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Investigating the cellular fate of a DNA-targeted platinum-based anticancer agent by orthogonal double-click chemistry

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Abstract

Confocal fluorescence microscopy was used to study a platinum-based anticancer agent in intact NCI-H460 lung cancer cells. Orthogonal copper-catalyzed azide–alkyne cycloaddition (click) reactions were used to simultaneously determine the cell-cycle-specific localization of the azide-functionalized platinum–acridine agent **1** and monitor its effects on nucleic acid metabolism. Copper-catalyzed postlabeling showed advantages over copper-free click chemistry using a dibenzocyclooctyne (DIBO)-modified reporter dye, which produced high background levels in microscopic images and failed to efficiently label platinum adducts in chromatin. Compound **1** was successfully labeled with the fluorophore DIBO to yield **1*** (characterized by in-line high-performance liquid chromatography/electrospray mass spectrometry). **1** and **1*** show a high degree of colocalization in the confocal images, but the ability of **1*** to target the (compacted) chromatin was markedly reduced, most likely owing to the steric bulk introduced by the DIBO tag. Nuclear platinum levels correlated inversely with the ability of the cells to synthesize DNA and cause cell cycle arrest, as confirmed by bivariate flow cytometry analysis. In addition, a decrease in the level of cellular transcription, shrinkage of the nucleolar regions, and redistribution of RNA into the cytosol were observed. Postlabeling in conjunction with colocalization

experiments is a useful tool for studying the cell killing mechanism of this type of DNA-targeted agent.

Keywords

Click chemistry; Confocal fluorescence microscopy; DNA synthesis; Platinum agents; Transcription

Introduction

Permanent DNA adducts are the major cause of cancer cell death triggered by platinum-based pharmaceuticals [1, 2]. Various methods have been used to gain insight into the subcellular distribution of platinum-based agents in intact cells. These include element- and isotope-specific techniques based on X-ray absorption/emission and mass spectrometry [3, 4], as well as microscopic detection of fluorescent or fluorescently labeled complexes [5]. Labeling techniques based on bioorthogonal click and ligation chemistries are powerful tools for studying cellular processes at spatial and temporal resolution [6]. Unlike strategies that involve labeling with a reporter molecule prior to incubation with cells, postlabeling allows the detection of an exogenous molecule of interest without perturbing its molecular mechanism and metabolism [7]. This is an important strategy if fluorescent tagging changes the cellular uptake, selectivity for subcellular compartments, and target interactions of the molecule to be probed. To achieve this in platinum–acridine compounds, a class of potent, DNA-targeted antitumor agents [8], we have adopted a method based on copper-catalyzed azide–alkyne cycloaddition (CuAAC) (click) chemistry [9]. This method allows fluorescent labeling of platinum–acridine in fixed cancer cells, in particular the adducts it forms in chromosomal DNA [10].

In a previous study, we developed and validated the postlabeling technique using quantitative sampling of fluorescence intensities in treated cells and appropriate condition-matched controls [10]. The method involves detection of an azide-modified platinum–acridine derivative (compound **1**, Fig. 1a) by means of an alkyne-modified fluorescent dye. Although subtle differences between **1** and azide-free analogues may exist in terms of their cellular uptake and efflux (among other pharmacological parameters), the compound faithfully mimics the structure and mechanism of formation of DNA adducts of the parent hybrid agents [10] and can be considered a viable probe for the current application. Although compound **1**, like other ligatable platinum–acridine compounds, does not exhibit the low-nanomolar half-maximal inhibitory concentrations observed for the most potent hybrids, the compound also maintains submicromolar activity in lung cancer cell lines [11].

We have now used this technique to compare the subcellular localization of postlabeled platinum with that of fluorescently (pre)labeled compound **1** (**1***, Fig. 1a) in NCI-H460 lung cancer cells during interphase and mitosis. We have also combined the detection of platinum with a second orthogonal click reaction. The second labeling step was used to monitor DNA and RNA synthesis in untreated and platinum-treated cells via incorporation of 5-ethynyl-2'-deoxyuridine (EdU) and 5-ethynyluridine (EU), respectively [12, 13]. Since the conditions vary substantially between experiments in cell-based systems, it would be desirable to

determine platinum and DNA/RNA synthesis levels in a given population of cells simultaneously in one imaging session. We were able to achieve this by microscopic colocalization studies and confirmed the results by bivariate flow cytometry analysis. The present study describes the first successful case of orthogonal postlabeling chemistry in whole cells involving a platinum-based pharmacophore. Inhibition of DNA synthesis and inhibition of RNA synthesis were confirmed as two major consequences of the nuclear damage caused by platinum–acridine compounds.

Materials and methods

Labeling of compound **1** with Alexa Fluor 488 DIBO to generate **1***

The copper-free click reaction was performed by combining solutions of compound **1** (synthesized according to a published procedure [10]; 2.5 μ L, 25 mM) and Alexa Fluor 488 DIBO (2.5 μ L, 2.5 mM; purchased from Invitrogen) in dimethylformamide, followed by adjustment of the volume to 200 μ L, vortexing, and incubation of the mixture at room temperature for 3 h. The progress of the reaction was monitored by reverse-phase high-performance liquid chromatography using the liquid chromatograph module of an Agilent Technologies 1100 LC/MSD trap system equipped with a multiwavelength diode-array detector. Analytical separations were performed on an Agilent 4.6 mm $\times$ 150 mm reverse-phase ZORBAX SB-C18 (5 μ m) column at 25 $^{\circ}$ C using the following conditions: solvent A, Optima water/0.1 % ammonium formate; solvent B, methanol/acetonitrile (1:1, v/v); flow rate, 0.5 mL min⁻¹; gradient, 0–5 min 95 % solvent A, 5–20 min 95 % solvent A to 5 % solvent A, 20–30 min 5 % solvent A. High-performance liquid chromatography traces were recorded over wavelength ranges from 363 to 463 nm (for compound **1**) and 480 to 496 nm (for labeled **1***). Electrospray mass spectra of the liquid chromatography fractions were recorded in negative-ion mode. All data were processed with the LC/MSD Trap Control 4.0 data analysis computer program.

Cell culture and incubations

The human non-small-cell lung cancer cell line NCI-H460 (American Type Culture Collection, Rockville, MD, USA) was maintained as described previously [10]. Cells were seeded into poly(D-lysine)-coated glass-bottom cell culture dishes (MatTek, Ashland, MD, USA) with 10⁵ cells per milliliter suspended in 2 mL of medium per dish. Cells were incubated overnight and then treated with compound **1** (5 μ M; synthesized according to a published procedure [10]), compound **1** in the presence of 10 % of its labeled form (**1***), or medium for controls, at 37 $^{\circ}$ C for 3 h. After incubation, cells were washed with phosphate-buffered saline (PBS; 3 $\times$ 2 mL). To detect newly synthesized DNA and RNA, incubations were performed at 37 $^{\circ}$ C for an additional 1 h in 2 mL of prewarmed fetal-bovine-serum-free medium containing 10 μ M EdU and 1 mM EU (Invitrogen), respectively. After treatment with DNA/RNA precursor, cells were exhaustively rinsed with chilled PBS (three times). Finally, cells were fixed by treatment with 3.7 % formaldehyde solution (in PBS, pH 7.4) for 15 min at room temperature, washed twice with a solution of bovine serum albumin (BSA; 3 % in PBS; Sigma) for 10 min, and permeabilized by treatment with 0.5 % Triton X-100 (in PBS, pH 7.4) at room temperature for 20 min. The reaction was quenched with 3 % BSA in PBS (2 $\times$ 10 min) and the cells were incubated with 250 μ L of click reaction

mixture [1 mM CuSO₄; 0.5 μM Alexa Fluor 488 alkyne or Alexa Fluor 647 alkyne (Invitrogen); 10 mM sodium ascorbate; 50 mM tris(hydroxymethyl) aminomethane (Tris)-HCl, pH 7.3] at room temperature for 30 min. Prior to the second click reaction, cells were washed using the following procedure: (1) 3 % BSA in PBS (5 min); (2) 0.5 % Triton X-100 in PBS (2 × 10 min); (3) PBS (2 × 10 min). Incorporated cellular EdU and EU were then labeled by reacting them with labeling mix [1 mM CuSO₄; 0.5 μM Alexa Fluor 647 - azide (Invitrogen); 10 mM sodium ascorbate; 50 mM Tris-HCl, pH 7.3] at room temperature for 30 min. Before addition of nuclear stain in colocalization experiments, cells were subjected to extensive washes: (1) 3 % BSA in PBS (5 min); (2) 0.5 % Triton X-100 in PBS (2 × 10 min); (3) PBS for (3 × 10 min). Nuclei were stained with 5 μg mL⁻¹ Hoechst 33342 (Sigma) in PBS (5 min), followed by three additional PBS washes prior to image acquisition.

Confocal microscopy

Images were recorded with a Zeiss LSM 710 confocal microscope (Carl Zeiss, Thornwood, NY, USA) using either a ×40 (PLAN APO, 0.95 numerical aperture) or a ×63 (PLAN APO, 1.2 numerical aperture) objective lens. All images were acquired in multitrack configuration mode to minimize excitation cross talk and emission bleed-through. A 405-nm laser line with an emission range of 410–478 nm was used for Hoechst 33342, a 488-nm laser line (emission 489–553 nm) was used for Alexa Fluor 488, and a 633-nm laser line (emission 655–732 nm) was used for Alexa Fluor 647. For all images, the pinhole value was kept at or below 1.2 airy units. Images were acquired with eight times line averaging at 1,024 × 1,024 pixel resolution and 12-bit sampling to provide a wide dynamic intensity range for analysis (0–4,096). The computer program ZEN was used for image acquisition and processing. Confocal image planes for each channel were not contrast-adjusted or otherwise changed and were assembled in Adobe Photoshop CS without further manipulation.

Cell treatments and CuAAC ligation procedures for flow cytometry analysis

For flow cytometry analysis, 10⁶ NCI-H460 cells were seeded into 35-mm cell culture dishes in 2 mL of medium and preincubated in 5 % CO₂ at 37 °C overnight. The medium was replaced with 2 mL of fresh medium for untreated cells or 2 mL of medium containing 2.5 and 5 μM compound **1**. After 24 h of incubation, cells were rinsed with PBS (3 × 2 mL) and treated with 2 mL of EdU-containing medium (10 μM) for 1 h. For no-EdU controls, cells were treated with EdU-free medium for 1 h. Cells were rinsed with PBS and detached with trypsin. Cell pellets were collected by centrifugation (1,500 rpm, 5 min), washed twice with PBS, and resuspended in 200 μL PBS. Cells were fixed by adding the suspension to 1.8 mL ice-cold ethanol with gentle pipetting. Fixed cells were stored at –20 °C for at least 2 h but no longer than 2 weeks. Immediately prior to the flow cytometry experiments, the cells were centrifuged at 1,500 rpm for 5 min, the supernatant was removed, and the cells were washed twice with PBS. After the cells had been rinsed with PBS, the cell pellets were resuspended in 200 μL of click reaction mixture (1 mM CuSO₄; 5 μM Alexa Fluor 488 alkyne; 10 mM sodium ascorbate; 50 mM Tris-HCl, pH 7.3) and incubated in the dark at room temperature for an additional 30 min. The click reaction was quenched by adding 1 mL of 1 % BSA in PBS (pH 7.2), and the cells were washed with PBS (3 × 1 mL) before being incorporated with the second click cocktail. Cells were then pelleted and incubated with the second click cocktails (1 mM CuSO₄; 5 μM Alexa Fluor 647 azide; 10 mM sodium

ascorbate; 50 mM Tris-HCl, pH 7.3) at room temperature for 30 min. After incubation, cells were centrifuged, washed with PBS, mixed with 500 μ L propidium iodide staining buffer (10 μ g mL⁻¹, 10 μ g mL⁻¹ RNase A, 1 % BSA in PBS, pH 7.2) and incubated in the dark for 30 min at 37 °C.

Flow cytometry

The cells were analyzed using a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) to determine cellular platinum levels, total DNA content, and EdU incorporation. For each sample, 20,000 events were counted. The Alexa Fluor 488, propidium iodide, and Alexa Fluor 647 fluorescence intensities were measured using the FL-1 (533 nm/30 nm), FL-2 (585 nm/40 nm), and FL-4 (675 nm/25 nm) channels of the instrument, respectively. Experiments for each condition were performed in quadruplicate. Data analysis was performed using BD Accuri C6 software.

Results and discussion

Detection of platinum–acridine in nuclear DNA during different phases of the cell cycle

Prior to investigating cellular events and colocalization using orthogonal labeling, we undertook a detailed analysis of platinum accumulation in nonsynchronized NCI-H460 lung cancer cells. Platinum–acridine compounds target double-stranded DNA by intercalation and rapidly ($t_{1/2} \approx 20$ min) induce highly cytotoxic permanent adducts with guanine nitrogen [8]. In previous work, we used inductively coupled plasma mass spectrometry of DNA extracted from NCI-H460 cells and found that platinum levels reached a maximum after 3 h of incubation with a hybrid agent [14]. To detect platinum in intact subcellular structures, cells were incubated with compound **1** under conditions that did not induce apoptosis and the DNA fragmentation and changes in cell morphology associated with it [15]. These conditions were met when cells were treated with compound **1** at a concentration of 5 μ M, but only for a short time.

The experiments pursued in this study required the use of two fluorophores emitting at different wavelengths. Using the same setup reported previously for green-fluorescent Alexa Fluor 488 alkyne, we demonstrated that the red-fluorescent dye Alexa Fluor 647 alkyne is equally compatible with CuAAC-based detection of intracellular platinum. For fluorescent postlabeling of the azide-modified platinum–acridine agent, cells were fixed, treated with copper-based click reaction mix in the presence of Alexa Fluor 647 alkyne (Fig. 1b), and co-stained with the DNA-specific dye Hoechst 33342.

Views of single confocal image planes of NCI-H460 cells (Fig. 2) show high levels of Alexa Fluor-associated fluorescence in the chromatin, consistent with a high platinum content in DNA in all phases of the cell cycle. Cells undergoing mitosis show the highest fluorescence intensity in the replicated chromosomal DNA. Although most of the cancer cells were in the growth phase at the time of treatment, a small population was captured while dividing. In cells that have entered mitosis (prophase), which show individual sister chromatids, the highest platinum–Alexa Fluor 647 (red) fluorescence colocalizes with the area of Hoechst-stained DNA (Fig. 2, row A). Likewise, use of Alexa Fluor 647 enabled us to detect high levels of platinum in the separated chromatids and reassembling chromosomes during

mitosis (Fig. 2, rows B, C). By contrast, the highest level of platinum-related fluorescence in cells in interphase was typically observed in the nucleolus, as previously reported [10]. The nucleolus is a transient, non-membrane-bound subnuclear structure, whose primary function involves ribosome biogenesis (Fig. 2, row D) [16]. High levels of fluorescence are also detected across the entire nuclear region within the loosely packaged chromatin. In all phases of the cell cycle, Alexa Fluor 647-based fluorescence is also observed in the cytoplasm, although at a much lower intensity.

To investigate if the detection of intracellular platinum–acridine can also be achieved by copper-free click chemistry, similar labeling experiments were performed with Alexa Fluor 488 DIBO, which contains an azide-reactive dibenzocyclooctyne (DIBO) group (Fig. 1b) [17]. The confocal images obtained from these experiments (see the electronic supplementary material) show unfavorable high background fluorescence in all cytosolic organelles of cells not treated with compound **1**. In addition, the DIBO-based fluorophore, unlike simple copper/alkyne, was unable to label platinum adducts in the DNA of dividing cells. This observation suggests that the steric bulk of DIBO may prohibit reaction with platinum-based azide in the compacted chromatin. Thus, copper-free click chemistry using DIBO-modified dye was dismissed as a postlabeling technique in experiments requiring orthogonal reactions with two fluorophores.

Colocalization studies of compound **1** and a fluorescently tagged derivative, **1***

To determine the effects of tagging the azide-modified complex **1** with a fluorophore prior to treatment of cancer cells, we attempted to label this compound with Alexa Fluor alkyne using CuAAC chemistry. Unfortunately, these reactions, which had to be performed in (partly) aqueous media, led to substantial decomposition of compound **1** and products indicating aquation of the complex and undesired substitution of platinum-coordinated chloride by the alkyne moiety (data not shown). By contrast, Alexa Fluor 488 DIBO reacted cleanly with compound **1** in dimethylformamide solution to produce the desired conjugate **1*** (Fig. 1a). When the DIBO-modified dye was reacted with excess platinum (1:10) at room temperature, complete conversion to cycloadduct was observed within 3 h, as confirmed by in-line liquid chromatography–mass spectrometry (Fig. 3). Reaction mixtures generated this way, in which no unreacted Alexa Fluor 488 DIBO was detectable, were directly used for colocalization studies in NCI-H460 cells. Because conjugate **1*** was generated in situ on a microgram scale, it was not possible to isolate sufficient amounts of the pure conjugate for imaging and cell proliferation assays.

Cells exposed to a mixture of **1** and **1*** (~9:1) were imaged after permeabilization and treatment with Alexa Fluor 647 alkyne in the absence or presence of copper. The former conditions were chosen to selectively detect green-fluorescent **1*** and as a negative control for non-platinum-associated Alexa Fluor 647 (red) fluorescence. Copper-based click chemistry with the red-fluorescent dye was used to detect platinum–azide (**1**) and **1*** simultaneously. Confocal images taken under both conditions show the highest level of green fluorescence in the nucleolar regions of the cells, confirming that the fluorophore-labeled platinum–acridine (**1***) accumulates in this subnuclear structure (Fig. 4). This is an important observation, since Alexa Fluor 488 DIBO itself shows no significant affinity for

the nucleoli in cells in interphase (see the controls for DIBO postlabeling experiments in the electronic supplementary material). By comparison, the nuclear and cytosolic levels of **1*** are considerably lower. As expected, under copper-free conditions only insignificant background levels of red fluorescence are observed in the Alexa Fluor 647 channel (Fig. 4, top panel). When copper is introduced for the purpose of postlabeling intracellular compound **1**, high levels of red fluorescence are observed across the entire nuclear region of the cells (Fig. 4, bottom panel). This observation is consistent with the previously observed (Fig. 2) accumulation of the hybrid agent in the nuclear DNA of cells in both interphase and mitosis. A high degree of colocalization is observed for compounds **1** and **1*** in the merged-channel image, suggesting that labeled and unlabeled platinum–acridine share qualitatively similar subcellular distribution patterns. However, closer inspection of individual cells reveals that the fluorescence level in the chromatin (relative to that observed in the nucleolus and cytosol) is significantly higher in the red channel than in the green channel (see the electronic supplementary material for an enlarged image of a single cell). A possible explanation for this effect is the increased steric bulk in **1*** introduced by the Alexa Fluor 488 DIBO moiety, which can be expected to affect the ability of the conjugate to associate and form adducts with double-stranded DNA of the chromatin.

Dual detection of platinum–acridine and nucleic acid synthesis in cells by confocal fluorescence microscopy

In another set of experiments, NCI-H460 cells were pulsed (60 min) with fluorescently detectable DNA/RNA precursors after treatment with compound **1** to assess the effect of intracellular platinum on DNA and RNA synthesis. Simultaneous detection of platinum and newly synthesized nucleic acids required orthogonal labeling of platinum–acridine by Alexa Fluor 488 alkyne and incorporated EdU or EU (Fig. 5a) by Alexa Fluor 647 azide, respectively (Fig. 5b). A potential complication in this assay might arise from cross-reactivities as a result of direct copper-mediated reaction of platinum–azide with the alkyne-modified precursors EU and EdU. For instance, if in the labeling steps after fixation unincorporated EdU underwent a direct click reaction with the DNA adducts of the platinum–azide, the latter would become undetectable by Alexa Fluor dye. Likewise, incorporated EU/EdU could potentially undergo click chemistry with excess platinum–azide. To avoid perturbation of the imaging results by such undesired cross-reactions between functionalized platinum, precursors, and labeling reagents, the following measures were taken: (1) incubations with compound **1** and EU/EdU were performed under optimized conditions yielding the highest levels of platinum–DNA adduct formation and DNA/RNA precursor incorporation [8] and (2) great care was taken to minimize undesired cross-reactions by performing extensive dialyzing washes of the fixed, permeabilized cells after each treatment step.

Confocal images were taken of untreated cells and platinum-treated cells in the presence of EdU (Fig. 6a) and EU (Fig. 6b) after orthogonal dual labeling. In both cases, cells treated with compound **1**, followed by postlabeling with Alexa Fluor 488, show a marked increase in green fluorescence relative to controls, consistent with accumulation of hybrid agent in the cells' nuclei. The opposite effect is observed in the Alexa Fluor 647 (red) channel. A relatively smaller number of cells in interphase stained positive for EdU incorporation,

consistent with the notion that the de novo synthesis of DNA in cells treated with compound **1** is greatly suppressed. Characteristically, cells that remain sufficiently viable to synthesize DNA show relatively low levels of platinum-related green fluorescence (Fig. 6a). The relative fluorescence intensities measured in platinum-treated cells show the most intense red fluorescence in cells with the lowest platinum-associated green fluorescence, whereas no significant red fluorescence is detected at the highest nuclear platinum levels (see the electronic supplementary material). However, because the cells in these experiments were not synchronized, cell-cycle-specific effects have to be taken into consideration, which makes it difficult to establish a true inverse proportionality between platinum and DNA synthesis levels.

A similar situation is observed in cells that were incubated with the nucleoside analogue EU to probe the effects of platinum on RNA synthesis (Fig. 6b). EU is a valuable probe since it is incorporated into RNA by RNA polymerase I (Pol I) and RNA polymerase II, but is not reduced intracellularly and incorporated into DNA [13]. In control cells, Alexa Fluor 647 (red) fluorescence is confined to the nucleus and shows the highest intensity in the nucleolar region, consistent with the high rate of preribosomal RNA synthesis required for ribosome biogenesis in the rapidly proliferating NCI-H460 cells (the doubling time was less than 20 h). Accumulation of compound **1** in the nuclei and nucleoli of treated cells causes a decrease in RNA synthesis, as evidenced by the significantly reduced fluorescence intensity in the red channel. It also results in a dramatic decrease in the size of the nucleolar region and a redistribution of EU-labeled RNA from the nucleus into the cytoplasm. This observation supports the notion that the nuclear and nucleolar accumulation of platinum–acridine compounds has a major effect on RNA metabolism (see the discussion later).

Dual detection of platinum–acridine and DNA synthesis using flow cytometry

In addition to confocal microscopy, we used flow cytometry analysis to assess the utility of the postlabeling strategy for monitoring cell cycle effects. In essence, we combined the orthogonal platinum–azide/EdU postlabeling assay with propidium iodide staining of the total cellular DNA, which serves as a measure of the number of cells present in each phase of the cell cycle [18]. Both cells exposed to compound **1** under conditions that do not induce apoptosis and untreated controls were subjected to a 60-min EdU pulse followed by labeling using CuAAC chemistry and the appropriate fluorescent dyes (see “Materials and methods” for details).

The bivariate analysis of intracellular platinum and DNA content/cell distribution shows a pronounced increase in green fluorescence for cells treated with compound **1** relative to the background determined for controls not treated with platinum but treated with green-fluorescent Alexa Fluor 488 alkyne (Fig. 7a). Elevated fluorescence is observed in the two major cell populations representing cells with an unreplicated (G_1) and a replicated (G_2/M) genome. Thus, flow cytometry, like confocal microscopy, in conjunction with postlabeling is a viable method for determining platinum levels in cells of varying DNA content during all phases of the cell cycle.

The same samples of treated and untreated cells were examined for incorporation of EdU using gated bivariate analysis (Fig. 7b). Untreated, replicating cells (control) proficiently

incorporate EdU into newly synthesized DNA. Approximately 27 % of the cells counted are in S phase, and G₁ and G₂/M phase cells account for 59 % and 9 % of the total cell population, respectively. By contrast, S phase analysis of the cells treated with compound **1** reveals an almost complete quenching of DNA synthesis. Less than 1 % of the cells are detected in S phase, and a distinct buildup of cells in G₁ phase (80 %) is observed. Cells treated with compound **1** show very similar platinum–azide versus DNA content and cellular EdU incorporation versus DNA content distributions (Fig. 7, right panels). The two distinct major cell populations assigned to cells in G₁ phase and cells in G₂/M phase, which show low levels of EdU incorporation (Fig. 7b), mimic those observed for the platinum–azide-related fluorescence (Fig. 7a). This correlation suggests that the latter elevated signal in counted cells is primarily associated with the DNA adducts formed by compound **1**, which is supported by the confocal microscopy data.

Biological implications

Platinum–acridine compounds form adducts in the genomic DNA of NCI-H460 cells at a 25-fold higher frequency than the clinical drug cisplatin under the same conditions [14]. The hybrid adduct, which causes extensive damage to replication forks and DNA double-strand breaks, is an inherently severer form of DNA damage than the cross-links formed by cisplatin [8]. Treatment of NCI-H460 cells with platinum–acridine compounds has previously been demonstrated to cause a buildup of cells at the G₁/S border of the cell cycle and an arrest of cells in early S phase [8]. Our dual-labeling strategy in combination with fluorescence imaging and flow cytometry suggests that these cell cycle effects, which ultimately lead to apoptotic cancer cell death, are a result of DNA-associated platinum.

The reduced level of EU incorporation into RNA in NCI-H460 cells treated with compound **1** across the entire nucleus supports the notion that inhibition of transcription plays a major role in the mechanism of this agent. On the other hand, the relatively high level of EU-related fluorescence in the cytosol of platinum-treated cells (Fig. 6b) was quite unexpected. We speculate that this observation may indicate redistribution and degradation by the cytosolic exosome of damaged RNA due to faulty transcription [19]. It has been proposed that stalling of Pol II by platinum–DNA adducts contributes to the cell killing effect of both cisplatin-type drugs and nontraditional platinum-based agents [1, 8]. Thus, inhibition of Pol II-mediated (messenger RNA) transcription was an expected outcome of the current postlabeling experiments. In addition, the cytomorphological changes of the nucleoli observed after treatment with compound **1** seem to indicate that Pol I-mediated ribosomal RNA synthesis is also affected. A potential mechanism would involve localization of platinum to the nucleolus and inhibition of the Pol I transcription machinery by adducts formed in the transcribed ribosomal RNA genes (ribosomal DNA). Although reversibly DNA binding drugs, such as actinomycin D, have shown dual Pol I/Pol II inhibition [13, 20], this would be an unprecedented mechanism for a platinum-based agent. Cisplatin also affects Pol I transcription but in an indirect way by recruiting transcription factors away from the nucleolus [21, 22]. Additional functional studies are warranted to validate Pol I transcription as a cellular target of platinum–acridine compounds.

Concluding remarks

Copper-mediated cycloaddition chemistry is a viable strategy for the detection of a suitably functionalized DNA-targeted platinum-based pharmacophore in intact cells. We have demonstrated that this technique can be applied in tandem with other orthogonal postlabeling reactions to study the intracellular fate and effects of these agents. Recent work by White et al. [23], who used the same postlabeling strategy to detect an azide-modified cisplatin derivative in whole cells, suggests that this method may also have broader applicability to studying clinically relevant cross-linking agents. On the other hand, introduction of the bulky DIBO-based fluorescent dye as a label and its use for postlabeling applications seem to be less promising approaches. Nevertheless, the potential advantages of incorporating less perturbing fluorophores, for instance, for imaging applications in live cells, remain to be explored. Future work will also address if the technique can be extended to immunocytochemical applications [24, 25] that will provide additional details on the DNA damage and repair mechanisms associated with these agents. The study has also provided insight into a previously unexplored potential cellular target of platinum-containing pharmacophores, the cell's nucleolus. Additional functional studies at the cellular level are required to validate this subnuclear structure and its Pol I machinery as a direct target of platinum-acridine compounds. Pol I inhibition would present an intriguing opportunity for designing more selective agents targeted at aberrant nucleolar ribosome biogenesis in cancer cells [26, 27].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CuAAC	Copper-catalyzed azide-alkyne cycloaddition
DIBO	Dibenzocyclooctyne
EdU	5-Ethynyl-2'-deoxyuridine
EU	5-Ethynyluridine
PBS	Phosphate-buffered saline
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Tris	Tris(hydroxymethyl)aminomethane

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Fig. 1.

a Structures of probes used in this study. **b** Detection of azide-modified platinum–acridine (DNA adducts) with a complementary alkyne-modified fluorescent dye. The *star* indicates Alexa Fluor 488 (*green*) and Alexa Fluor 647 (*red*) fluorophores. **c** Simultaneous detection of platinum–acridine in cells incubated with a mixture of compound **1** and its Alexa Fluor 488 DIBO conjugate, **1***. Postlabeling of **1** was performed with the alkyne form of Alexa Fluor 647 (*red star*)

Fig. 2.

Cell-cycle-dependent distribution of compound **1** in NCI-H460 cells. Confocal images of cells in early mitosis (prophase, *A*), late mitosis (*B*, *C*), and interphase (*D*) after treatment with compound **1** (5 μ M) and after labeling with copper-catalyzed azide–alkyne cycloaddition/clickable fluorophore Alexa Fluor 647 (*red*). Hoechst 33342 (*blue*), merged-channel, and bright field channel images are also shown. (For additional views of cells labeled with Alexa Fluor 647 alkyne and condition-matched controls, see the electronic supplementary material)

Fig. 3.

Labeling of compound **1** with Alexa Fluor 488 DIBO in dimethylformamide solution monitored by in-line liquid chromatography–mass spectrometry. High-performance liquid chromatography traces are shown for an Alexa Fluor-specific (λ_{max} 488 nm) and an acridine-specific (λ_{max} 413 nm) wavelength range. High-performance liquid chromatography fractions are labeled **1** and **2** for the two constitutional isomers of cycloadduct **1*** and **3** for compound **1**. Molecular ions ($[M - nH]^-$) are observed for each species at m/z values of 1,556, 1,557, and 721, respectively. Note the absence of unreacted Alexa Fluor 488 DIBO under these reaction conditions (tenfold excess of compound **1**)

Fig. 4.

Colocalization study of platinum-acridine **1**, postlabeled with Alexa Fluor 647 alkyne (*red channel*), and its Alexa Fluor 488 - DIBO-labeled derivative, **1***, in NCI-H460 cells. Cells were costained with Hoechst 33342 nuclear dye (*blue channel*). Note the highest degree of colocalization of the two species in the nucleolar regions of postlabeled cells, which appear as *bright-yellow spots* in the merged-channel image. *Scale bars* represent a length of 10 μm

Fig. 5.

a Structures of the alkyne-modified nucleic acid precursors used in this study. **b** Combined detection of intracellular platinum–acridine and DNA/RNA synthesis using orthogonal, sequential copper-catalyzed azide–alkyne cycloaddition (CuAAC) reactions. Labeling was performed with the alkyne form of Alexa Fluor 488 (*green star*) and the azide form of Alexa Fluor 647 (*red star*)

Fig. 6.

Dual detection of platinum-acridine with Alexa Fluor 488 - alkyne (*green channel*) and DNA synthesis (**a**) or RNA synthesis (**b**) with Alexa Fluor 647 (*red channel*) in NCI-H460 cells by means of orthogonal CuAAC click chemistry. Cells were co-stained with Hoechst 33342 nuclear dye (*blue channel*). The *white arrows* in **a** indicate cells in interphase that show detectable levels of DNA synthesis activity after treatment owing to relatively low levels of intranuclear platinum. The *white arrows* in **b** highlight nucleolar regions before and after treatment. The red fluorescence in cells treated with compound **1** is no longer confined to the nuclei. *Scale bars* represent a length of 10 μ m

Fig. 7.

Simultaneous detection of cellular levels of compound **1** (a) and DNA replication (b) using orthogonal postlabeling in conjunction with bivariate flow cytometry analysis. The *vertical arrows* indicate the buildup of cells at the G₁/S border, and the *horizontal arrows* indicate cells in G₂/M phase. For alternative representations of correlations and control experiments, see the electronic supplementary material