Published in final edited form as:

Chem Res Toxicol. 2015 November 16; 28(11): 2170–2178. doi:10.1021/acs.chemrestox.5b00327.

Cellular Recognition and Repair of Monofunctional–Intercalative Platinum–DNA Adducts

Fang Liu, Jimmy Suryadi, and Ulrich Bierbach*

Department of Chemistry, Wake Forest University, Winston-Salem, North Carolina 27109, United States

Abstract

The cellular recognition and processing of monofunctional–intercalative DNA adducts formed by [PtCl(en)(L)](NO₃)₂ (**P1-A1**; en = ethane-1,2-diamine; L = *N*-[2-(acridin-9-ylamino)ethyl]-*N*-methylpropionamidinium, acridinium cation), a cytotoxic hybrid agent with potent anticancer activity, was studied. Excision of these adducts and subsequent DNA repair synthesis were monitored in plasmids modified with platinum using incubations with mammalian cell-free extract. On the basis of the levels of [α -³²P]-dCTP incorporation, **P1-A1**–DNA adducts were rapidly repaired with a rate approximately 8 times faster ($t_{1/2} \approx 18$ min at 30 °C) than the adducts (cross-links) formed by the drug cisplatin. Cellular responses to **P1-A1** and cisplatin were also studied in NCI-H460 lung cancer cells using immunocytochemistry in conjunction with confocal fluorescence microscopy. At the same dose, **P1-A1**, but not cisplatin, elicited a distinct requirement for DNA double-strand break repair and stalled replication fork repair, which caused nuclear fluorescent staining related to high levels of MUS81, a specialized repair endonuclease, and phosphorylated histone protein γ -H2AX. The results confirm previous observations in yeast-based chemical genomics assays. γ -H2AX fluorescence is observed as a large number of discrete foci signaling DNA double-strand breaks, pan-nuclear preapoptotic staining, and unique circularly shaped staining around the nucleoli and nuclear rim. DNA cleavage assays indicate that **P1-A1** does not act as a typical topoisomerase poison, suggesting the high level of DNA double-strand breaks in cells is more likely a result of topoisomerase-independent replication fork collapse. Overall, the cellular response to platinum–acridines shares striking similarities with that reported for DNA adduct-forming derivatives of the drug doxorubicin. The results of this study are discussed in light of the cellular mechanism of action of platinum–acridines and their ability to overcome resistance to cisplatin.

Abstract

*Corresponding Author. Tel: 336-758-3507. bierbau@wfu.edu.

The authors declare no competing financial interest.

INTRODUCTION

DNA-targeted cytotoxic agents continue to play a major role in cancer chemotherapy. Among the most widely used drugs are cisplatin (Figure 1a) and several of its second-generation derivatives.¹ It is generally accepted that the mechanism of action of these agents involves the formation of permanent intrastrand and interstrand cross-links in chromosomal DNA (Figure 1b), which cause cancer cell death by stalling DNA-processing enzymes.² Unfortunately, cisplatin-type cross-links are also recognized and efficiently removed by the nucleotide excision repair (NER) machinery, which may confer resistance to platinum in DNA repair-proficient forms of the disease, such as chemoresistant nonsmall cell lung cancer (NSCLC).^{3,4} To overcome this drawback, novel platinum-containing pharmacophores have been designed.^{5,6} These agents produce not only a unique spectrum of antitumor activity, but their DNA adducts also have the potential to evade detection by the cell's DNA damage response (DDR). Several classes of nonclassical platinum agents have been described whose unique DNA adducts are processed differently than the typical cross-links. These include polynuclear agents,⁷ monofunctional complexes, and complexes exhibiting trans-coordination geometries,⁸ as well as hybrid agents comprising DNA platinating and intercalating functionalities.⁶

The hybrid adducts formed by platinum–acridines represented by $[\text{PtCl}(\text{en})(\text{L})](\text{NO}_3)_2$ (**P1-A1**; en = ethane-1,2-diamine; L = *N*-[2-(acridin-9-ylamino)ethyl]-*N*-methylpropionamidine, acridinium cation; Figure 1a,c) do not distort DNA significantly and leave the thermodynamic stability of the damaged biopolymer unchanged due to complete enthalpy–entropy compensation.^{9,10} This contrasts the situation for cisplatin, which kinks and destabilizes the DNA duplex significantly,¹¹ rendering its adducts ideal substrates for NER.^{12,13} On the basis of these observations, we rationalized that the novel hybrid adducts might be less susceptible to NER removal than the cross-links, which could explain the exquisite potency of platinum–acridines in NSCLC. Compounds based on the prototype **P1-A1** are 100–1000-fold more cytotoxic in this form of cancer than cisplatin and maintain high activity in cell lines expressing high levels of NER proteins.^{14–16} At equitoxic doses (IC_{90}), **P1-A1** kills NCI-H460 lung cancer cells at concentrations 150-fold lower than those required for cisplatin while producing only 3-fold higher platinum levels in nuclear DNA compared to that of the clinical drug.¹⁷ The hybrid agent also proved to be an effective

quencher of DNA synthesis¹⁸ and a significantly more potent inhibitor of RNA polymerase II-mediated transcription than cisplatin.⁹ Cells treated with **P1-A1** undergo apoptosis triggered by a robust cell cycle arrest in early S phase, whereas under the same conditions cisplatin-treated cells accumulate in G2/M phase without apparent cell death.¹⁸ Together, these observations seem to suggest that the monofunctional–intercalative adducts formed by **P1-A1** are more detrimental to the cell's replisome and transcriptome than the cross-links formed by cisplatin.

Using a validated yeast-based chemogenomic screening platform based on a comprehensive library of *S. cerevisiae* deletion strains, platinum–acridines have recently been studied for their cellular mechanism of action.¹⁹ The damage response and sensitivity profiles observed in this assay faithfully mimic structure–(re)activity relationships established in human cancer cells and confirm that DNA is the major target of these compounds. The results also demonstrated that the platinum–acridines damage DNA differently than cisplatin, requiring not only NER but also a high level of double-strand break and stalled replication fork repair.²⁰ Here, we demonstrate that the responses observed in yeast also play a role in human lung cancer cells.

MATERIALS AND METHODS

General Procedures and Supplies

The platinum–acridine derivative **P1-A1** was synthesized according to a published procedure.²¹ *cis*-Diamminedichloroplatinum(II) (cisplatin) was purchased from Sigma. Stock solutions of both agents were prepared in phosphate-buffered saline (PBS) and stored at –20 °C. Cisplatin and **P1-A1** in solution were quantified spectrophotometrically using $\epsilon_{300} = 132 \text{ M}^{-1}\cdot\text{cm}^{-1}$ and $\epsilon_{413} = 10,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$, respectively. Milli-Q ultrapure water was used for the preparation of all assay solutions. pUC19 (2686 bp) and pET16b (5711 bp) plasmids were prepared by heat-shock transformation of chemically competent *E. coli* (NovaBlue, Novagen) followed by extraction of the DNA from the cells and purification using a Plasmid-Plus Midi kit (Qiagen, Valencia, CA). DNA concentrations were determined using a Thermo Scientific NanoDrop ND-2000 spectrophotometer, and platinum in drug-modified DNA (to confirm drug-to-DNA base ratios, r_b) was determined by inductively coupled plasma mass spectrometry (ICP-MS) as described previously.¹⁷ The human cancer cell line NCI-H460 (large-cell lung carcinoma) was obtained from the American Type Culture Collection (Rockville, MD, USA). Cell-free extract (CFE) from Chinese hamster ovary (CHO-K1) cells was generated according to a protocol reported by Li et al.²² [α -³²P]-Deoxycytidine-5'-triphosphate ([α -³²P]-dCTP) was purchased from PerkinElmer (Waltham, MA). The primary antibodies, anti-MUS81 (ab97391, rabbit polyclonal) and anti- γ -H2AX (ab22551, mouse monoclonal), and the corresponding secondary antibodies, Alexa Fluor-488-labeled IgG (ab150073, donkey antirabbit) and Alexa Fluor-647-labeled IgG (ab150115, goat antimouse) were obtained from Abcam (Cambridge, MA). The topoisomerase I screening kit was purchased from TopoGen Inc. (Columbus, OH). All other reagents were of biochemical-grade and DNase-free, where available, and used as supplied.

DNA Repair Assay

pUC19 plasmid was linearized with *Bam*HI restriction enzyme (New England Biolabs Inc., Ipswich, MA), purified by agarose gel electrophoresis, and extracted using a MinElute gel extraction kit (Qiagen, Valencia, CA). Platinum-modified DNA samples ($r_b = 0.03$, ICP-MS) were generated by reacting the linearized plasmid with cisplatin or **P1-A1** for 24 h at 37 °C, followed by microdialysis of the samples for 12 h at 4 °C against water. Repair reactions were performed using a protocol described by Kosthunova et al.⁹ with modifications. Typical repair reactions were assembled in a volume of 50 μ L containing 300 ng of platinated DNA and 5 μ L of CHO CFE along with the following buffers and reagents: 40 mM HEPES-KOH at pH 7.5, 30 mM KCl, 5 mM MgCl₂, 22 mM creatine phosphate, 50 mg/mL creatine phosphokinase, 2 mM ATP, 0.4 mg/mL BSA, 20 μ M each of dATP, dGTP, and dTTP, 8 μ M dCTP, and 5 μ Ci of [α -³²P]-dCTP (3000 Ci/mmol). Reactions were allowed to proceed at 30 °C for 15, 30, 60, and 120 min and quenched at each time point by the addition of 0.6% SDS. The reaction mixtures were then treated with 2 μ g of Proteinase K at 37 °C for 15 min. The DNA was extracted using phenol/chloroform and precipitated by adding ethanol, 1/10 v/v of 3 M sodium acetate (pH 5.2), and 40 μ g of glycogen. Radiolabeled, linearized pET16b (5711 bp) was added during phenol/chloroform extraction as an internal “no-repair” control. The recovered DNA was centrifuged for 30 min at 14,000 rpm, washed with 70% ethanol, centrifuged for an additional 15 min, and air-dried for 30 min at room temperature. The DNA pellets were dissolved in 1 $\times$ DNA loading dye and loaded onto 1% agarose gels. Electrophoretic separations were performed in 1 $\times$ TAE buffer at 50 V for 5 h. The DNA bands were first visualized and photodocumented after ethidium bromide (5 μ g/mL) staining. Dried gels were then exposed to a Kodak K1 screen, which was scanned with a FX Molecular Imager (Biorad Laboratories Inc., Hercules, CA). Bands containing ³²P-labeled DNA were quantified using the Quantity One software (Biorad). Band intensities of repaired pUC19 were normalized to the intensity of control plasmid (pET16b) and are reported as the mean of three reactions $\pm$ the standard deviation.

Cell Culture/Dosing

NCI-H460 cells were cultured in RPMI-1640 media (HyClone) containing 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10 mM HEPES, and 110 mg/L sodium pyruvate supplemented with 10% fetal bovine serum (FBS), 10% penstrep (P&S), and 10% L-glutamine. Cells were incubated at 37 °C in humidified 5% CO₂ and subcultured every 2 to 3 days to maintain cells in logarithmic growth. To synchronize cells in S phase (double thymidine block), cells at a density of 10⁵ cells/well were plated into poly-D-lysine-coated glass bottomed Petri dishes (MatTek Corp., Ashland, MD) and allowed to attach overnight. Cells were then incubated for 18 h in the presence of 2.5 mM thymidine. Thymidine was removed by washing with 1 $\times$ PBS, and fresh media was added. Incubation was continued for 8 h followed by another treatment with 2.5 mM thymidine for 15 h, washing with 1 $\times$ PBS, and addition of fresh media. After 1.5 h, cells were treated with 100 nM cisplatin or **P1-A1** for 3 and 6 h. Cells treated with media served as the control.

Immunocytochemistry

After the removal of media, plated NCI-H460 cells were washed twice with 1× Tris-buffered saline/Tween-20 surfactant (TBST) and fixed with 3.7% paraformaldehyde in TBST at room temperature for 15 min. Cells were then washed three times with TBST to remove excess paraformaldehyde, permeabilized by treatment with 0.5% Triton-X 100 in TBST for 15 min, followed by three additional washes with TBST buffer. The permeabilized cells were first treated with 5% bovine serum albumin (BSA) for 1 h followed by three TBST washes and then incubated with appropriately diluted primary antibodies (1:300 anti-MUS81, or 1:200 anti- γ -H2AX) in 5% BSA solution at 4 °C for 14 h. After five TBST washes, the appropriate secondary antibody (1:500 Alexa Fluor-488 antirabbit IgG or 1:500 Alexa Fluor-647 antimouse IgG) was added to the cells, and incubations were continued for 1 h. Cells were washed five times with TBST and finally treated with Hoechst 33342 nuclear stain (5 μ g/mL) for 15 min. Prior to the confocal imaging sessions, the cell culture dishes were washed three times with PBS. Incubations with 5 μ M of platinum-acridine for up to 24 h and the corresponding cell-based assays, as described in the Supporting Information, were performed in the same manner.

Confocal Microscopy

Confocal images were collected on a Zeiss LSM-710 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) using a 40× (PLAN APO, 0.95 NA) or a 63× (PLAN APO, 1.2 NA) objective lens. Excitation cross-talk and emission bleed-through were minimized by acquiring image data in multitrack configuration mode. The following laser lines were used for fluorophore excitation: 405 nm (Hoechst 33342 dye) with an emission range of 424–466 nm, 488 nm (Alexa Fluor-488) with an emission range of 489–553 nm, and 633 nm (Alexa Fluor-647) with an emission range of 655–732 nm. To allow comparison of fluorescence intensities between samples, the excitation power, pinhole settings, PMT gain, and offset values for each respective channel were strictly maintained at the same values across and within imaging sessions. For all images, a pinhole value of 1.2 airy units or less was used, and images were acquired with 8 × line averaging as 3 × 3 tiles at 1024 × 1024 pixel resolution. Images were collected with 12-bit sampling to provide a wide dynamic intensity range for analysis (0–4096). The Zen software was used for image acquisition. The fluorescence intensities of Alexa Fluor-488 and Alexa Fluor-647 were quantified using Zen (blue edition) software (Zeiss, 2011). Fluorescence signals for individual cells in each channel were measured by drawing a region of interest (ROI) around the nuclear regions, with the Hoechst 33342 or bright-field images used to define nucleus location. The fluorescence intensities were corrected by subtracting background fluorescence, which was determined by drawing an ROI of the same size in an empty region of the image. A total of >100 cells in 9 different fields were analyzed in this manner for each treatment (duplicates).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](https://doi.org/10.1021/acs.chemrestox.5b00327) at DOI: [10.1021/acs.chemrestox.5b00327](https://doi.org/10.1021/acs.chemrestox.5b00327).

Additional confocal images of NCI-H460 cells dosed with cisplatin and **P1-A1** for 3 and 24 h; confocal images captured of individual apoptotic cells treated with **P1-A1**; gel mobility shift experiments for pHOT1 plasmid modified with **P1-A1**; confocal images showing cytomorphological differences in the nucleolar regions of NCI-H460 cells treated with cisplatin and **P1-A1**; and results of the topoisomerase II assays ([PDF](#))

Statistical analysis was done using Welch's two-sample *t* test for unequal variances (Microsoft Excel 2010).

Topoisomerase Assay

Human topoisomerase I (TOP1) cleavage activity was monitored in pHOT1 plasmid (TopoGen Inc., Columbus, OH) modified with **P1-A1**. To generate the platinum-modified substrate, the plasmid was incubated with platinum at varying r_b values for 24 h at 37 °C. Assays were carried out in 20- μ L of reaction buffer with 0.15 mg of modified supercoiled plasmid and 6 units of topoisomerase enzyme. Reactions were assembled on ice and incubated for 30 min at 37 °C. Prior to electrophoretic separations of the reactions mixtures, DNA-enzyme complexes were denatured by adding 1% sodium dodecyl sulfate (SDS), and the enzyme was digested (unless stated otherwise) with 50 μ g/mL Proteinase K for 30 min at 37 °C. Reaction mixtures were loaded onto 1% agarose gels containing 0.1% SDS in 1 $\times$ TAE buffer. Gels were electrophoresed first in the absence of ethidium bromide and then for 45–60 min with ethidium bromide (0.5 μ g/mL) in the running buffer to resolve nicked from covalently closed circles of DNA. Finally, gels were destained and photodocumented. DNA unwinding assays (for **P1-A1**) were performed as described previously.²³

RESULTS

In Vitro DNA Repair Synthesis

To determine the relative rates of excision repair for the DNA adducts formed by cisplatin and **P1-A1**, an in vitro assay (Figure 2A) was performed using cell-free extract (CFE) isolated from DNA repair-proficient Chinese hamster ovary (CHO) cells that produce high levels of NER proteins.²² Linearized pUC19 plasmid DNA served as the double-stranded DNA repair template. Great care was taken that the globally modified plasmid samples contained the same number of cisplatin or **P1-A1** adducts ($r_b = 0.03$, or 3 adducts per 100 DNA bases) and that samples were treated with the same batch of CFE to allow for a direct comparison between the two agents. The progress of the repair process was monitored by measuring ³²P incorporation into the newly synthesized patches of DNA at the sites of damage excision. Briefly, excision repair and DNA synthesis were monitored by quantifying ³²P-modified plasmid after 15, 30, 60, and 120 min of continuous incubation densitometrically after separation of the reaction mixtures on agarose gels (Figure 2B). To generate reliable kinetic data, it was necessary to start monitoring the repair reaction at early time points and only for a short period of time to avoid nucleolytic degradation of plasmid in the CFE (observed after 180 min of incubation; data not shown). For each time point, the relative amounts of repaired plasmid were then expressed as picomoles of incorporated [α -³²P]-dCTP and plotted vs time (Figure 2C).

A dramatic difference in repair kinetics is observed for the DNA adducts formed by cisplatin and **P1-A1**. At each time point, significantly more plasmid damaged with the latter hybrid agent has been repaired than plasmid modified with adducts of the clinical drug (Student's *t* test, $P < 0.01$). The data acquired for **P1-A1** suggest a rapid onset of excision and efficient resynthesis of the DNA immediately after plasmid and CHO CFE were mixed. After only 15 min of exposure to CFE, the level of removal/repair of adducts proves to be 4 times higher

for **P1-A1** compared with that of cisplatin. DNA repair synthesis during nucleotide excision repair can be modeled as a slow first-order process.²⁴ To estimate the relative rates of repair, the time-dependent data generated in this assay were fitted to first-order reaction kinetics. On the basis of this treatment, **P1-A1** adducts are repaired with a rate approximately 8 times faster than that of cisplatin adducts under the same conditions.

Immunocytochemical Detection of a DNA Damage Response in Lung Cancer Cells

Chemogenomic screening of platinum–acridines in gene deletion strains of *S. cerevisiae* indicated that the DNA-damaging effects of the most cytotoxic hybrid agents triggered responses from both NER and HRR repair pathways.¹⁹ Unlike cisplatin, **P1-A1** showed a distinct requirement for DNA double-strand break repair in conjunction with replication fork repair (MUS81-MMS4). MUS81-MMS4/EME1 is a structure-specific heterodimeric endonuclease involved in the repair of disrupted replication forks and DNA double-strand breaks by homologous recombination.²⁰ The nuclease has been shown to process stalled and collapsed replication forks that have resulted from specific types of DNA damage, such as interstrand cross-links²⁵ and single-strand breaks resulting from cleavable DNA–topoisomerase I complexes.²⁶

To investigate if the above observations in budding yeast translate to DDR pathways in mammalian cells, we used confocal fluorescence microscopy in conjunction with immunocytochemical detection of MUS81 and γ -H2AX. The latter phosphorylated histone protein is known to accumulate at the sites of DNA double-strand breaks as an early signal of DNA repair initiation in the form discrete foci, which can be visualized immunocytochemically.²⁷ In this assay, we treated NCI-H460 nonsmall cell lung cancer cells in S phase with 100 nM cisplatin or **P1-A1** for 3 and 6 h and fixed the cells post-treatment. (Cells were incubated at a concentration at which **P1-A1** kills 90% of NCI-H460 cancer cells after 72 h of incubation (IC₉₀) but only for 6 h with no apparent signs of cell death.^{17,18}) We then used appropriate antibodies and the corresponding fluorophore-labeled secondary antibodies to study simultaneously intracellular levels and localization of MUS81 and γ -H2AX.

Confocal images of NCI-H460 cells treated with cisplatin and **P1-A1** for 3 h show strong MUS81-related green fluorescence across the cytoplasm and a less intense staining of the nuclei with no significant differences between treated and control cells ($P > 0.05$). The red fluorescence associated with anti- γ -H2AX antibodies is observed in the cells' nuclei, where it is localized to discrete repair foci in approximately 40% of the cells treated with cisplatin (Supporting Information). By contrast, a major population of cells treated with **P1-A1** reveals more extensively stained nuclei and a larger number of repair foci per nucleus relative to the cisplatin group (Supporting Information). These differences persist after 6 h of incubation. When the nuclear regions of >100 cells in each group were examined at this time point, **P1-A1**, but not cisplatin, produced significantly higher levels of green and red fluorescence relative to that of control cells ($P < 0.01$, Figure 3).

In cells treated with **P1-A1**, redistribution of MUS81-related green fluorescence from the cytosol to the perinuclear region and nuclear envelope (3 h) and from the nuclear envelope into the nucleus (6 h) is observed (Figure 4A, green channel). This leads to green staining

across the entire nucleus with the exception of the nucleoli. In addition to the discrete γ -H2AX repair foci, which persist throughout treatment with **P1-A1**, a pronounced increase in γ -H2AX-related fluorescence (Figure 4A, red channel) due to pan-nuclear staining is observed for the hybrid agent in a large population of cells. While generally no major colocalization is observed for the green and red emission signals in these cells (Figure 4A, merged channel), several large, discrete γ -H2AX foci do colocalize with areas of high MUS81-related green fluorescence (Figure 4B).

In images (red channel) recorded after incubation with **P1-A1** for 3 h, two major populations of cells are observed: cells exhibiting discrete γ -H2AX repair foci and cells in which γ -H2AX-related fluorescence is confined to areas around the nuclear rim and around the nucleoli (Figure 4C). After 6 h of incubation, cells showing extensive pan-nuclear staining become predominant (Figure 4C). The latter two γ -H2AX distribution patterns are virtually absent in control cells and cells treated with cisplatin. It appears that the relatively higher red fluorescence levels detected in the nuclei of cells incubated with **P1-A1** (Figure 3) for 6 h compared to those exposed to cisplatin are primarily caused by the extensive circularly shaped and pan-nuclear staining observed for the former agent. Nucleus-wide phosphorylation of H2AX histone protein also persists in apoptotic cells (giving rise to “apoptotic rings”²⁸) generated by treatment with platinum–acridine for longer time intervals and at higher doses of the cytotoxic agent (Figures S4 and S5).

Processing of DNA Modified with **P1-A1** by Human Topoisomerase I (TOP1)

The high levels and persistence of H2AX phosphorylation and foci formation confirmed in the immunocytochemical assay suggest that **P1-A1** is a strong inducer of DNA double-strand breaks. While platinum–acridines do not cause spontaneous DNA strand breaks, one potential mechanism might involve topoisomerase-mediated cleavage. The dual requirement for HRR and MUS81 observed in yeast,¹⁹ which mimics that of the clinical TOP1 poison camptothecin,²⁹ prompted us to investigate this mechanism for **P1-A1** in a cell-free assay.

The ability of DNA adducts of **P1-A1** to poison TOP1 and produce permanent single-strand breaks was monitored in negatively supercoiled pHOT1 plasmid DNA on agarose gels (Figure 5). Initially, in cleavage assays performed with platinum–acridine, the disappearance of the supercoiled plasmid (Form I) and relaxed topoisomers (Form I_r) and the concomitant dose-dependent increase in Form II were interpreted as camptothecin-like stimulation of DNA single-strand breaks (Figure 5A).³⁰ **P1-A1** alone in the absence of enzyme is also able to convert Form I of the plasmid completely into relaxed Form II as a consequence of the DNA unwinding properties of the intercalated acridine chromophores.²³ However, this conversion occurs at a significantly higher degree of modification ($r_b = 0.054$; see the Supporting Information) than the highest ratio used in the cleavage assay. This observation appeared to be consistent with enzyme-mediated cleavage rather than adduct-mediated unwinding. Nevertheless, to rule out simple unwinding of the plasmid as the cause of the band shift and to screen for the formation of trapped covalent DNA–topoisomerase complexes, the assay was performed with modifications. As in the previous experiment, when plasmid modified at $r_b = 0.02$ was exposed to TOP1, virtually complete conversion of plasmid to Form II and no remaining partially relaxed topoisomers are observed (Figure 5B,

lane 3). However, when samples were treated with NaCN (either before or after the digestion of topoisomerase enzyme with Proteinase K), which rapidly reverses platinum–DNA adducts,³¹ the majority of DNA molecules returned to supercoiled topoisomers (Figure 5B, lanes 4 and 5). This observation confirms that Form II generated in this assay mainly consists of relaxed, underwound closed circles³² and only a minor amount of the comigrating nicked open-circular form. Bands on the gel assigned to the latter form of DNA after cleavage by TOP1 (lanes 4, 5, and 7 in Figure 5B) are significantly enhanced compared to those of the unreacted control (lane 1 in Figure 5B). This observation supports the notion that **P1-A1** adducts may indeed have caused single-strand breaks in a small fraction of TOP1-catalyzed cleavage events. This outcome is not surprising as the high-affinity TOP1 recognition sequence engineered into the pHOT1 plasmid, AGACTT↓AGAAAAATTT³³ (where the arrow marks the TOP1 cleavage site), contains the preferred binding sites for platinum–acridines, 5'-TA and 5'-AG (see Figure 1). Local unwinding of the duplex by a hybrid adduct formed near the cleavage site can be expected to cause strand misalignment resulting in the inhibition of the enzyme-catalyzed resealing step. Reactions performed in this assay without Proteinase K digestion aimed at detecting trapped covalent DNA–enzyme complexes as bands of reduced mobility on the gels were unsuccessful. Using a similar assay, we also studied DNA relaxation by human topoisomerase II in plasmid containing adducts of **P1-A1**. On the basis of the results of these experiments (see the Supporting Information), a mechanism by which our hybrid agent acts as a topoisomerase II (TOP2) poison to stimulate permanent DNA double-strand breaks can be firmly ruled out.

DISCUSSION

Excision Repair of P1-A1–DNA Adducts by Mammalian NER Is a Rapid Process

In cell-free extracts, the monofunctional–intercalative DNA adducts formed by hybrid agent **P1-A1** are readily recognized and repaired by the mammalian excision repair machinery. While this result was unexpected based on the biophysical properties of these adducts, the fast rate of repair synthesis explains why NER-deficient yeast strains are so sensitive to this agent.¹⁹ The yeast-based assays suggested that the removal of hybrid adducts is dominated by the classical global genomic (GG) NER pathway, which involves incisions by XPF-ERCC1 (RAD1–RAD10) and XPG (RAD2) on the 5' and 3' sides of the adduct, respectively.¹² The rapid (initial) rate of repair synthesis is also in agreement with the level of global repair observed previously in HeLa cell-free extracts.⁹

The level of DNA adducts in cells continuously dosed with platinum is controlled by (i) the rates of cellular uptake/efflux of compound, (ii) the rate of trafficking of reactive complex between cellular reservoirs (lysosomes and cytoplasm) and the nucleus, (iii) the rate of covalent adduct formation in chromatin, and (iv) the rate of adduct reversal. **P1-A1** is a significantly more efficient DNA-platinating agent than cisplatin.¹⁷ The DNA-targeted hybrid agent induces cytotoxic intercalative monoadducts in one single binding event with $t_{1/2} \sim 20$ min.³⁴ By contrast, cisplatin produces biologically inactive monoadducts with $t_{1/2} \sim 2$ h, of which more than 80% convert to cytotoxic 1,2-intrastrand cross-links on the same time scale.³⁵ Despite this kinetic advantage and the 60-fold higher intracellular accumulation of **P1-A1** compared to that of cisplatin in NCI-H460 cells,¹⁷ the frequency of

adducts produced by **P1-A1** in chromatin is only 3–10-fold higher than that observed for cisplatin (at 0.1 μ M drug concentrations after 3 h of continuous dosing). These observations support the notion that the hybrid adducts are removed from DNA at a faster rate than the cisplatin adducts in NCI-H460 cells.

Of the entire array of bifunctional adducts formed by cisplatin, the 1,2-intrastrand cross-links (Figure 1b) are least readily repaired by NER in cell-free extracts.³⁶ This has been attributed to the shielding of the major cisplatin–DNA lesion from NER activity by high-mobility group (HMG) proteins, which tightly bind to the distorted DNA at the site of the crosslink.³⁷ This contrasts the situation for **P1-A1**, whose structurally distinct DNA adducts are not recognized by HMG proteins.⁹ Thus, while the hybrid adducts per se may not be better substrates for NER than the cross-links, they may be more readily detectable by DDR proteins. Despite this unfavorable feature, the remaining unrepaired **P1-A1** adducts appear to be responsible for a significantly stronger cytotoxic effect than the cisplatin-type cross-links.

P1-A1–DNA Adducts Lead to Double-Strand Breaks and Require Specialized Replication Fork Repair

From the screening of the yeast genome,¹⁹ specific genes emerged that buffer the cellular effects of platinum–acridines (genes whose deletion sensitize treated cells to these agents, resulting in reduced cell viability). Heterozygous deletion strains treated with **P1-A1**, the most cytotoxic molecule screened in this assay, showed the highest dependency on DDR modules. Contrary to our expectation, cells had a high requirement for the expression of NER genes, *RAD1–RAD10* and *RAD2* (orthologues of human *XPF–ERCC1* and *XPG*), and also required the expression of genes involved in DNA double-strand break repair by homologous recombination (HRR), including *RAD51* and *RAD54*. The viability of cells treated with **P1-A1** also depended on *MUS81–MMS4* (orthologue of human *MUS81–EME1*) for survival, a heterodimeric structure-specific endonuclease involved in the repair of stalled replication forks and DNA double-strand breaks. Our results reported here indicate that both DNA repair pathways are also involved in the recognition and processing of the intercalative monoadducts produced by **P1-A1** in mammalian cells.

The high levels of nuclear *MUS81* and γ -H2AX in lung cancer cells treated with **P1-A1** suggest that replication fork arrest and accumulation of DNA double-strand breaks promote cell cycle arrest and subsequent apoptotic cell death. Cell death occurs most likely after initiation of a replication stress-mediated checkpoint response.³⁸ In particular, the early onset of pan-nuclear H2AX phosphorylation observed for **P1-A1**, but not for cisplatin, can be attributed to preapoptotic signaling during S phase.³⁹ These findings are consistent with the observation that cisplatin–DNA adducts slow, but do not arrest, DNA synthesis, which delays cell-cycle progression.⁴⁰ Likewise, cisplatin was unable to stall DNA synthesis and induce replication arrest in NCI-H460 cells.¹⁸ One mechanism, which prevents replication fork arrest and allows platinum-treated cells to transition through S phase, is the bypass of adducts by trans-lesion synthesis (TLS) DNA polymerases.⁴¹ TLS by DNA polymerase η (Pol η) is a potential source of cellular tolerance (and tumor resistance) to cisplatin.⁴² Yeast chemogenomics demonstrated that this pathway is not at all involved in mitigating the

genotoxic effects of the hybrid adducts formed by **P1-A1** and other platinum–acridines.¹⁹ If these results can be confirmed in mammalian cells, this important difference may help explain why platinum–acridines show high activity in cisplatin-resistant cancers. It is noteworthy to mention that the novel platinum complex *cis*-diammine-(phenanthridine)chloroplatinum(II) nitrate (“phenanthriplatin”) forms nonintercalative DNA monoadducts that do not permit efficient TLS.⁴³ It has been suggested that the lack of lesion bypass may contribute to the promising activity of this analogue in cisplatin-resistant cell lines.

The specific roles of MUS81 in the repair of the hybrid adducts remain elusive. The pan-nuclear accumulation and partial colocalization with γ -H2AX repair foci observed in confocal images suggest that the endonuclease may be required for both early repair of replication forks blocked by the hybrid adducts and for resolving Holliday junctions during subsequent DNA double-strand repair by HRR. Two interesting observations with potential relevance to the mechanism of action of platinum–acridines should be noted: the distinct accumulation of circularly shaped γ -H2AX staining around the cells’ nucleoli and the absence of MUS81-related fluorescence in this transient subnuclear structure. We have previously demonstrated using orthogonal fluorescent postlabeling techniques⁴⁴ that platinum–acridines accumulate in the nucleolar regions in cells, the sites of transcription of rRNA genes (rDNA) by RNA polymerase I (Pol I).⁴⁵ Furthermore, in cells treated with platinum–acridines the nucleoli show distinct morphological changes and appear considerably smaller than those in cisplatin-treated cells (Supporting Information). In experiments measuring the incorporation of fluorescently detectable ribonucleotide during transcription, NCI-H460 cells treated with **P1-A1** showed signs consistent with disrupted rRNA synthesis.⁴⁶ Excision repair in actively transcribed nucleolar rDNA is a highly inefficient process.^{47,48} This lack of repair capability and the inability of MUS81 to localize to the nucleolus demonstrated in this study corroborate the possibility that irreparable damage to rRNA genes contributes to the cytotoxicity of platinum–acridines.

Damage Responses to P1-A1–DNA Adducts and Anthracycline–DNA Adducts Are Strikingly Similar

DNA-alkylating derivatives of the classical intercalator and topoisomerase II poison doxorubicin produce highly cytotoxic covalent–intercalative DNA adducts⁴⁹ reminiscent of the dual binding mode of platinum–acridines. In cell cultures, these compounds proved to be more cytotoxic than the parent intercalators.⁴⁹ Similar to platinum–acridines, their adducts, which are repaired by the classical NER and HRR pathways, stimulate DNA double-strand breaks by a nontopoisomerase-mediated mechanism, which causes replication and cell cycle arrest.^{50,51} The authors also demonstrated that MUS81 is required for the repair of these adducts,⁵¹ despite their inability to induce enzyme-mediated DNA damage or interstrand crosslinks, which typically trigger repair by this endonuclease. It appears that both types of hybrid agents share similar cell-kill mechanisms, which are triggered by a unique type of DNA lesion currently not exploited in cancer chemotherapies.

SUMMARY

The present study has provided new insights into the cellular response to the DNA damage produced by platinum–acridine hybrid agents. Our results confirm that the monofunctional–intercalative DNA adducts are readily removed by excision repair but if unrepaired constitute a more severe threat to cell survival than the DNA cross-links. The 150-fold higher cytotoxicity of **P1-A1** compared to that of cisplatin in NCI-H460 lung cancer cells cannot be explained with higher DNA adduct levels alone (approximately 10-fold in cells treated with 0.1 μ M of platinum¹⁷) but likely also reflect the more detrimental effects of the hybrid adducts on progressing replication forks. These differences may also help explain why platinum–acridines are more effective than conventional platinum-based therapies in chemoresistant forms of cancer. Additional experiments are warranted to validate the cell-cycle effects, DDR pathways, and cell-kill mechanisms triggered by platinum–acridines. These include sensitivity screening and knockdown experiments in MUS81, TOP1, and TLS deficient cells lines.

ACKNOWLEDGMENTS

We thank Dr. Glen Marrs (WFU, Department of Biology) for assistance with the confocal microscopy experiments and Dr. Keith Levine (RTI International, Research Triangle Park, NC) for the acquisition of the ICP-MS data.

Funding

This work was supported by the National Institutes of Health (R01 CA101880). We also gratefully acknowledge support through the Tumor Tissue Core Laboratory of the Comprehensive Cancer Center of Wake Forest University (NIH center core grant P30 CA012197).

ABBREVIATIONS

CFE	cell-free extract
CHO	Chinese hamster ovary
DDR	DNA damage response
HMG	high-mobility group
HRR	homologous recombination repair
NER	nucleotide excision repair
NSCLC	non-small-cell lung cancer
TLS	trans-lesion synthesis
TOP1	human topoisomerase I
TOP2	human topoisomerase II

REFERENCES

1. Kelland L. The resurgence of platinum-based cancer chemotherapy. *Nat. Rev. Cancer.* 2007; 7:573–584. [PubMed: 17625587]
2. Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nat. Rev. Drug Discovery.* 2005; 4:307–320. [PubMed: 15789122]

3. Burris HA 3rd. Shortcomings of current therapies for non-small-cell lung cancer: unmet medical needs. *Oncogene*. 2009; 28(Suppl 1):S4–S13. [PubMed: 19680296]
4. Fujii T, Toyooka S, Ichimura K, Fujiwara Y, Hotta K, Soh J, Suehisa H, Kobayashi N, Aoe M, Yoshino T, Kiura K, Date H. ERCC1 protein expression predicts the response of cisplatin-based neoadjuvant chemotherapy in non-small-cell lung cancer. *Lung Cancer*. 2008; 59:377–384. [PubMed: 17905465]
5. Momekov G, Bakalova A, Karaivanova M. Novel approaches towards development of non-classical platinum-based antineoplastic agents: Design of platinum complexes characterized by an alternative DNA-binding pattern and/or tumor-targeted cytotoxicity. *Curr. Med. Chem*. 2005; 12:2177–2191. [PubMed: 16178779]
6. Suryadi J, Bierbach U. DNA metalating-intercalating hybrid agents for the treatment of chemoresistant cancers. *Chem. Eur. J*. 2012; 18:12926–12934. [PubMed: 22987397]
7. Farrell N. Polynuclear platinum drugs. *Met. Ions Biol. Syst*. 2004; 42:251–296. [PubMed: 15206105]
8. Coluccia M, Natile G. Trans-Platinum Complexes in Cancer Therapy. *Anti-Cancer Agents Med. Chem*. 2007; 7:111–123.
9. Kostrhunova H, Malina J, Pickard AJ, Stepankova J, Vojtiskova M, Kasparkova J, Muchova T, Rohlfing ML, Bierbach U, Brabec V. Replacement of a thiourea with an amidine group in a monofunctional platinum-acridine antitumor agent. Effect on DNA interactions, DNA adduct recognition and repair. *Mol. Pharmaceutics*. 2011; 8:1941–1954.
10. Baruah H, Bierbach U. Biophysical characterization and molecular modeling of the coordinative-intercalative DNA monoadduct of a platinum-acridinylthiourea agent in a site-specifically modified dodecamer. *J. Biol. Inorg. Chem*. 2004; 9:335–344. [PubMed: 15024635]
11. Poklar N, Pilch DS, Lippard SJ, Redding EA, Dunham SU, Breslauer KJ. Influence of cisplatin intrastrand crosslinking on the conformation, thermal stability, and energetics of a 20-mer DNA duplex. *Proc. Natl. Acad. Sci. U. S. A*. 1996; 93:7606–7611. [PubMed: 8755522]
12. Wood RD. Nucleotide excision repair in mammalian cells. *J. Biol. Chem*. 1997; 272:23465–23468. [PubMed: 9295277]
13. 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; 59:3968–3971. [PubMed: 10463593]
14. Ding S, Pickard AJ, Kucera GL, Bierbach U. Design of enzymatically cleavable prodrugs of a potent platinum-containing anticancer agent. *Chem. Eur. J*. 2014; 20:16164–16173. [PubMed: 25303639]
15. Graham LA, Wilson GM, West TK, Day CS, Kucera GL, Bierbach U. Unusual Reactivity of a Potent Platinum-Acridine Hybrid Antitumor Agent. *ACS Med. Chem. Lett*. 2011; 2:687–691. [PubMed: 21927647]
16. Ma Z, Choudhury JR, Wright MW, Day CS, Saluta G, Kucera GL, Bierbach U. A non-cross-linking platinum-acridine agent with potent activity in non-small-cell lung cancer. *J. Med. Chem*. 2008; 51:7574–7580. [PubMed: 19012390]
17. Qiao X, Zeitany AE, Wright MW, Essader AS, Levine KE, Kucera GL, Bierbach U. Analysis of the DNA damage produced by a platinum-acridine antitumor agent and its effects in NCI-H460 lung cancer cells. *Metallomics*. 2012; 4:645–652. [PubMed: 22456976]
18. Smyre CL, Saluta G, Kute TE, Kucera GL, Bierbach U. Inhibition of DNA Synthesis by a Platinum-Acridine Hybrid Agent Leads to Potent Cell Kill in Non-Small Cell Lung Cancer. *ACS Med. Chem. Lett*. 2011; 2:870–874. [PubMed: 22328962]
19. Cheung-Ong K, Song KT, Ma Z, Shabtai D, Lee AY, Gallo D, Heisler LE, Brown GW, Bierbach U, Giaever G, Nislow C. Comparative chemogenomics to examine the mechanism of action of dna-targeted platinum-acridine anticancer agents. *ACS Chem. Biol*. 2012; 7:1892–1901. [PubMed: 22928710]
20. Ciccica A, McDonald N, West SC. Structural and functional relationships of the XPF/MUS81 family of proteins. *Annu. Rev. Biochem*. 2008; 77:259–287. [PubMed: 18518821]

21. Ding S, Qiao X, Kucera GL, Bierbach U. Using a build-and-click approach for producing structural and functional diversity in DNA-targeted hybrid anticancer agents. *J. Med. Chem.* 2012; 55:10198–10203. [PubMed: 23074987]
22. Li YC, Ross J, Scheppler JA, Franza BR Jr. An in vitro transcription analysis of early responses of the human immunodeficiency virus type 1 long terminal repeat to different transcriptional activators. *Mol. Cell. Biol.* 1991; 11:1883–1893. [PubMed: 2005886]
23. Graham LA, Suryadi J, West TK, Kucera GL, Bierbach U. Synthesis, aqueous reactivity, and biological evaluation of carboxylic acid ester-functionalized platinum-acridine hybrid anticancer agents. *J. Med. Chem.* 2012; 55:7817–7827. [PubMed: 22871158]
24. Verbruggen P, Heinemann T, Manders E, von Bornstaedt G, van Driel R, Hofer T. Robustness of DNA repair through collective rate control. *PLoS Comput. Biol.* 2014; 10:e1003438. [PubMed: 24499930]
25. Hanada K, Budzowska M, Modesti M, Maas A, Wyman C, Essers J, Kanaar R. The structure-specific endonuclease Mus81-Eme1 promotes conversion of interstrand DNA crosslinks into double-strands breaks. *EMBO J.* 2006; 25:4921–4932. [PubMed: 17036055]
26. Regairaz M, Zhang YW, Fu H, Agama KK, Tata N, Agrawal S, Aladjem MI, Pommier Y. Mus81-mediated DNA cleavage resolves replication forks stalled by topoisomerase I-DNA complexes. *J. Cell Biol.* 2011; 195:739–749. [PubMed: 22123861]
27. Bonner WM, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, Pommier Y. γ -H2AX and cancer. *Nat. Rev. Cancer.* 2008; 8:957–967. [PubMed: 19005492]
28. Solier S, Pommier Y. The nuclear γ -H2AX apoptotic ring: implications for cancers and autoimmune diseases. *Cell. Mol. Life Sci.* 2014; 71:2289–2297. [PubMed: 24448903]
29. Lee W, St Onge RP, Proctor M, Flaherty P, Jordan MI, Arkin AP, Davis RW, Nislow C, Giaever G. Genome-wide requirements for resistance to functionally distinct DNA-damaging agents. *PLoS Genet.* 2005; 1:e24. [PubMed: 16121259]
30. Champoux JJ. DNA Topoisomerase I-Mediated Nicking of Circular Duplex DNA. In: Osheroff, N.; Bjornsti, M-A., editors. *DNA Topoisomerase Protocols*. Totowa, NJ: Humana Press; 2001. p. 81–87.
31. Kane SA, Lippard SJ. Photoreactivity of platinum(II) in cisplatin-modified DNA affords specific cross-links to HMG domain proteins. *Biochemistry.* 1996; 35:2180–2188. [PubMed: 8652559]
32. Shen, LL. DNA Unwinding Test Using Eukaryotic DNA Topoisomerase I. In: Osheroff, N.; Bjornsti, M-A., editors. *DNA Topoisomerase Protocols*. Totowa, NJ: Humana Press; 2001. p. 149–160.
33. Bonven BJ, Gocke E, Westergaard O. A high affinity topoisomerase I binding sequence is clustered at DNAase I hypersensitive sites in Tetrahymena R-chromatin. *Cell.* 1985; 41:541–551. [PubMed: 2985282]
34. Choudhury JR, Rao L, Bierbach U. Rates of intercalator-driven platination of DNA determined by a restriction enzyme cleavage inhibition assay. *J. Biol. Inorg. Chem.* 2011; 16:373–380. [PubMed: 21086002]
35. Gelasco, A.; Lippard, SJ. Anticancer Activity of Cisplatin and Related Complexes. In: Clarke, MJ.; Sadler, PJ., editors. *Topics in Biological Inorganic Chemistry*. New York: Springer; 1999. p. 1–43.
36. Calsou P, Frit P, Salles B. Repair synthesis by human cell extracts in cisplatin-damaged DNA is preferentially determined by minor adducts. *Nucleic Acids Res.* 1992; 20:6363–6368. [PubMed: 1475197]
37. Zamble DB, Mu D, Reardon JT, Sancar A, Lippard SJ. Repair of cisplatin–DNA adducts by the mammalian excision nuclease. *Biochemistry.* 1996; 35:10004–10013. [PubMed: 8756462]
38. Hustedt N, Gasser SM, Shimada K. Replication checkpoint: tuning and coordination of replication forks in S phase. *Genes.* 2013; 4:388–434. [PubMed: 24705211]
39. de Feraudy S, Revet I, Bezrookove V, Feeney L, Cleaver JE. A minority of foci or pan-nuclear apoptotic staining of γ -H2AX in the S phase after UV damage contain DNA double-strand breaks. *Proc. Natl. Acad. Sci. U. S. A.* 2010; 107:6870–6875. [PubMed: 20351298]
40. Sorenson CM, Eastman A. Mechanism of cis-diamminedichloroplatinum(II)-induced cytotoxicity: role of G2 arrest and DNA double-strand breaks. *Cancer Res.* 1988; 48:4484–4488. [PubMed: 3395999]

41. Sale JE. Competition, collaboration and coordination - determining how cells bypass DNA damage. *J. Cell Sci.* 2012; 125:1633–1643. [PubMed: 22499669]
42. Chaney SG, Campbell SL, Temple B, Bassett E, Wu YB, Faldu M. Protein interactions with platinum-DNA adducts: from structure to function. *J. Inorg. Biochem.* 2004; 98:1551–1559. [PubMed: 15458816]
43. Gregory MT, Park GY, Johnstone TC, Lee YS, Yang W, Lippard SJ. Structural and mechanistic studies of polymerase η bypass of phenanthriplatin DNA damage. *Proc. Natl. Acad. Sci. U. S. A.* 2014; 111:9133–9138. [PubMed: 24927576]
44. Ding S, Qiao X, Suryadi J, Marrs GS, Kucera GL, Bierbach U. Using fluorescent post-labeling to probe the subcellular localization of DNA-targeted platinum anticancer agents. *Angew. Chem. Int. Ed.* 2013; 52:3350–3354.
45. Pickard AJ, Bierbach U. The cell's nucleolus: an emerging target for chemotherapeutic intervention. *Chem Med Chem.* 2013; 8:1441–1449. [PubMed: 23881648]
46. Qiao X, Ding S, Liu F, Kucera GL, Bierbach U. Investigating the cellular fate of a DNA-targeted platinum-based anticancer agent by orthogonal double-click chemistry. *J. Biol. Inorg. Chem.* 2014; 19:415–426. [PubMed: 24407462]
47. Balajee AS, May A, Bohr VA. DNA repair of pyrimidine dimers and 6–4 photoproducts in the ribosomal DNA. *Nucleic Acids Res.* 1999; 27:2511–2520. [PubMed: 10352180]
48. Christians FC, Hanawalt PC. Lack of transcription-coupled repair in mammalian ribosomal RNA genes. *Biochemistry.* 1993; 32:10512–10518. [PubMed: 8399197]
49. Swift LP, Rephaeli A, Nudelman A, Phillips DR, Cutts SM. Doxorubicin-DNA adducts induce a non-topoisomerase II-mediated form of cell death. *Cancer Res.* 2006; 66:4863–4871. [PubMed: 16651442]
50. Spencer DM, Bilardi RA, Koch TH, Post GC, Nafie JW, Kimura K, Cutts SM, Phillips DR. DNA repair in response to anthracycline-DNA adducts: a role for both homologous recombination and nucleotide excision repair. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* 2008; 638:110–121.
51. Bilardi RA, Kimura KI, Phillips DR, Cutts SM. Processing of anthracycline-DNA adducts via DNA replication and interstrand crosslink repair pathways. *Biochem. Pharmacol.* 2012; 83:1241–1250. [PubMed: 22326903]

(a)

(b)

(c)

Figure 1.

Structures of cytotoxic platinum agents tested (a) and summary of major DNA adducts formed by cisplatin (b) and platinum-acridines (c). “Py” stands for pyrimidine base and “Pu” stands for purine base. Intercalation of acridine, represented by the vertical rectangular shape in c, in the latter hybrid adducts is observed on the 5' face of the damaged guanine (G) base (see ref 6).

Figure 2.

In vitro DNA synthesis monitored by [α - ^{32}P]-dCTP incorporation. (A) Assay design. (B) Representative autoradiogram of an agarose gel showing the progress of the repair of platinum-acridine-modified pUC19 plasmid DNA after 15, 30, 60, and 120 min. pET16b is a no-platinum control. (C) Progress of repair synthesis for DNA treated with cisplatin (blue trace) and **P1-A1** (red trace), expressed as amount of incorporated radiolabeled DNA precursor. Estimated rates: $k_{\text{obs}, 30^\circ\text{C}}(\text{P1-A1}) = 4 \times 10^{-2} \text{ min}^{-1}$, $t_{1/2} = 18 \text{ min}$, $R^2 = 0.998$; $k_{\text{obs}, 30^\circ\text{C}}(\text{cisplatin}) = 5 \times 10^{-3} \text{ min}^{-1}$, $t_{1/2} = 135 \text{ min}$, $R^2 = 0.987$; $^{32}\text{P}_{\text{max}}$ is 0.085 pmol for both agents. Error bars represent $\pm$ standard errors for three experiments.

Figure 3.

Results of the cell-based immunocytochemical assay: (A) confocal images captured of fixed NCI-H460 cells (stitched 3×3 tiles) from the two treatment groups and control cells after 6 h of continuous dosing. Images were recorded for fluorescence in the blue (Hoechst 33342), green (Alexa Fluor 488), and red (Alexa Fluor 647) channels. Scale bars represent a distance of 50 μ m. (B) Plot of background-subtracted fluorescence intensities in the region of interest (ROI, nucleus, and perinuclear region) measured for treatment and control groups ($n > 100$). ROIs in cells treated with platinum–acridine show significantly higher fluorescence intensity ($*P < 0.01$, Welch's t test) when compared to that of either cisplatin-treated cells or control cells.

Figure 4.

(A) Confocal images of NCI-H460 cells treated with platinum-acridine for 3 and 6 h. Note the redistribution of MUS81-related fluorescence (green channel) from the cytoplasm to the nucleus and the increase in, and pan-nuclear distribution of, γ -H2AX-related fluorescence after 6 h of continuous dosing. Scale bar: 10 μ m. (B) Enlarged colocalization image captured of a segment of a single NCI-H460 cell after 3 h of continuous dosing with platinum-acridine. Note the distinct localization of the green (MUS81) fluorescence to the nuclear membrane. Colocalization of γ -H2AX foci with areas of high MUS81-related fluorescence is highlighted by arrows. Scale bar: 5 μ m. (C) Confocal images of three types of γ -H2AX staining showing discrete foci (3 h, panel I), circularly shaped staining (3 h, panel II), and pan-nuclear staining (6 h, panel III). Scale bar: 5 μ m.

Figure 5.

TOP1 cleavage assays for **P1-A1** in pHOT1 plasmid DNA. (A) Lane assignments: 1, plasmid alone; 2, relaxed DNA marker; 3, plasmid + topoisomerase; 4, plasmid +100 μ M camptothecin + topoisomerase; 5–7, plasmid + **P1-A1** at $r_b = 0.005, 0.01, 0.02$ + topoisomerase. (B) Modified assay with and without NaCN addition and Proteinase K digestion. NaCN was typically added before denaturing/digestion of topoisomerase, except for lane 5, where NaCN was added after Proteinase K digestion. For details, see the Materials and Methods section.