCl_2(NH_3)_2$] [8–10]. In general, cytotoxicity is equivalent to that of cisplatin, with IC_{50} values falling in the 1–10 μM range, with activity expressed in both cisplatin and oxaliplatin-resistant cells [10]. Further, the TPA compounds demonstrate a unique profile of cytotoxicity across the NCI tumor panel with TPA compounds, as a class, showing comparatively more activity in breast and colon cancers compared to cisplatin [10].

For mechanistic studies, the prototypical compound has been *trans*-amminedichloro(thiazole)platinum(II) (*trans*-[$PtCl_2(NH_3)$(thiazole)], ATZ), which is moderately water-soluble and moderately active *in vivo* [11]. The DNA binding profile of TPA compounds is typified by a significantly higher level of DNA–DNA interstrand cross-linking than cisplatin [12], although the cross-link formation between two adjacent guanines of a (GC) base pair is similar to that of cisplatin [6,13–15].

In the context of drug development, modification of the *trans*-platinum structure to an N_2O_2 donor set *trans*-[$Pt(O_2CR)_2(L)(L')$] (where O_2CR is a carboxylate ligand) enhances solubility while retaining the cytotoxicity of the parent chloride compounds [16]. The N_2O_2 series interestingly retains activity in cisplatin and oxaliplatin-resistant cell lines [8]. The ''carboxylate strategy'' has proven successful for clinically available compounds such as carboplatin and oxaliplatin and in the present case, the placing of two carboxylates in a mutually *trans* positions produces a system which is remarkably stable to hydrolysis. Further, modulation of the chemical properties such as hydrolysis and rates of substitution can be achieved by varying the nature of the carboxylate ligand as well as the planar amine [16]. Given the distinct differences in chemical reactivity between compounds with chloride and carboxylate leaving groups, it is of interest to compare their cellular effects.

The demonstrated cytotoxicity of novel *trans*-platinum compounds implies activation of cellular pathways associated with growth arrest and cell death, pathways once thought to be restricted to c-DDP. This paper reports on the cellular pharmacology of selected N_2O_2 compounds including their cytotoxicity, cellular accumulation, p53 induction, apoptosis and DNA cross-linking. Our studies show that structural modification of the *trans*-platinum geometry can lead to compounds that are effective against ovarian carcinoma cells and that may ultimately prove to be useful in the clinical treatment of ovarian cancer.

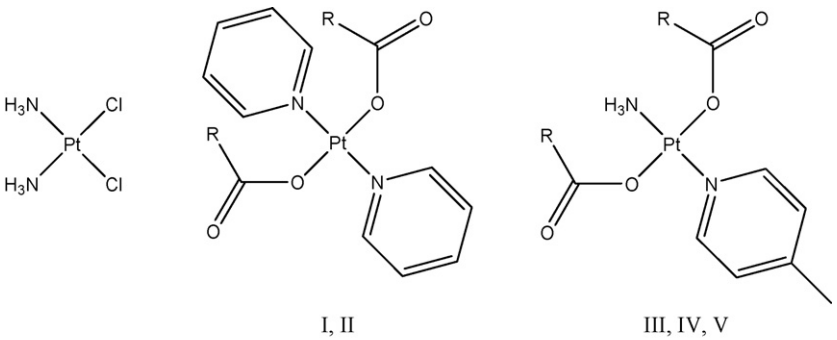
2. Materials and methods

2.1. Compound synthesis

The platinum compounds utilized in the current studies were synthesized using methods previously reported [8,16] and are shown in Fig. 1

2.2. Cell lines, growth and viability assays

A2780 human ovarian carcinoma cells were cultured in RPMI (Gibco, Carlsbad, CA) 5% FBS, 5% BCS (Bovine Calf Serum), 0.25% penicillin/streptomycin. Cells were routinely passaged at 80–90% confluency. For the MTT assay, cells were plated at a density of 6500 cells per well in a 96-well plate and incubated overnight at 37 °C in a 5% CO_2 atmosphere. Cells were treated with varying drug concentrations for 72 h. After drug removal, cells were washed twice with cold PBS and incubated with MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (2 mg/ml in PBS) for 3 h. The excess MTT (Sigma, St.Louis, MO) was then removed, the dye dissolved in 100 μl of DMSO (Sigma, St.Louis, MO), and absorbance was read on a Molecular Devices Vmax kinetic microplate reader at 540 nm.



	R =
I, III	CH_3
II, IV	CH_2OH
V	H

Fig. 1 – Structures of the platinum compounds utilized in the current study.

Trypan blue exclusion was used to determine cells viability. Briefly, 3.5×10^5 cells were plated and incubated overnight. Cells were then treated with the IC_{90} concentration of the appropriate compound and viability monitored as a function of time. After 4 days, remaining cells were washed and fresh media added to the plates. Cell viability was monitored for an additional two days to assess proliferative recovery.

2.3. Detection of apoptotic cells based on subdiploid DNA

Vybrant apoptosis kit #3 was obtained from Invitrogen (Carlsbad,CA) and utilized as per the manufacturers' protocol. Briefly, cells were fixed in an ethanol and fetal bovine serum solution, washed with PBS, and stained with a solution of Propidium Iodide (PI) and Rnase A, as previously described [17]. Annexin V staining was performed with the TACS Annexin V-FITC kit from Trevigen, according to the manufacturer's specifications. Samples were analyzed for subdiploid DNA on a Becton Dickinson FACScan flow cytometer (BD Biosciences, San Jose, CA).

2.4. Caspase activation assay

Staining for active caspases was performed using caspase kits (Immunochemistry Technologies, LLC, Bloomington, MN) as per manufacturer's instructions.

2.5. Western blot analysis of protein expression

After treatment of cells at the IC_{90} concentrations of the indicated drugs, cells were harvested, lysed (2% SDS, 0.06 M Tris–HCl pH 6.8), and protein concentrations determined by the Lowry Method. Protein (30 μg) was electrophoresed on a 12% polyacrylamide gel and the gels were transferred to a nitrocellulose membrane and incubated with the appropriate antibodies: p53 (1:5000 BD Biosciences, San Jose, CA), p21 (1:2000 BD Biosciences, San Jose, CA), γH2AX clone JBW301 (1:1000 Upstate, Charlottesville, VA), PARP (1:2000, Trevigen, MD). After incubation with Anti-mouse secondary antibody (1:3000 Sigma, St. Louis, MO), signals were detected by enhanced chemiluminescence (Pierce, WI)

2.6. DNA damage detection by alkaline unwinding

Total DNA damage was determined by the alkaline unwinding assay 12 h post treatment as described previously [18]. DNA damaged induced by *trans*-platinum compounds was expressed in terms of radiation doses required to induce a similar degree of unwinding.

2.7. Cellular platinum uptake

8×10^6 cells were incubated overnight at 37 °C. Drug was then added at equimolar (25 μM) concentrations and incubated for a further 16 h. The inoculum was removed and the cells were washed twice with 1X PBS. The pellets were digested with 12N HNO_3, 30% H_2O_2 and 12.1 N HCl. Digestion was in accordance to United States Environmental Protection Agency procedure 3050b (all volumes reduced by 1/10) and diluted with Milli-Q Water (Millipore Corporation, Billerica, MA). The samples were analyzed by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry)(Varian).

2.8. Assessment of DNA cross-link formation

DNA cross-link formation can be monitored based on the reduction in radiation-induced Comet tail formation detected after drug treatment. The Comet assay kit was obtained from Trevigen (Gaithersburg, MD). For the cross-linking assay, cells were treated and upon harvesting, diluted to 1×10^5 cells/ml in Ca^{2+}, Mg^{2+} free 1X PBS (Gibco, Carlsbad, CA), and then exposed to 5 Gy radiation from a ^{137}Cs source. Images were obtained on an Olympus IX70 microscope with 40X magnification using SYBR Green at an excitation wavelength of 494 nm and an emission wavelength of 521 nm. Comets were scored from three independent experiments with a total of 100 cells being analyzed. Comets were analyzed using comet-score freeware obtained from TriTek Corporation. Calculations were based on the Olive Tail Moment [19]. The percentage cross-linking was calculated based on the following formula:

$$\%\ \text{decrease MOTM} = \left[\frac{1-(\text{MOTM}_{\text{di}}-\text{MOTM}_{\text{cu}})}{(\text{MOTM}_{\text{ci}}-\text{MOTM}_{\text{cu}})} \times 100\right];$$

where $MOTM_{di}$ is drug treated irradiated sample, $MOTM_{cu}$ is untreated unirradiated control and $MOTM_{ci}$ is untreated irradiated control [20].

2.9. Statistical analysis

Statistical analysis was carried out using Graphpad Prism 4.

3. Results

3.1. Growth inhibition and cell death

Sensitivity of A2780 human ovarian carcinoma cells to the compounds shown in Fig. 1 was found to range between 6.2 μM and 19.8 μM for the IC_{50}, and between 15 μM and 58 μM for the IC_{90} (Table 1), consistent with previous reports [8]. Notably, compound V, *trans*-[Pt $(O_2CH)_2(NH_3)$(4-pic)], demonstrated considerably lower IC_{50} and IC_{90} values (6.2 μM and 15 μM, respectively) than the other transplatinum compounds

Table 1 – IC_{50} and IC_{90} values were determined after 72 h of drug exposure by the MTT dye assay

Drug	IC_{50} (μM)	IC_{90} (μM)	% Apoptosis (IC_{90})
cDDP	0.8 ± 0.2	7.8 ± 1.8	74.9 ± 6.5
I	18 ± 2.3	40 ± 3.2	70.8 ± 2.3
II	10 ± 4.1	23 ± 1.6	74.3 ± 3.4
III	19.8 ± 3	58 ± 5	67.5 ± 3.3
IV	11 ± 2.4	20 ± 3	64.4 ± 12.5
V	6.2 ± 1.2	15 ± 1.3	79.3 ± 5.1

The % apoptosis was determined by assessment of Annexin V positive/Propidium Iodide negative cell populations by FACS analysis.

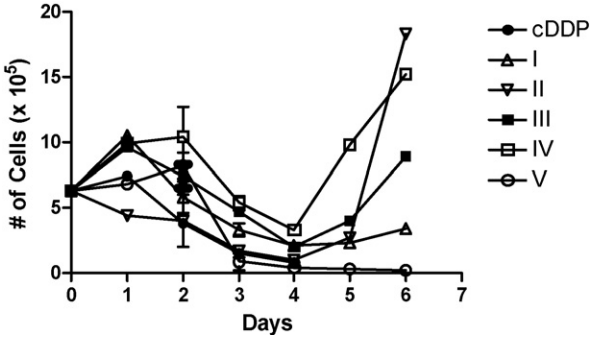


Fig. 2 – Cell death and growth inhibition in A2780 cells treated with *trans*-platinum compounds. Cells were treated at the IC_{90} concentration of drug continuously for 4 days and viable cell number was monitored by trypan blue exclusion. Each point represents the mean ± S.E.M. for three independent experiments performed in duplicate. Where S.E.M. bars are not visible, the error is encompassed within the symbol.

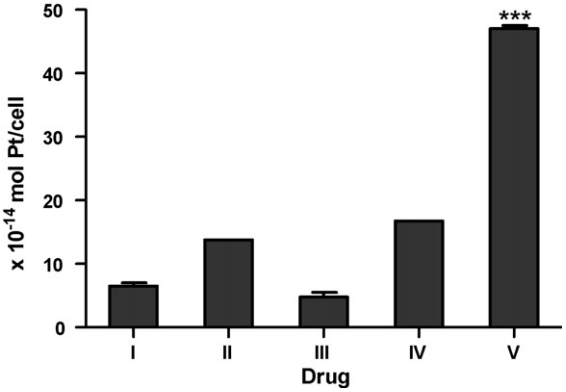


Fig. 3 – Platinum accumulation in A2780 cells. Cellular platinum accumulation was measure by ICP after 16 h of treatment at equimolar concentration of the indicated compound. Each point represents the mean ± S.E.M. for three independent experiments. Where S.E.M. bars are not visible, the error is encompassed within the bar. Compound V shows significantly higher intracellular levels than compounds I-IV * indicates $P < 0.0001$.**

tested, demonstrating the enhancement of cytotoxicity within this series using the more reactive formate ligand.

To distinguish between growth arrest and cell death effects of the *trans*-platinum compounds, cell viability was monitored by trypan blue exclusion over the course of 6 days. Fig. 2 indicates that at the IC_{90} concentrations, all of the tested compounds, including c-DDP, induced cell death. Interestingly, some of the compounds (notably II, III and IV) appeared to allow proliferative recovery over the time frame of this study; in contrast, cells treated with cDDP, I and V remained in an arrested state. Propidium Iodide/Annexin V staining of drug treated cells demonstrated apoptosis 24 h post treatment (Table 1) confirming the ability of these compounds to exert cytotoxic effects, observations that will be further developed in a subsequent section.

3.2. Cellular uptake of platinum

The factors that are usually thought to contribute to platinum drug cytotoxicity are cellular uptake, DNA binding and adduct formation. To examine the effect of different carboxylate leaving groups on drug accumulation, the cellular levels of the *trans*-platinum compounds I–V were measured after a 16 h exposure to the drugs. Consistent with its enhanced cytotoxicity, the uptake of compound V was approximately 3-fold greater than that of the other compounds tested, as shown in Fig. 3.

3.3. Promotion of apoptosis

Cytotoxic *trans*-platinum compounds including *trans*-$[PtX_2(NH_3)(pyr)]$ and *trans*-$[PtX_2(pyr)_2]$ (X = Cl, O_2CCH_3) induce apoptosis in a variety of cell types [21–23]. The structurally related compounds III, IV and V were chosen for more detailed study and to examine the effect of the leaving group within an isostructural series. As caspase cleavage is an important indicator of apoptosis, we assessed both caspase 3 and 9 cleavage as these are two of the most important pro-apoptotic caspases. Caspase 9 plays an integral role in the apoptotic process, and makes up the apoptosome complex along with cytochrome c and apaf1, that cleaves caspase 3 [24]. Fig. 4A indicates that compounds III, IV and V all promoted cleavage (i.e. activation) of caspase 3 and 9, as did cDDP. Fig. 4A and B further indicate that the pattern of both caspase 3 and caspase 9 cleavage for *trans* compounds generally paralleled that of cDDP. The reason for the slight decrease in the levels of caspase 3 cleavage for compound IV, *trans*-$[Pt(O_2C\text{-}CH_2OH)_2(NH_3)(4\text{-pic})]$, at the 96 h time point is not clear but could represent an effort by the cells to evade apoptosis. The PI staining for sub G_0 cells in Fig. 4C is further consistent with an apoptotic mode of cell death.

To further establish that apoptotic pathways were similar for the *trans*-platinum complexes and cDDP, poly(ADP-ribose) polymerase (PARP) cleavage induced by cDDP and compounds III–V was assayed. PARP is a downstream substrate of caspase 3 that is required for cellular integrity [25] and its *in vivo* cleavage by caspase 3 serves as a key indicator of apoptosis [25]. In Fig. 4D, we show that all the compounds induced moderate levels of PARP cleavage at 48 h and significantly higher levels at 72 h. These data are consistent with the caspase cleavage/activation data that show increasing levels of cleavage between hours 48 h and 72 h.

3.4. The DNA damage response

The promotion of apoptosis is likely to be related to the induction of DNA damage by the transplatinum compounds. One hallmark of the DNA damage response pathway is the induction of p53, which plays a pro-apoptotic role, in part by inducing bax and downregulating bcl-2 [26]. The ability of the *trans*-$[Pt(O_2CR)_2(L)(L')]$ compounds to induce the p53 protein was assessed after 24 h of continuous treatment (Fig. 5). All drugs tested induced p53 after 24 h, with levels similar to that of cisplatin. As would be expected, drug treatment also

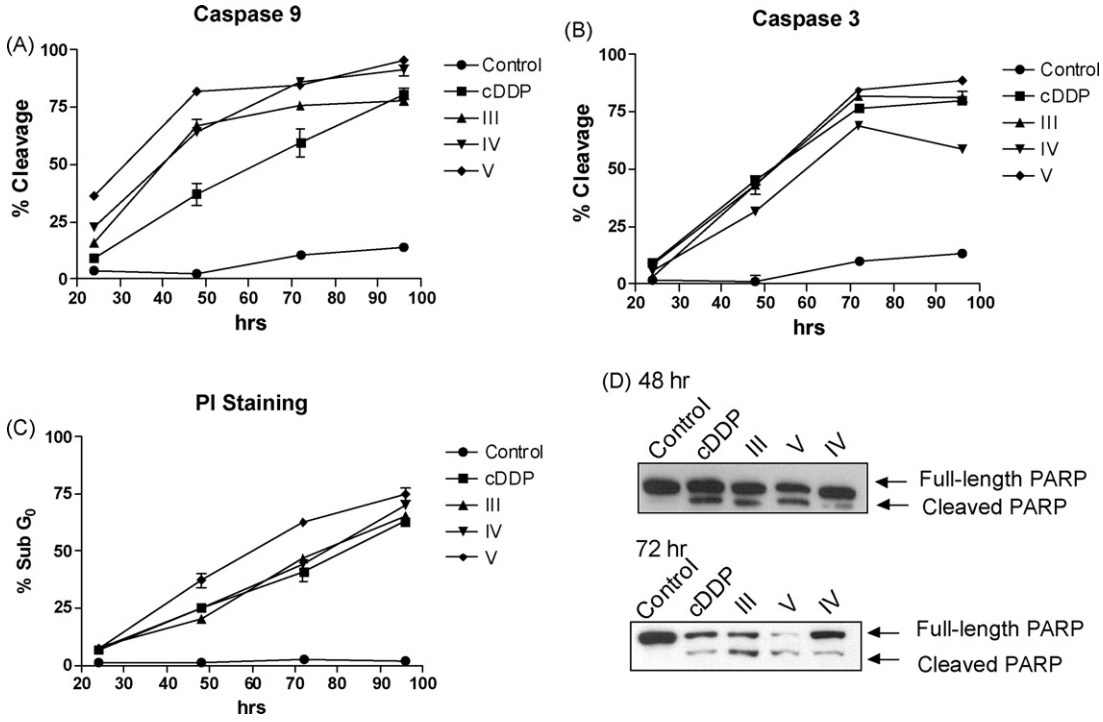


Fig. 4 – Promotion of apoptosis in A2780 cells. Apoptosis was determined by caspase and PARP activation (cleavage). (A) Caspase 9 cleavage induced by TPAs. (B) Caspase 3 cleavage induced by TPAs. (C) % sub G_0 cells as determined by FACS analysis. (D) PARP cleavage was assessed at 48 h and 72 h post treatment with drugs. Data presented in Panels A, B and C are representative of two independent experiments ± range. Where range bars are not visible, the error is encompassed within the symbol.

induced p21, one primary transactivational target of p53 [26]. The induction of p21 could reflect the fact that the cells undergo cell cycle arrest at 24 h. This is the first demonstration that cytotoxic *trans*-platinum compounds induce expression of the p53 tumor suppressor protein as an initial response to cytotoxic stress, similar to cDDP, indicating that p53 may play a role in the apoptotic death induced by these *trans*-platinum drugs.

As the induction of p53 may reflect the generation of DNA lesions, our subsequent studies addressed the type(s) of DNA damage induced by the *trans* planar amines. Previous studies have shown that *trans*-[$PtCl_2(NH_3)$(thiazole)] (ATZ) induces

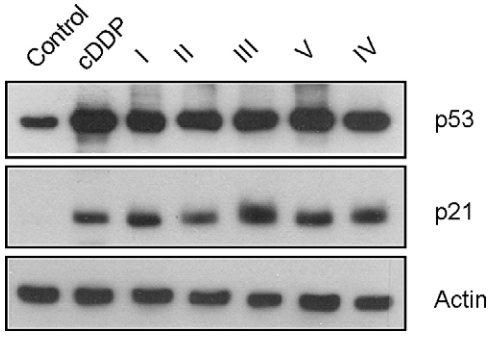


Fig. 5 – p53 expression in A2780 cells. A2780 cells were treated for 24 h with IC_{90} concentration of each drug. Western blots are representative of three independent experiments.

DNA strand breaks in A2780 and MCF-7 cells [27]. In parallel, *in vitro* work has shown that transplatinum compounds in general form interstrand cross-links [6,12,27]. In principle, interstrand cross-links might lead to double-strand breaks as the cells attempt to repair these lesions.

Bulk damage to DNA was evaluated using the alkaline unwinding assay [18]. Fig. 6 indicates that compound V showed significant levels of DNA strand breakage while compounds II and IV showed only baseline levels of damage. DNA breakage induced by ATZ was utilized as a positive control. The more pronounced damage evident with compound V may reflect its more rapid uptake as well as the fact that the faster hydrolysis rate of the formate compound could lead to more and faster DNA binding.

To determine whether the DNA damage was characteristic of double strand breaks, we evaluated the expression of γH2AX, a DNA damage repair protein that is specifically recruited to sites of double strand breaks and which may play an important role in the recruitment of DNA repair proteins [28,29]. γH2AX expression was induced by c-DDP within 16 h, and by the *trans* platinum compounds at 24 h (Fig. 6B). The trend for the *trans* compounds was qualitatively consistent with the data in Fig. 6A, where compound V produced more damage at earlier time points. Again, these observations are not surprising, as the hydrolysis and DNA binding of cDDP is faster than that of the carboxylate compounds; furthermore, within a carboxylate series, the presence of the formate ligand as leaving group results in a more rapid rate of aquation [8,16].

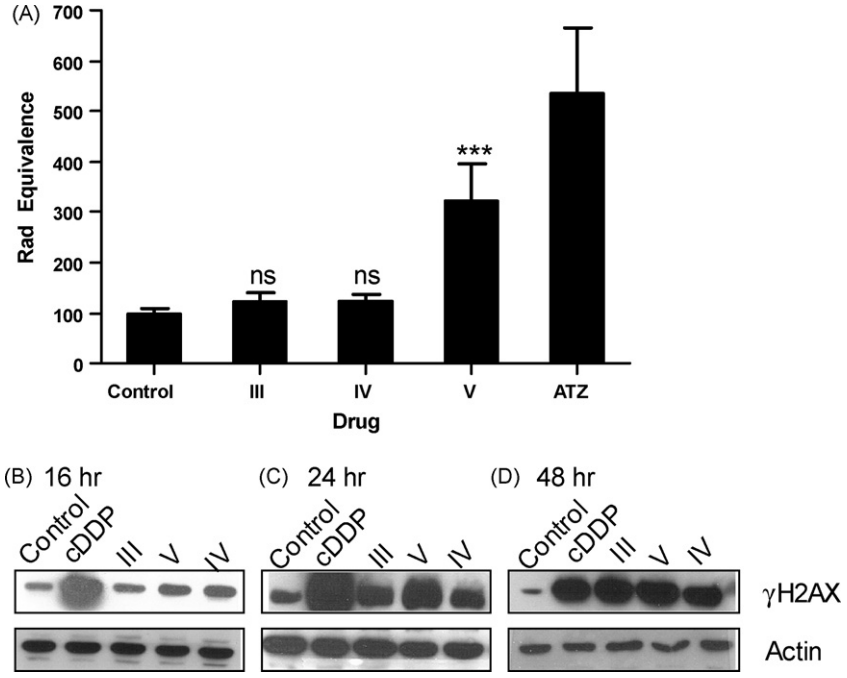


Fig. 6 – (A) Induction of DNA breaks in A2780 cells. Total strand breakage was assessed by alkaline unwinding at 16 h. Data presented represents means ± standard errors. *P*-values were calculated in comparison to control (untreated cells). For compound V, * indicates $P < 0.0001$; (B–D) γH2AX expression in response to TPA treatment. A2780 cells treated at the IC_{90} concentration were analyzed at 16 h (B), 24 h (C) and 48 h (D) post treatment. Western blots are representative of three independent experiments.**

3.5. *DNA interstrand cross-linking*

To assess the levels of interstrand cross-linking, the alkaline comet assay was used to quantify and visualize the cross-links formed after treatment with compound V, *trans*-[Pt$(O_2CH)_2(NH_3)$(4-pic)]. The drug-induced reduction in tail length in irradiated cells observed in Fig. 7A (3 lower panels), which presents representative images of the 12 h, 16 h and 24 h comets, respectively, is indicative of increased cross-linking. The data presented in Fig. 7B indicate that compound V promoted the time-dependent formation of DNA interstrand cross-links in A2780 cells.

4. Discussion

The activation of the *trans* geometry by the replacement of an NH_3 with a planar amine is a relatively novel strategy for the development of platinum based drugs with biological activity [10,30]. Such complexes, and similar ones containing alicyclic or aliphatic heterocyclic amines generally show similar cytotoxicity to cDDP and, in addition, retain activity in cDDP-resistant cell lines [21,31–33]. To date, most mechanistic work has been carried out in studies using transplatinum compounds where the leaving groups have been chlorides. Recently, novel kinetically inert but water-soluble *trans*-platinum compounds containing an N_2O_2 donor set, *trans*-[Pt$(O_2CR)_2$(L)(L′)], have been described [16,23,27]. Despite much mechanistic work on DNA binding of the various cytotoxic *trans*-platinum complexes, there have been few efforts to evaluate the biological effects of these compounds in tumor cell models. The current studies demonstrate that suitable chemical modification within the *trans* geometry does generate transplatinum compounds that produce similar biological responses to that of cDDP in a human ovarian cancer cell line.

The cytotoxicity results are consistent with previous work involving this series of *trans*-platinum compounds [8,10,23] in that the tumor cells were most sensitive to compound V, *trans*-[Pt$(O_2CH)_2(NH_3)$(4-pic)]. It has previously been noted that acetate derivatives with mixed picoline and amine ligands may differ from those with two picoline ligands [23] in terms of their cellular pharmacokinetics. This aspect of drug structure is supported by the enhanced accumulation of compound V, *trans*-[Pt$(O_2CH)_2(NH_3)$(4-pic)], compared to the isostructural acetate and hydroxyacetate analogs (compounds III and IV, respectively). For purposes of comparison, the accumulation of compound V exceeds that of cDDP, which is $5.45 \pm 1.06 \times 10^{-14}$ mol Pt/cell. Cellular uptake generally correlates with water-solubility in this closely related series. Interestingly, the *trans*-[Pt$(O_2CCH_3)_2(NH_3)$(4-pic)] compound showed similar levels of uptake in Pam 212-*ras* cells [23] as in the A2780 cells assessed here.

In addition to its enhanced uptake, compound V, which has a faster rate of hydrolysis, ($k_1 = 10.7 \times 10^{-6}\ s^{-1)}$ than III and IV ($k_1 = 3.21$ and $5.61 \times 10^{-6}\ s^{-1}$), respectively [16], does not permit proliferative recovery to occur; in contrast, extensive recovery is noted with III and IV. Since aquation is considered the rate-limiting step for reaction of platinum compounds with target DNA, it is likely that the more rapid accumulation

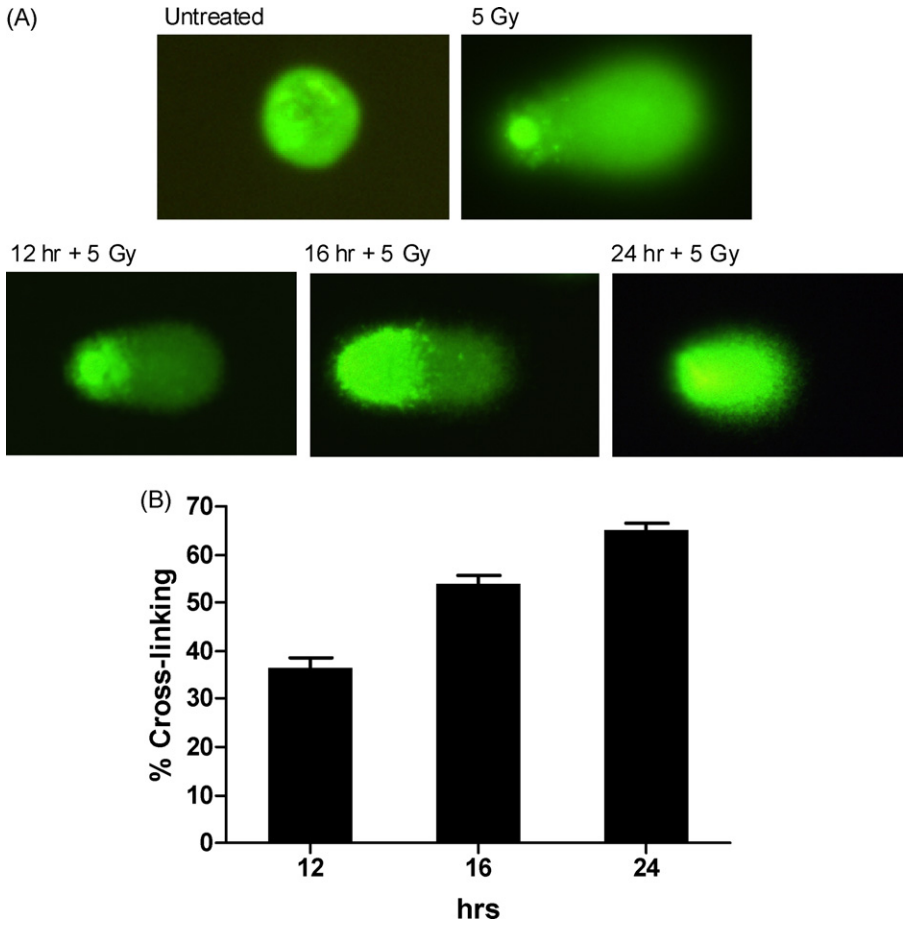


Fig. 7 – DNA interstrand cross-link formation. (A) Representative images of each time point. (B) Time course of cross-linking induced by compound V. Each point represents the mean ± S.E.M. for three independent experiments with a total of over 100 cells examined at each time point. Mean Olive Tail Moments were 4.57 ± 1.89 for untreated controls and 51.57 ± 10.01 for cells irradiated with 5 Gy.

of compound V in the cell contributes to its capacity to promote more extensive DNA damage. This line of reasoning can also be applied to the cytotoxicity (IC_{50} and IC_{90}) values observed for the *trans*-platinum compounds as compared to cDDP. The determined rates of hydrolysis of trans-[Pt(amine)$_2$(O_2CR)$_2$] are, in general, at least an order of magnitude slower than that of cDDP, $k_1 = 5.18 \times 10^{-5}\ s^{-1}$ [16,34].

cDDP mediated DNA damage has been shown to up-regulate p53 in A2780 cells [35]. This paper demonstrates for the first time that *trans*-platinum compounds can also induce p53 up-regulation in human tumor cells, raising the possibility that p53 contributes to the cytotoxic effect of these compounds, particularly the promotion of apoptosis.

The induction of p53 may be related to DNA interstrand cross-linking and/or the generation of DNA breaks. In general, introduction of a planar amine in TPA compounds produces different distortions in DNA because of the steric hindrance and stacking properties of the planar ligand [6,36]. Furthermore, the structural consequences of bending and unwinding are different from the cDDP interstrand cross-links; specifically, the bending angle towards the minor groove is 45° for cisplatin compared to 22° for ATZ [12]. Further, the TPA interstrand cross-links are poor substrates for nucleotide excision repair [37]. Thus, the p53 response may be related, in part, to these diverse structural distortions. Recognition by p53 of *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)interactions with DNA has been documented by *in vitro* gel shift assays in cell free systems [38].

The ability of *trans*-platinum compounds to induce apoptosis has previously been reported by generalized Propidium Iodide or Annexin V assays [22,23]. Caspase 3 is a critical effector caspase that leads to PARP cleavage [24], one of the final steps in the caspase cascade and execution of apoptosis. Our current work demonstrates the cleavage (activation) of caspases 3 and 9 as well as PARP.

Induction of strand breaks and interstrand cross-link formation [27,39] are a well-known feature of *trans*-platinum complexes. The gross assessment of strand break formation in the current work does not address the origin of the damage; however, as noted earlier, the distortion in DNA formed by these types of compounds is such that drug-induced strand breaks cannot be discounted. The relatively high degree of strand breakage induced by *trans*-[Pt(O_2CH)$_2$(NH_3)(4-pic)], and subsequent up-regulation of γH2AX further substantiate the capacity of *trans*-platinum to induce DNA damage. Though γH2AX formation does not differentiate between drug induced

and repair mediated double strand breaks, these results support the possibility of a relationship between the kinetics of DNA damage and the ability of the cells to recover.

As previously stated, the interstrand cross-links induced by *trans*-platinum compounds of the type *trans*-[$PtCl_2(NH_3)(L)$] (L = pyridine, thiazole) are between adjacent GC base pairs [23]. The ability of these *trans*-platinum compounds to form interstrand cross-links may serve to both promote γH2AX formation and apoptosis. It is plausible that the DNA cross-linking serves as the initial insult to the replicative ability of the cells that is followed by strand breaks as measured by γH2AX and alkaline unwinding assays as the cell attempts to repair the cross-links. Subsequently, apoptosis is induced by the cleavage of caspases, possibly as a consequence of the inability of the cells to repair the damage. It is further noteworthy that DNA lesions produced by the *trans*-platinum compounds are generally not repaired as efficiently as those for cisplatin [37,40]. In particular, the greater frequency of interstrand cross-links observed for typical transplanaramine compounds compared to cisplatin [12,41] could produce a greater challenge to cell viability than intrastrand cross-links since interstrand cross-link repair is futile in most attempts [42–44]. H2AX phosphorylation has also been shown to act in conjunction with JNK and the caspase-3/CAD pathway for promotion of apoptosis [45]. In summary, our current work reinforces the potential utility of the *trans*-platinum compound to act as antitumor drugs.

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