Rapid fluorescence-based reporter-gene assays to evaluate the cytotoxicity and antitumor drug potential of platinum complexes

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Background: The need for new platinum antitumor drugs is underscored by the usefulness of cisplatin and carboplatin in chemotherapy and the resistance of many tumors to these compounds. Combinatorial chemistry could aid in the search for cisplatin analogs if fast, high-throughput assays were available. Our goal was to develop rapid cell-based assays suitable for high-throughput screening that accurately predict the cytotoxicity of platinum complexes. We examined the effects of platinum complexes and other agents on reporter-gene expression in cancer cells.

Results: HeLa Tet-On cells with inducible enhanced green fluorescent protein (EGFP) were prepared. Cisplatin and other *cis*-disubstituted platinum complexes inhibited EGFP expression, with a strong positive correlation between EGFP inhibition and cytotoxicity. By contrast, *trans*-$[Pt(NH_3)_2Cl_2]$, other *trans*-platinum complexes, methyl methanesulfonate or heat shock stimulated EGFP expression. Northern and nuclear run-on analyses revealed that the changes in EGFP expression were at the level of transcription. In another reporter-gene assay in Jurkat cells, cisplatin, but not *trans*-$[Pt(NH_3)_2Cl_2]$ or $K_2[PtCl_4]$, inhibited β-lactamase expression, as measured by hydrolysis of the fluorescent substrate CCF2.

Conclusions: The EGFP results indicate that cytotoxic stress enhances transcription from the inducible promoter, whereas compounds able to form the 1,2-intrastrand platinum–DNA cross-links repress transcription. Both fluorescence-based reporter-gene assays afford promising new approaches to platinum anticancer drug discovery.

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Introduction

The success of *cis*-$[Pt(NH_3)_2Cl_2]$ (*cis*-DDP or cisplatin) and carboplatin as chemotherapeutic agents, combined with the resistance of many tumors to these compounds, underscores the need for new platinum drugs [1]. Although combinatorial chemistry is a key component of many drug discovery efforts, there is only one report of its application to the search for cisplatin analogs [2]. In order to facilitate the screening of combinatorial libraries of platinum complexes, fast, high-throughput assays are needed. Methods utilizing cultured cancer cells are desirable because they provide essential information about the uptake and metabolism of new compounds. The commonly used cytotoxicity assays, however, are not ideal. The clonogenic or colony-counting method can take over one week to yield results, and the assay is not suitable for high-throughput screening [3]. Assays employing dyes such as neutral red [4], MTT [5] or sulforhodamine B [6] can be performed with the aid of robotics for high throughput, but the cells must be incubated for several days before quantitation. The goal of this research, therefore, was to develop rapid cell-based assays suitable for high-throughput screening that accurately predict the cytotoxicity of platinum complexes.

Transcription, a key step in gene expression, seemed at the outset to be a suitable focal point for assay development. The *in vitro* inhibition of replication and transcription by platinum–DNA adducts is well documented [7–9]. Platinum complexes can also inhibit gene expression in mammalian cells. In one study [10], plasmids containing the β-galactosidase reporter gene under the control of the human cytomegalovirus (CMV) or simian virus 40 (SV40) early region (SVER) promoter, were modified with *cis*- or *trans*-DDP and transfected into mammalian cell lines. Cisplatin inhibited β-galactosidase transcription 2–3-fold better than the clinically ineffective *trans*-DDP at equal adduct levels, a result attributed to more efficient bypass of *trans*-DDP adducts by RNA polymerase. In a series of transient transfections of CV-1 monkey cells, *cis*- and *trans*-DDP both inhibited reporter-gene expression from various promoters [11]. Cisplatin, unlike *trans*-DDP, inhibited expression from strong promoters better than from weak promoters, suggesting that

the targeting of highly expressed genes could contribute to the cytotoxicity of the cis isomer. In mouse tumor cells cisplatin, but not an equitoxic dose of *trans*-DDP, blocked transcription from the mouse mammary tumor virus (MMTV) promoter [12]. It was suggested that an alteration in DNA structure upon the formation of cisplatin adducts prevents chromatin remodeling and transcription-factor binding, both essential for transcriptional activation, an explanation consistent with the results of recent X-ray and nuclear magnetic resonance (NMR) studies [13–15].

Cisplatin and other DNA-damaging agents can also stimulate gene expression from promoters implicated in the cytotoxicity or resistance pathways. In mammalian cells, DNA damage can induce p53-dependent apoptosis or a SOS DNA repair type response that results in cell-cycle arrest, DNA repair and enhanced survival [16,17]. In mouse erythroleukemia cells, *c-myc*-driven chloramphenicol acetyltransferase (CAT) expression was enhanced by cisplatin [18]. Activation of *c-myc* has been implicated in cisplatin resistance [19]. In melanoma cells, cisplatin and UV light both induce *c-jun* promoter activity, which is correlated with apoptosis in many cell lines [20]. The effect of cisplatin on genes involved in the regulation of cell growth is reviewed in [21].

DNA damage also induces gene expression from promoters with no obvious role in cytotoxicity or resistance. Transient and stable transfections of appropriate vectors in HeLa cells revealed that cisplatin inhibited CAT expression from the SVER promoter and the adenovirus 2 major late promoter (MLP), but induced expression from the HIV-1 long terminal repeat (LTR) and adenovirus 5 E3 promoters [22]. LTR induction was also observed in cisplatin-treated human and rat fibroblasts [23] and in HeLa cells treated with mitomycin C or UV light [24].

The promoter-specific effects of *cis*- and *trans*-DDP in mammalian cells imply that a reporter-gene assay could be a fast, convenient way to screen novel platinum complexes for biological activity. There have been only a few reports linking reporter-gene expression with cytotoxicity [25–27], and none of these directly correlates transcriptional effects with mammalian cell death. Until recently, reporter-gene assays were not convenient for high-throughput screening.

A better alternative is the recently cloned *Aequoria victoria* green fluorescent protein (GFP) [28–30]. GFP has a stable, species-independent post-translational fluorophore that requires no cofactors or substrates and can be visualized in intact cells and tissues with readily available fluorescein isothiocyanate (FITC) excitation and emission filters. To improve upon existing fluorescence-based reporter-gene technologies, a method using β-lactamase as a reporter enzyme was recently developed [31]. The fluorogenic substrate CCF2/AM is readily taken up into the cytoplasm of a cell, where esterases convert it to the membrane-impermeable substrate CCF2. Upon excitation with violet light, intact CCF2 emits green fluorescence, whereas the product of β-lactamase-catalyzed CCF2 hydrolysis emits blue light. It is therefore possible to distinguish expressing from nonexpressing cells by the colour of their fluorescence. The ratio of blue to green fluorescence intensities is a measure of β-lactamase expression, and the enzymatic amplification of the signal allows very low levels of expression to be detected.

Because of the long (20–30 h) half-life of GFP in mammalian cells [32], we examined the effect of platinum complexes on inducible GFP expression. A Tet-On system was chosen [33] where the gene of interest, enhanced GFP (EGFP), was cloned into a plasmid downstream of the minimal early promoter of the CMV (P_{minCMV}). In the presence of doxycycline, the tet-responsive transcriptional activator (rtTA) binds and activates the tetracycline-responsive element (TRE), which in turn activates the CMV promoter. In this manner, the addition of doxycycline to the growth medium induces EGFP expression. Inducible β-lactamase expression in Jurkat cells was achieved by using a previously reported cell line [31] with the β-lactamase reporter under the control of a tandem trimer of nuclear factor of activated T-cells (NF-AT) binding sites and the G-protein-coupled muscarinic receptor under the control of the CMV promoter (CMV-M_1-GPCR/NFAT-bla Jurkat or C2 Jurkat). Treatment of the cells with the muscarinic agonist carbachol induced β-lactamase expression. A Jurkat cell line (CMV–bla) with constitutive β-lactamase expression was also used.

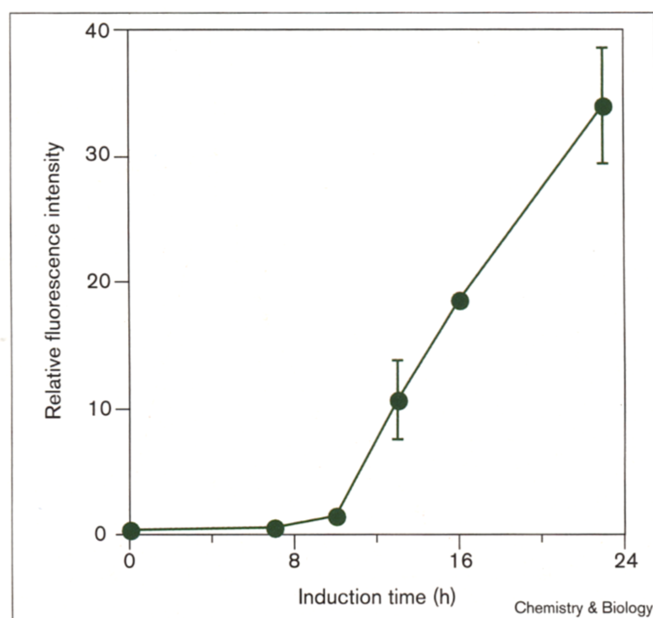
We demonstrate in this paper that the cytotoxicity of platinum complexes can be predicted from their effect on either EGFP or β-lactamase expression in human cancer cells. The implications of the effect of different types of damaging agents on inducible EGFP expression are also discussed.

Results

Inducible EGFP expression in HeLa Tet-On cells

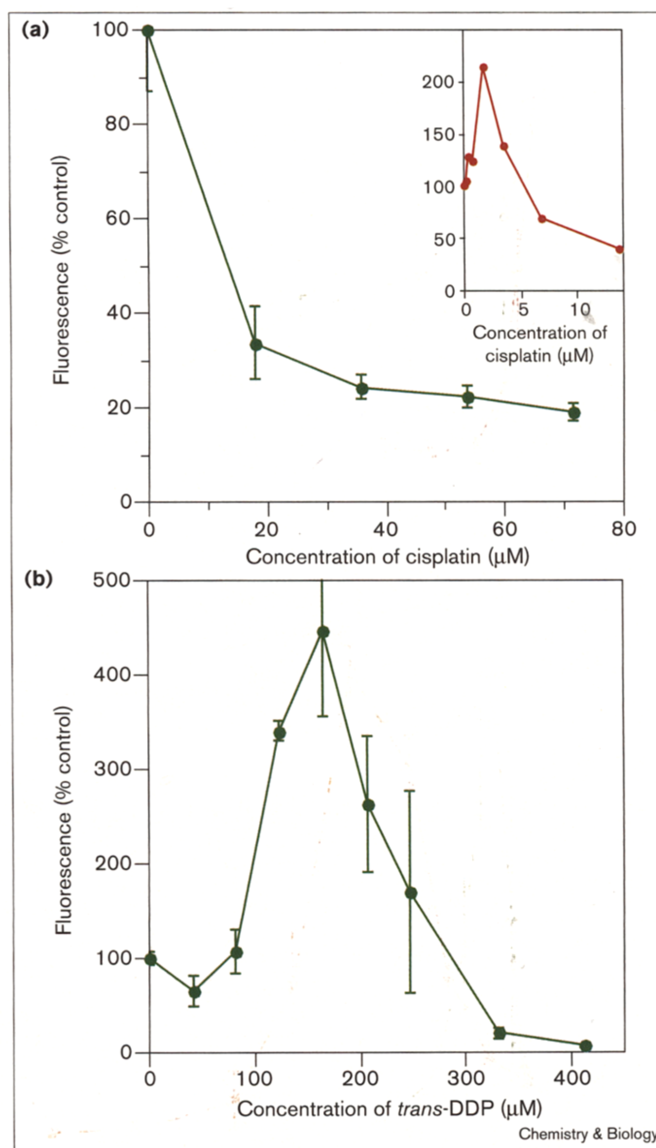
To generate human cancer cells with inducible EGFP expression, HeLa Tet-On cells were stably transfected with pTRE-EGFP, with the hygromycin resistance plasmid pTK-Hyg added for selection. Fluorescence microscopy and western blotting (data not shown) confirmed that several clones including C27 expressed EGFP in the presence of doxycycline. The time course of doxycycline induction of EGFP fluorescence in HeLa C27 is plotted in Figure 1. A 13.5 h induction period provided sufficiently high fluorescence readings, which were independent of the doxycycline concentration over a range of 2.2–216 μM (data not shown).

Figure 1



Time course of EGFP induction in HeLa C27 cells. Cells were treated with 21.6 μM doxycycline. Error bars represent ± 1 esd.

Figure 2



Effect of **(a)** cisplatin and **(b)** *trans*-DDP treatment on EGFP expression in HeLa C27 cells. All samples were induced with 21.6 μM doxycycline for 13.5 h. Error bars represent ± 1 esd.

Effect of platinum complexes on EGFP expression in HeLa C27 cells

A dose-dependent decrease in EGFP expression was observed when HeLa C27 cells were treated simultaneously with cisplatin and doxycycline and examined after 13.5 h (Figure 2a). *trans*-DDP affected EGFP expression in a manner distinct from cisplatin. At the lower *trans*-DDP concentrations used, there was a dose-dependent increase in EGFP, followed by a sharp decrease at very high concentrations (Figure 2b). This effect was not observed in cells treated with *trans*-DDP alone; doxycycline induction was essential. Similar results were obtained using another clone (data not shown), indicating that the results were not unique to C27 cells.

Whereas cisplatin treatment resulted in a dose-dependent decrease in EGFP expression, at extremely low concentrations the drug also caused a modest increase (twofold) in EGFP expression (see inset, Figure 2a). These results were consistently reproducible although the error remained large.

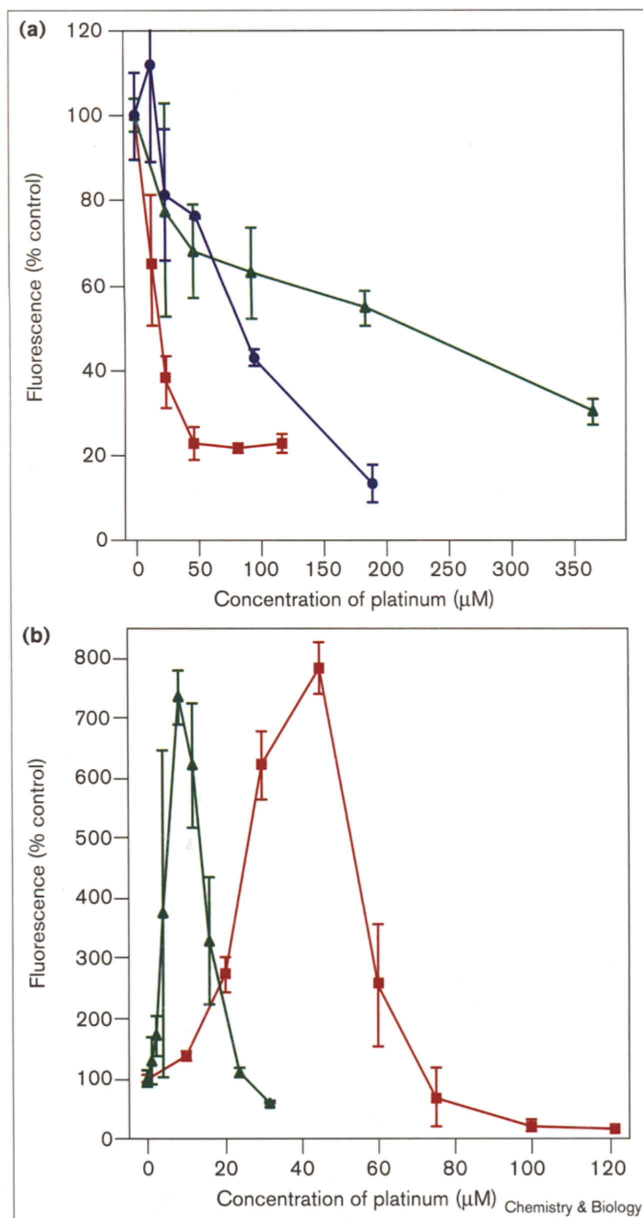
The effect of a 13.5 h co-treatment with doxycycline and two major types of platinum complexes was tested. *cis*-Platinum complexes having chloride ligands in cis positions mimicked cisplatin by inhibiting EGFP expression in a dose-dependent manner (Figure 3a). Complexes having chloride ligands in trans positions induced EGFP expression at low doses and inhibited it at high doses in a manner similar to *trans*-DDP (Figure 3b). Treatment with the noncytotoxic compound [Pt(dien)Cl]Cl had no effect on EGFP expression (data not shown).

DAPI (4′,6-diamidino-2-phenylindole) staining (data not shown) revealed that a 16.5 h treatment with 20 μM cisplatin induced apoptosis in 50% of the C27 cells, that is, AC_{50} = 20 μM. After a 20 h treatment, the AC_{50} for cisplatin was 16 μM. Although trypan blue staining showed that a 20 h treatment with 360 μM *trans*-DDP killed more than 90% of the cells, under no conditions did *trans*-DDP induce apoptosis in more than 15% of the HeLa C27 cells.

Effect of other cytotoxic treatments on EGFP expression in HeLa C27

To assess the generality of the platinum compound results, HeLa C27 cells were treated with several nonplatinum

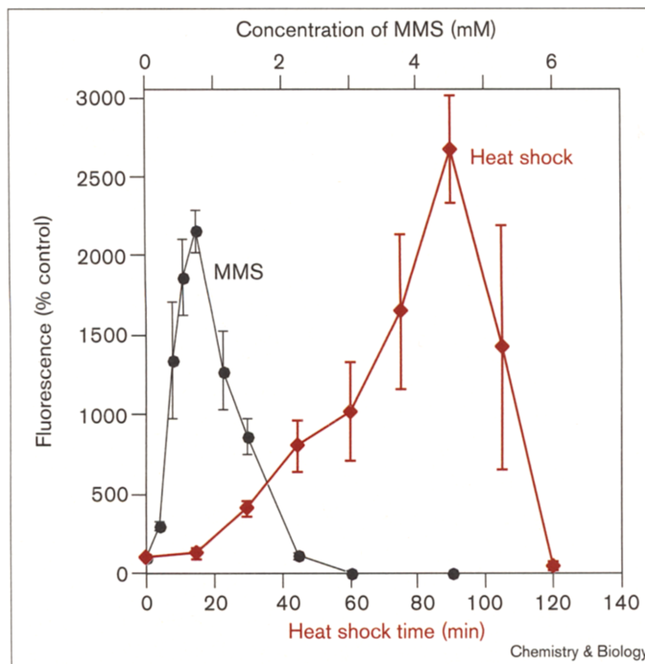
Figure 3



Effect of *cis-* and *trans-*platinum complexes on EGFP expression in HeLa C27 cells. **(a)** *cis-*$[Pt(NH_3)(NH_2C_6H_{11})Cl_2]$ (red squares); *cis-*$[Pt(C_3H_7NH_2)_2Cl_2]$ (blue circles); [Pt(lysine)Cl_2] (green triangles). **(b)** *t,t,t-*$[PtCl_2(OH)_2(NH_3)(C_6H_{11}NH_2)]$ (or JM335) (green triangles); *trans-*$[Pt(CH_3NH_2)_2Cl_2]$ (red squares). Error bars represent ± 1 esd.

cytotoxic agents. Methyl methanesulfate (MMS), an alkylating agent, behaved like *trans*-DDP, stimulating EGFP expression at low doses and inhibiting it at high doses (Figure 4). Similarly, a short (< 90 min) 45°C heat shock caused a time-dependent increase in EGFP expression, whereas a 120 min treatment caused a decrease (Figure 4). Calicheamicin, on the other hand, was similar to cisplatin in causing only a decrease in EGFP expression (data not shown).

Figure 4



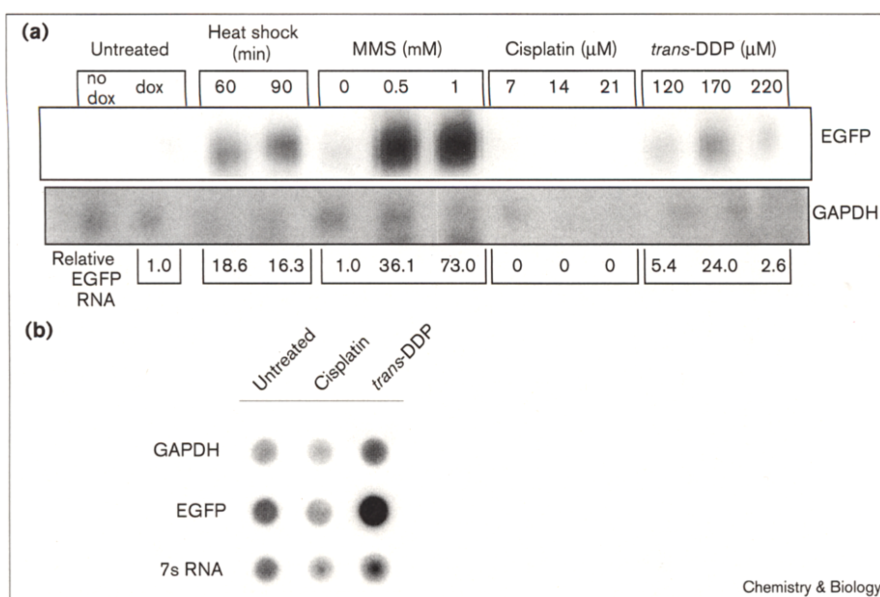
Effect of methyl methanesulfonate (MMS) or 45°C heat shock on EGFP expression in HeLa C27. Cells were treated with MMS in serum-free medium for 1.5 h, then washed and induced in complete medium. After the heat shock cells were induced at 37°C. Error bars represent ± 1 esd.

Northern and run-on analysis of EGFP transcription in HeLa C27 cells

To determine whether changes in EGFP expression occur at the transcriptional or post-transcriptional level, HeLa C27 cells were treated with cytotoxic agents and subjected to northern analysis. A separate control was prepared for MMS, with cells treated in serum-free media for 1.5 h and then given fresh complete media before induction. As Figure 5a reveals, EGFP transcription was greatly enhanced by *trans*-DDP, MMS and heat-shock treatment and blocked by treatment with cisplatin. In addition to specific inhibition of the EGFP, there was also an overall decrease in transcription in the cisplatin-treated cells.

The nuclear run-on transcription assays show that changes in EGFP expression in HeLa C27 cells treated with cisplatin or *trans*-DDP are due to inhibition or enhancement of transcription, respectively (Figure 5b). Cells were treated with doxycycline for EGFP induction and co-treated with cisplatin (15 μM) or *trans*-DDP (120 μM). Nuclei isolated from these cells were used in the run-on assay. Labeled mRNA for EGFP in the dot blots corresponding to treatment with doxycycline alone confirms induction of EGFP. Co-treatment with *trans*-DDP (120 μM) resulted in a dramatic increase in EGFP transcription. A similar treatment with cisplatin (15 μM) caused a general decrease in transcription, however. Significantly, twice as many nuclei

Figure 5



(a) Northern blot of RNA prepared from HeLa C27 cells treated with various cytotoxic agents. RNA was quantitated by phosphorimager analysis, and all readings were normalized for loading by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) results. **(b)** Nuclear run-on transcription assay of HeLa C27 cells induced for EGFP production in the presence and absence of platinum compounds, cisplatin (15 μM) and *trans*-DDP (120 μM). Isolated nuclei were subjected to *in vitro* run-on transcription and the labeled nascent mRNA transcripts hybridized to dot blots containing EGFP and the control genes, GAPDH and 7s RNA. dox, doxycycline.

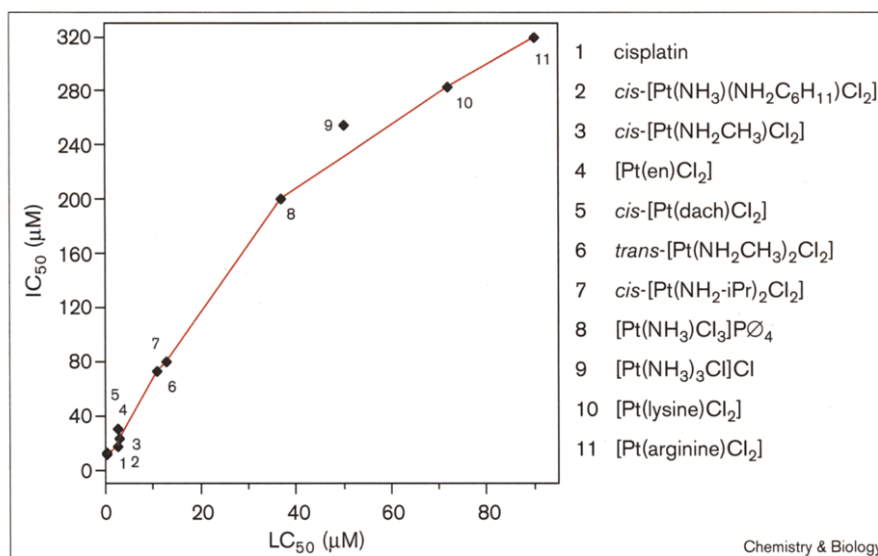
(~10^8) were required in the run-on assay to achieve radiolabel incorporation levels similar to those for the untreated cells and for cells treated with *trans*-DDP.

Screening of platinum complexes by reporter-gene assays

The 13.5 h co-treatment EGFP protocol and the colony counting cytotoxicity assay were used to determine the IC_{50} and LC_{50} values, respectively, for a series of platinum complexes in HeLa C27 cells. The results, which show a strong positive correlation between EGFP inhibition and cell death, are plotted in Figure 6. Compounds such as cisplatin and *cis*-$[Pt(NH_3)(NH_2C_6H_{11})Cl_2]$, both used in the clinic, score best and would clearly have been selected as antitumor drug candidates using this reporter-gene assay. The platinum–amino-acid compounds, on the other hand, do not appear to be worth pursuing on the basis of these results.

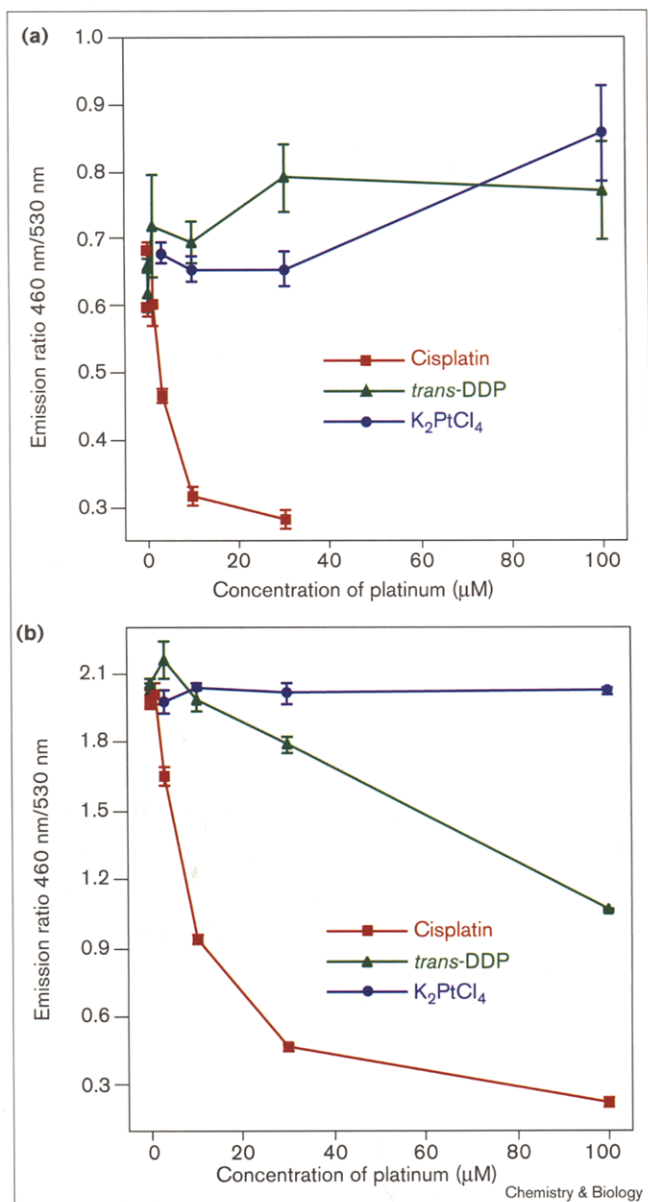
To study the activity of platinum complexes with another reporter-gene assay, Jurkat C2 cells were treated with *cis*-DDP, *trans*-DDP and K_2PtCl_4. The carbachol-dependent induction of β-lactamase was quantitated by measuring the ratio of intact to cleaved CCF2 substrate (Figure 7a). The IC_{50} value was 8 μM for cisplatin, but neither *trans*-DDP nor K_2PtCl_4 blocked β-lactamase induction at concentrations up to 100 μM. Trypan blue

Figure 6



Comparison of IC_{50} with LC_{50} for treatment of HeLa C27 cells with a series of platinum complexes.

Figure 7



Effect of platinum-compound treatment on β-lactamase expression, as measured by CCF2 cleavage, in two Jurkat cell lines. **(a)** C2 Jurkat cells were treated with platinum for 20 h and then induced with carbachol for 4 h. **(b)** CMV-bla Jurkat cells were treated with platinum for 24 h. Error bars represent ± 1 esd.

and DAPI staining of Jurkat C2 cells revealed an LC_{50} value of 30 μM and an AC_{50} value of 15 μM for a 19.5 h cisplatin treatment. *trans*-DDP had an LC_{50} of 100 μM after 19.5 h, but never caused apoptosis in more than 35% of the cells. The CCF2 cleavage assay was repeated (Figure 7b) in the constitutively expressing CMV–bla Jurkat cell line. In that case, the IC_{50} was 10 μM for cisplatin, 100 μM for *trans*-DDP and K_2PtCl_4 did not affect β-lactamase expression. The images in Figure 8 compare CMV–bla Jurkat cells treated with cisplatin and *trans*-DDP to the dye-loaded, unplatinated control cells.

Discussion

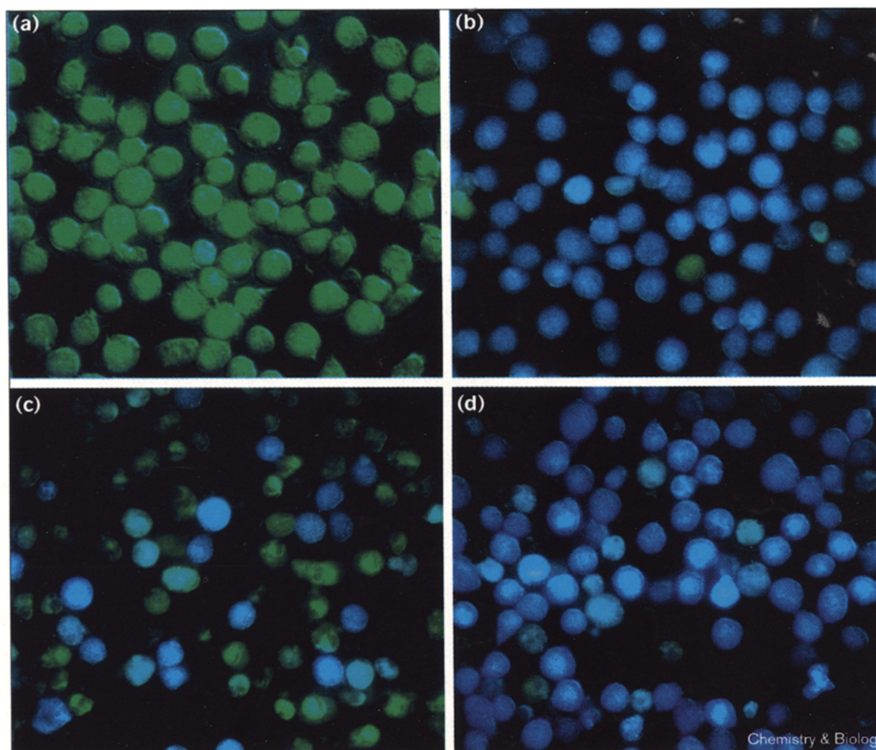
Inducible expression of EGFP in HeLa Tet-On cells

The effects of cytotoxic agents on EGFP expression in HeLa C27 cells discovered in the present study are unprecedented in that different agents yielded two distinct types of responses. For one class of cytotoxic agent, including *trans*-DDP, other *trans*-platinum complexes, and, to a greater extent, MMS and heat shock, EGFP expression rose sharply, reached a maximum, and then fell with increasingly toxic treatments. The second class included cisplatin, other *cis*-platinum complexes, and calicheamicin, in which a dose-dependent decrease in EGFP expression occurred. Very low (< 2 μM) concentrations of cisplatin caused an ~200% increase in EGFP expression relative to the untreated control, quite modest compared to the maximal values of 800%, 2100% and 2500% for *trans*-platinum complexes, MMS and heat shock, respectively. Northern analysis revealed a correlation between EGFP fluorescence and transcription of the gene; furthermore, cisplatin-treated cells had a lower total RNA content than others, indicating an overall decrease in transcription. Run-on analysis indicated that these changes occur at the level of transcription rather than mRNA stabilization. Although various forms of DNA damage can influence reporter-gene expression positively or negatively, there are no reports in which different genotoxic agents cause opposite effects on transcription from the same promoter. There are also no examples of a single cytotoxic agent both enhancing and inhibiting gene expression from the same promoter in a dose-dependent manner.

The contrasting effects were not a function of overall cytotoxicity. For example, *trans*-$[Pt(NH_2CH_3)_2Cl_2]$ caused a dose-dependent increase in EGFP expression from 1–5 times its LC_{50} value of 8 μM. By contrast, $[Pt(lysine)Cl_2]$, brought about a dose-dependent decrease at values ranging from 0.3–5 times its 70 μM LC_{50} value. Apoptosis cannot explain the different effects either. Heat shock [34,35], MMS [36] and JM335 [37] all induce both apoptosis and EGFP expression, whereas *trans*-DDP stimulates EGFP expression but kills cells using a nonapoptotic mechanism. The results are also unlikely to be a consequence of the specific site of integration of pTRE-EGFP into the host genome, because similar results were obtained with an independent HeLa clone.

The upregulation of the EGFP by heat shock, as well as by several DNA-damaging agents, suggests that it is a general response to stress. The mammalian stress response involves the induction of many types of gene products including heat-shock proteins [25,38], stress-activated protein kinases (JNK/SAPK) [39,40], the p53-dependent cell-cycle arrest and apoptosis pathway

Figure 8



Cisplatin inhibition of gene expression visualized by β-lactamase reporter-catalyzed hydrolysis of CCF2. **(a)** Wild-type Jurkat, untreated. **(b)** CMV–bla Jurkat, untreated. **(c)** CMV–bla Jurkat treated with 40 μM cisplatin at 37°C for 24 h. **(d)** CMV–bla Jurkat treated with 40 μM trans-DDP at 37°C for 24 h. Panels are 140 μm wide.

proteins and DNA repair enzymes [16,17]. Although the Tet-On gene expression system is not endogenous to mammalian cells, this artificial construct, once activated by doxycycline, could nonetheless also be upregulated by stress. The damage induced by *cis*-platinum complexes and calicheamicin must be different from other forms of stress. It is not surprising that calicheamicin blocks gene expression, for it is a highly toxic compound that rapidly enters cells, cleaves DNA and induces apoptosis in less than 4 hours [41,42]. The inhibition of EGFP transcription by many *cis*-platinum complexes, of variable toxicity but all capable of forming 1,2-intrastrand DNA cross-links, suggests a structural effect with mechanistic consequences. The bending and unwinding of DNA (reviewed in [43]) induced by the 1,2-intrastrand cross-link could lead to differential recognition by proteins such as SSRP1 [44,45], which is involved in the elongation of transcription through nucleosomes (G. Orphansides, W-H. Lu, W.S. Lane, M. Hampsey and D. Reinberg, personal communication), or the TATA-binding protein [46], both of which recognize and bind to this platinum adduct but not to the *trans*-DDP–DNA adducts. Such binding would account for the transcriptional consequences of *cis*-platinum adducts compared to most other types of damage. The modest increase in EGFP at low doses of cisplatin could result from the conflicting influences of stress response and the unique structural consequences of its DNA adducts.

Cisplatin–DNA adducts are recognized by a variety of proteins [47], including those involved in nucleotide-excision repair (NER), DNA-mismatch repair, and the high mobility group (HMG)-domain proteins. It seems unlikely that either of the repair systems is responsible for the different transcriptional effects, because NER processes recognize *trans*-DDP intrastrand cross-links [48] and the mismatch-repair pathway recognizes MMS damage [49]. The HMG-domain proteins, which specifically recognize DNA damaged by *cis*-platinum complexes but not *trans*-platinum complexes or any of the other agents tested, could be involved. HMG-domain proteins might contribute to cisplatin cytotoxicity by blocking or shielding cisplatin–DNA adducts from repair [43,50–52]. Repair blockage would cause *cis*-platinum cross-links to be more persistent than other forms of DNA damage. If upregulation of the EGFP is a response to stress in HeLa C27 cells, however, then persistent DNA adducts might be expected further to induce rather than to block EGFP expression, unless the shielding is so effective that this damage is completely unrecognized.

It has also been proposed that cisplatin adducts could titrate HMG-domain proteins away from their natural binding sites [53]. Because many HMG-domain proteins serve as transcription factors, such an event could lead to cell death through the misregulation of transcription [54]. If the transcription factors necessary for EGFP induction

specifically recognize cisplatin–DNA adducts, then doxycycline- and stress/doxycycline-induced EGFP transcription would be prevented by cisplatin and its structural analogs. SSRP1 is a candidate for this activity ([44,45]; G. Orphanides, W-H. Lu, W.S. Lane, M. Hampsey and D. Reinberg, personal communication). The titration mechanism would also explain the modest increase in EGFP expression at low levels of cisplatin. Only when a sufficient number of adducts formed would titration be effective.

The titration hypothesis would also apply to non-HMG-domain transcription factors. The basal transcription factor TATA binding protein/TFIID (TBP) has been reported to recognize both cisplatin- and UV-damaged DNA [55]. In a subsequent study [46], DNA modified with a series of damaging agents was assayed for TBP binding and for transcription inhibition in HeLa whole-cell extracts. For each type of adduct tested, TBP binding correlated with transcription inhibition, with cisplatin and *cis*-[Pt(dach)Cl_2] (dach, 1,2-diaminocyclohexane) having strong effects. In some experiments, *trans*-DDP-modified DNA bound TBP and inhibited transcription slightly, whereas MMS and [Pt(dien)Cl]Cl (dien, diethylenetriamine) did neither. It was proposed that the kinked DNA structure formed by *cis*-platinum adducts resembles the TBP-binding site and can thus titrate the protein away from its normal transcriptional activator region. These results are entirely consistent with the present EGFP expression results, and the observation that *trans*-DDP enhances EGFP expression to a lesser extent than MMS or heat shock could be explained by low-level transcription factor binding, as observed in the TBP study.

The use of reporter-gene assays for screening platinum complexes

The EGFP and β-lactamase reporter-gene assays are both good methods for screening platinum complexes to predict cytotoxicity. The EGFP induction assay yielded a good correlation between LC_{50} and IC_{50} values for a series of known platinum complexes. Use of a range of sample concentrations in the EGFP assay reveals that gene expression is stimulated at low doses. Nevertheless, the IC_{50} value ultimately obtained correlated well with the LC_{50} results despite the initial increase. The EGFP method is much faster than any currently available cytotoxicity assay, but modest levels of EGFP induction at the 13.5 h time point require the cells to be lysed in a small volume of buffer and transferred to smaller wells in order to intensify the fluorescence signal. The β-lactamase CCF2/AM assay is the better alternative for high-throughput screening. This method, which showed a dose-dependent decrease in gene expression upon treatment with cisplatin but not with ineffective platinum complexes, also surpasses existing cytotoxicity assays in speed. The results are available within 24 h of platinum treatment, and the assay can be performed with the aid of robotics for convenient high-throughput screening. Inducible β-lactamase expression can be easily quantitated because of enzymatic amplification of the fluorescence signal. Because of the short (3.5 h) half-life of the enzyme, platinum-induced changes in constitutive β-lactamase expression can also be observed in less than 24 h.

An additional advantage to both reporter-gene assays is that they screen platinum complexes for DNA damage that inhibits gene expression, rather than measuring cell death as an endpoint. In that sense, they are mechanism-based screening methods. Because the construction of stable cell lines expressing reporter genes can take several months, the choice of cell type for platinum drug screening should be made judiciously.

Significance

The success of cisplatin and carboplatin as anticancer drugs, combined with the resistance of many tumors to platinum chemotherapy, have driven the search for new platinum antitumor drugs. With appropriate synthetic approaches and high-throughput screening methods, combinatorial chemistry could accelerate the drug discovery process. We have demonstrated the utility of two fluorescence-based methodologies for quickly screening platinum complexes in human cancer cells. These assays, which measure the inhibition of reporter-gene expression by cytotoxic platinum complexes, focus on biochemical processes rather than merely cytotoxic endpoints. Both methods, enhanced green fluorescent protein (EGFP) induction and hydrolysis of a cephalosporin substrate (CCF2), provide results in under 24 h, surpassing the speed of the commonly used cytotoxicity assays. Additionally, the EGFP induction assay is unique in that many forms of cytotoxic stress cause an upregulation of doxycycline-inducible EGFP transcription, whereas *cis*-platinum compounds inhibit EGFP transcription. This contrasting effect suggests that the bent and unwound structure of the 1,2-intrastrand platinum–DNA cross-link modulates the cellular response to *cis*-platinum compounds, possibly because components of the transcriptional initiation or elongation machinery bind to the kinked complex.

Materials and methods

Materials

Tissue culture media and antibiotics were purchased from Gibco BRL. Tet System Approved fetal bovine serum, pEGFP-1, pTRE, pTK-Hyg, HeLa Tet-On cells and anti-GFP antibody were obtained from Clontech. The plasmid containing 7s RNA was a gift from B. Panning and P.A. Sharp (MIT). *Escherichia coli* containing the plasmid for GAPDH was obtained from the American Type Culture Collection (ATCC). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. [α-^{32}P] UTP was obtained from du Pont and ATP, GTP and CTP from Boehringer Mannheim. Cisplatin and JM335 were provided by Johnson-Matthey. All other platinum complexes were available in the laboratory. Calicheamicin was donated by P. Dedon (MIT).

Physical measurements

EGFP fluorescence measurements were recorded by using a Molecular Devices Fmax instrument equipped with a 485 ± 14 nm excitation filter

and a 538 ± 30 nm emission filter. CCF2 fluorescence was measured by using a Cytofluor 4000 microtiter plate fluorimeter with a 395 ± 10 nm excitation filter and emission filters of 460 ± 20 nm (blue fluorescence) and 530 ± 15 nm (green fluorescence). In both cases the emission values were corrected by subtracting readings from blank wells. Images of cells plated onto poly-L-lysine coated glass cover slips were captured onto Kodak 400 ASA slide film with an inverted epifluorescence microscope (Zeiss) through a 63 × 1.25 numerical aperture (N.A.) oil immersion objective. Slides were scanned and the images digitized. Automated DNA sequencing was performed on an ABI Prism system at the MIT Biopolymers Laboratory. Platinum concentrations, relative to a potassium hexachloroplatinate(IV) standard, were determined using a Varian model AA-1475 atomic absorption spectrophotometer, equipped with a GTA-95 graphite tube atomizer. Phosphorimager analyses were performed on a BioRad Molecular Imager.

Cloning of pTRE-EGFP

The EGFP gene was PCR-amplified from pEGFP-1. The 36-nucleotide primer for the coding strand, 5′-TCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGT-3′, included the 5′ and 3′ sequences adjacent to the *Eco*RI restriction site preceding the EGFP start codon in pEGFP-1. The 36-nucleotide primer for the anticoding strand, 3′-ATTTCGCCGGCGCTGAGATCTAGTATTAGTCGG-TAT-5′, included the sequences adjacent to the *Xba*I site following the EGFP stop codon in pEGFP-1. PCR was performed in an EZStart 100 reaction tube (Molecular BioProducts) with 0.2 μg template DNA, 0.5 μg each primer and 5 units Pfu polymerase (Stratagene) in a total volume of 50 μl. The products were precipitated with ethanol, digested with *Eco*RI and *Xba*I and precipitated again. The pTRE vector was digested with *Eco*RI and *Xba*I, purified on a 1% agarose gel and ligated to the EGFP insert using T4 DNA ligase. The identity of the resulting plasmid pTRE-EGFP was verified by restriction mapping and automated DNA sequencing.

Transfection of HeLa Tet-On cells with pTRE-EGFP

HeLa Tet-On cells were cultured in D-MEM with 10% fetal bovine serum, 100 μg/ml of penicillin, 100 units/ml of streptomycin and 400 μg/ml of geneticin. The cells were transfected using the calcium phosphate method [56] with *Ssp*I-linearized pTRE-EGFP and to confer hygromycin resistance, *Hind*III-linearized pTK-Hyg. The selective medium contained all of the growth medium components and 0.2 mg/ml of hygromycin. The resistant colonies were grown to confluence on 35 mm plates, induced with 4.3 μM doxycycline and examined by fluorescence microscopy using an FITC filter pair. Extracts were prepared from five fluorescent clones and analyzed for inducible EGFP expression by Western blotting with a polyclonal anti-GFP antibody. The clone selected for high inducible expression with low background was C27.

EGFP expression assay in HeLa C27 cells

HeLa C27 cells were maintained in selective media. 24 h before induction, the cells were distributed in six-well plates such that they would be 60–70% confluent upon induction. Aqueous 0.4–2.0 mM platinum solutions were prepared no more than 2 h before use, and platinum concentrations were confirmed by atomic absorption spectroscopy. In a typical co-treatment experiment, the platinum complexes were added to each well together with 21.6 μM doxycycline. After a 13.5 h incubation, the cells were washed with PBS and lysed by vigorous shaking in 125 μl of PBS with 0.5% SDS. The lysate samples were transferred to black 96-well microplates for fluorescence plate reading. A 4 μl aliquot of lysate was removed from each sample and used in the Nano-Orange protein assay (Molecular Probes). The EGFP fluorescence values were divided by their respective protein assay readings to correct for variations in cell plating. The corrected fluorescence values were then normalized relative to the control (unplatinated) samples. All samples were prepared in triplicate, and the data were averaged with error bars representing one standard deviation.

Northern blotting of RNA from HeLa C27 extracts

For northern blotting analysis, HeLa C27 cells were distributed on 10 cm plates and grown to 70% confluence. Parallel samples for the EGFP expression assay were prepared in triplicate on six-well plates. The cells were treated with the indicated cytotoxic agents and induced for 13.5 h with doxycycline. RNA was isolated from cell extracts by using the Biotecx Ultraspec-II kit. After agarose-formaldehyde gel electrophoresis, the RNA was transferred to nitrocellulose and processed according to standard procedures [57]. The blot was probed with random primer-labeled DNA encoding EGFP and, as a control, human GAPDH. The blot was quantitated by phosphorimager analysis, and EGFP values were corrected for loading by normalizing to the ubiquitously expressed GAPDH.

Nuclear run-on transcription

HeLa C27 cells were grown in monolayers on 15 cm plates (Falcon) to ~60% confluence. EGFP induction was initiated by replacing the media and adding doxycycline (21.6 μM). The cells were co-treated with cisplatin (15 μM) or *trans*-DDP (120 μM) and harvested 12 h later by trypsinization. The nuclei were isolated as described [58] and either used immediately or stored in liquid nitrogen.

The *in vitro* run-on assay was performed by following a previously described protocol [58]. Briefly, run-on transcription was initiated by the addition of ATP, CTP and GTP and [α-^{32}P] UTP (3000 Ci/mM)] to 5–10 × 10^7 in suspension and incubated for 30 min at 30°C with slow shaking. The reactions were stopped by treatment with RNase-free DNase, proteinase K and SDS as described previously [58]. The solutions were extracted with phenol/chloroform/isoamyl alcohol (pH 5.2) until the interface was clear. The separated aqueous layer was supplemented with 100 μg carrier tRNA and precipitated by using trichloracetic acid. Acid insoluble material was filtered onto glass filters (Schleicher & Schuell) in a vacuum manifold and washed extensively. The filters were treated with RNase-free DNase and the labeled mRNA was extracted as described. After a proteinase K/SDS treatment, the solutions were extracted with phenol/chloroform/isoamyl alcohol and precipitated by using ethanol. Incorporated radiolabel varied between 2.5 and 5 × 10^6 cpm. The specific run-on transcripts were identified by hybridizing the labeled mRNA with dot blots containing genes of interest. Blots were prepared by immobilizing 10 μg of linearized and denatured plasmids with the genes for GFP, GAPDH and 7s RNA onto nitrocellulose filters with a dot-blot apparatus (Schleicher & Schuell). The filters were prehybridized for 12–16 h in a Hybaid oven at 55°C in a formamide buffer, 50% formamide, 5 × Denhardt's solution, 5 × SSC, 0.2% SDS, 100 μg/ml salmon sperm DNA. Equal counts of the labeled mRNA transcripts were added to the blots and incubated for ~36 h at 55°C. The blots were washed twice in 2 × SSC, each time for 1 h at 55°C, treated with RNase A (10 μg/ml) for 15 min at 37°C, washed and phosphorimaged.

Cytotoxicity and apoptosis assays

For HeLa C27 cells, colony counting cytotoxicity assays were performed as previously described [2]. All samples were prepared in triplicate, and platinum concentrations were verified by atomic absorption spectroscopy. For apoptosis assays, HeLa C27 cells were distributed on 24-well plates 24 h prior to platinum treatment. After treatment, the supernatant medium was transferred to microcentrifuge tubes, and the cells were washed with PBS, which was transferred to the same tubes. The cells were trypsinized, added to the tubes, pelleted and DAPI-stained.

Jurkat C2 cells were maintained at 10–50 × 10^4 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 100 units/ml streptomycin. Prior to platinum treatment, the cells were diluted to 10 × 10^4 cells/ml and distributed in 0.5 ml aliquots. Cell viability was measured by the trypan blue exclusion assay and apoptosis, by DAPI staining. All samples were prepared in duplicate.

β-Lactamase expression assay in Jurkat cells

Jurkat C2 cells in RPMI media were distributed into black 96-well clear-bottom microtiter plates at 100,000 cells per well. The platinum complexes were freshly dissolved at 1 mM concentration in water. Serial

dilutions of these solutions were prepared in water and added in 10% of final volume to the cells. After a 20 h platinum treatment, the cells were induced with 100 μM carbachol for 4 h. Cells were then loaded with 1 μM CCF2/AM at room temperature for 1 h, followed by plate reading for green and blue fluorescence. After background correction, the signal from the blue channel was divided by the signal from the green channel to obtain the final blue to green intensity ratio. With the gain settings used for this experiment, a population with > 95% of the cells expressing β-lactamase gave a ratio of greater than 3.0 and a population of with no cells expressing β-lactamase gave a ratio of 0.1–0.2. Jurkat cells constitutively expressing β-lactamase under the control of the CMV early promoter (CMV-bla Jurkat) were treated in the same manner as the C2 clone, but were kept 24 h in the incubator without any induction period prior to dye loading. All samples were prepared in quadruplicate, and the data were averaged with error bars representing standard error (standard deviation/square root of number of multiples).

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References

1. Pil, P. & Lippard, S.J. (1997). Cisplatin and related drugs. In *Encyclopedia of Cancer.* (Bertino, J.R., ed.), pp. 392-410, Academic Press, San Diego, CA.
2. Sandman, K.E., Fuhrmann, P. & Lippard, S.J. (1998). A mechanism-based, solution phase method for screening combinatorial mixtures of potential platinum anticancer drugs. *J. Biol. Inorg. Chem.* **3**, 74-80.
3. Sandman, K.E. & Lippard, S.J. (1999). Methods for screening the potential antitumor activity of platinum compounds in combinatorial libraries. In *30 Years of Cisplatin – Chemistry and Biochemistry of a Leading Anticancer Drug.* (Lippert, B., ed.), pp. 523-536, Verlag Helvetica Chimica Acta, Basel.
4. Borenfreund, E., Babich, H. & Martin-Alguacil, N. (1990). Rapid chemosensitivity assay with human normal and tumor cells *in vitro. In Vitro Cell. Dev. Biol.* **26**, 1030-1034.
5. Perez, R.P., Godwin, A.K., Handel, L.M. & Hamilton, T.C. (1993). A comparison of clonogenic, microtetrazolium and sulforhodamine B assays for determination of cisplatin cytotoxicity in human ovarian carcinoma cell lines. *Eur. J. Cancer* **29A**, 395-399.
6. Fricker, S.P. & Buckley, R.G. (1996). Comparison of two colorimetric assays as cytotoxicity endpoints for an *in vitro* screen for antitumour agents. *Anticancer Res.* **16**, 3755-3760.
7. Harder, H.C. & Rosenberg, B. (1970). Inhibitory effects of anti-tumor platinum compounds on DNA, RNA and protein syntheses in mammalian cells *in vitro. Int. J. Cancer* **6**, 207-216.
8. Pinto, A.L. & Lippard, S.J. (1985). Sequence-dependent termination of *in vitro* DNA synthesis by *cis-* and *trans*-diamminedichloroplatinum(II). *Proc. Natl Acad. Sci. USA* **82**, 4616-4619.
9. Bernges, F. & Holler, E. (1988). Effects of coordination of diammineplatinum(II) with DNA on the activities of *Escherichia coli* DNA polymerase I. *Biochemistry* **27**, 6398-6402.
10. Mello, J.A., Lippard, S.J. & Essigmann, J.M. (1995). DNA adducts of *cis*-diamminedichloroplatinum(II) and its trans isomer inhibit RNA polymerase II differentially *in vivo. Biochemistry* **34**, 14783-14791.
11. Evans, G.L. & Gralla, J.D. (1992). Differential effects of cisplatin on the expression of chimeric marker genes in CV-1 cells. *Biochem. Pharm.* **44**, 107-119.
12. Mymryk, J.S., Zaniewski, E. & Archer, T.K. (1995). Cisplatin inhibits chromatin remodeling, transcription factor binding, and transcription from the mouse mammary tumor virus promoter in vivo. *Proc. Natl Acad. Sci. USA* **92**, 2076-2080.
13. Takahara, P.M., Rosenzweig, A.C., Frederick, C.A. & Lippard, S.J. (1995). Crystal structure of double-stranded DNA containing the major adduct of the anticancer drug cisplatin. *Nature* **377**, 649-652.
14. Dunham, S.U., Dunham, S.U., Turner, C.J. & Lippard, S.J. (1998). Solution structure of a DNA duplex containing a nitroxide spin-labeled platinum d(GpG) intrastrand cross-link refined with NMR-derived long-range electron-proton distance restraints. *J. Am. Chem. Soc.* **120**, 5395-5406.
15. Gelasco, A. & Lippard, S.J. (1998). NMR solution structure of a DNA dodecamer duplex containing a *cis*-diammineplatinum(II) d(GpG) intrastrand cross-link, the major adduct of the anticancer drug cisplatin. *Biochemistry* **37**, 9230-9239.
16. Smith, M.L. & Fornace, A.J. (1996). Mammalian DNA damage-inducible genes associated with growth arrest and apoptosis. *Mutat. Res.* **340**, 109-124.
17. Smith, M.L. & Fornace, A.J. (1997). p53-mediated protective responses to UV irradiation. *Proc. Natl Acad. Sci. USA* **94**, 12255-12257.
18. Eliopoulos, A., Kerr, D.J. & Spandidos, D.A. (1991). The effect of cisplatin and carboplatin on c-*myc* promoter in erythroleukemic cells. *Anti-Cancer Drugs* **2**, 597-601.
19. Niimi, S., *et al.*, & Saijo, N. (1991). Resistance to anti-cancer drugs in NIH3T3 cells transfected with c-*myc* and/or c-Ha-*ras* genes. *Br. J. Cancer* **63**, 237-241.
20. Rabo, Y.B., Shoshan, M.C., Linder, S. & Hansson, J. (1996). Different mechanisms are responsible for c-*jun* mRNA induction by cisplatin and ultraviolet light. *Int. J. Cancer* **65**, 821-826.
21. Scanlon, K.J., Kashani-Sabet, M., Tone, T. & Funato, T. (1991). Cisplatin resistance in human cancers. *Phamacol. Ther.* **52**, 385-406.
22. Evans, G.L. & Gralla, J.D. (1992). Cisplatin-induced imbalances in the pattern of chimeric marker gene expression in HeLa cells. *Biochem. Biophys. Res. Comm.* **184**, 1-8.
23. Zoumpourlis, V., Kerr, D.J. & Spandidos, D.A. (1992). Carboplatin as opposed to cisplatin does not stimulate the expression of the human immunodeficiency virus long terminal repeat sequences. *Biochem. Pharmacol.* **43**, 650-654.
24. Valerie, K., Delers, A., Bruck, C., Thiriart, C., Rosenberg, H., Debouck, C. & Rosenberg, M. (1988). Activation of human immunodeficiency virus type 1 by DNA damage in human cells. *Nature* **333**, 78-81.
25. Schaefer, E.L., Morimoto, R.I., Theodorakis, N.G. & Seidenfeld, J. (1988). Chemical specificity for induction of stress response genes by DNA-damaging drugs in human adenocarcinoma cells. *Carcinogenesis* **9**, 1733-1738.
26. Collins, L.A., Torrero, M.N. & Franzblau, S.G. (1998). Green fluorescent protein reporter microplate assay for high-throughput screening of compounds against *Mycobacterium tuberculosis. Antimicrob. Agents Chemother.* **42**, 344-347.
27. Fink, D., *et al.*, & Howell, S.B. (1998). The effect of different chemotherapeutic agents on the enrichment of DNA mismatch repair-deficient tumour cells. *Br. J. Cancer* **77**, 703-708.
28. Cubitt, A.B., Heim, R., Adams, S.R., Boyd, A.E., Gross, L.A. & Tsien, R.Y. (1995). Understanding, improving and using green fluorescent proteins. *Trends Biochem.* **20**, 448-455.
29. Coxon, A. & Bestor, T.H. (1995). Proteins that glow in green and blue. *Chem. Biol.* **2**, 119-121.
30. Chalfie, M. (1995). Green fluorescent protein. *Photochem. Photobiol.* **62**, 651-656.
31. Zlokarnik, G., *et al.*, & Tsien, R.Y. (1998). Quantitation of transcription and clonal selection of single living cells with β-lactamase as reporter. *Science* **279**, 84-88.
32. ClonTechniques XIII (1998). Living colors destabilized EGFP vectors.
33. Freundlieb, S., Baron, U., Bonin, A.L., Gossen, M. & Bujard, H. (1997). Use of tetracycline-controlled gene expression systems to study mammalian cell cycle. *Methods Enzymol.* **283**, 159-173.
34. Boreham, D.R., Dolling, J.-A., Maves, S.R., Miller, S., Morrison, D.P. & Mitchel, R.E.J. (1997). Heat-induced thermal tolerance and radiation resistance to apoptosis in human lymphocytes. *Biochem. Cell Biol.* **75**, 393-397.
35. Robertson, J.D., Datta, K. & Kehrer, J.P. (1997). *Bcl-x_L* overexpression restricts heat-induced apoptosis and influences hsp70, bcl-2, and Bax protein levels in FL5.12 cells. *Biochem. Biophys. Res. Comm.* **241**, 164-168.
36. Kuo, M.-L., Chou, Y.-W., Chau, Y.-P. & Meng, T.-C. (1996). Differential induction of apoptosis in oncogene-transformed NIH 3T3 cells by methylmethanesulfonate. *Biochem. Pharmacol.* **52**, 481-488.
37. O'Neill, C.F., Ormerod, M.G., Robertson, D., Titley, J.C., Cumber-Walsweer, Y. & Kelland, L.R. (1996). Apoptotic and non-apoptotic cell death induced by *cis* and *trans* analogues of a novel ammine(cyclohexylamine)dihydroxodichloro-platinum (IV) complex. *Br. J. Cancer* **74**, 1037-1045.
38. Morimoto, R.I., *et al.*, & Sistonen, L.T. (1994). Regulation of heat shock gene transcription by a family of heat shock factors. In *The Biology of Heat Shock Proteins and Molecular Chaperones.* (Morimoto, R.I., Tissiere, A. & Georgopoulos, C., eds) pp. 417-455. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

39. Potapova, O., *et al.*, & Mercola, D. (1997). The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. *J. Biol. Chem.* **272**, 14041-14044.
40. Sanchez-Perez, I., Murguia, J.R. & Perona, R. (1998). Cisplatin induces a persistent activation of JNK that is related to cell death. *Oncogene* **16**, 533-540.
41. Nicolaou, K.C., Stabila, P., Esmaeli-Azad, B., Wrasidlo, W. & Hiatt, A. (1993). Cell-specific regulation of apoptosis by designed enediynes. *Proc. Natl Acad. Sci. USA* **90**, 3142-3146.
42. Nicolaou, K.C., Pitsinos, E.N., Theodorakis, E.A., Saimoto, H. & Wrasidlo, W. (1994). Synthetic calicheamicin mimics with novel initiation mechanisms: DNA cleavage, cytotoxicity, and apoptosis. *Chem. Biol.* **1**, 57-66.
43. Zamble, D.B., Mu, D., Reardon, J.T., Sancar, A. & Lippard, S.J. (1996). Repair of cisplatin–DNA adducts by the mammalian excision nuclease. *Biochemistry* **35**, 10004-10013.
44. Toney, J.H., Donahue, B.A., Kellett, P.J., Bruhn, S.L., Essigmann, J.M. & Lippard, S.J. (1989). Isolation of cDNAs encoding a human protein that binds selectively to DNA modified by the anticancer drug *cis*-diamminedichloroplatinum(II). *Proc. Natl Acad. Sci. USA* **86**, 8328-8332.
45. Bruhn, S.L., Toney, J.H. & Lippard, S.J. (1990). Biological processing of DNA modified by platinum compounds. *Prog. Inorg. Chem.* **38**, 477-516.
46. Coin, F., Frit, P., Viollet, B., Salles, B. & Egly, J.-M. (1998). TATA binding protein discriminates between different lesions on DNA, resulting in a transcription decrease. *Mol. Cell. Biol.* **18**, 3907-3914.
47. Zlatanova, J., Yaneva, J. & Leuba, S.H. (1998). Proteins that specifically recognize cisplatin-damaged DNA: a clue to anticancer activity of cisplatin. *FASEB J.* **12**, 791-799.
48. Hansson, J., Grossman, L., Lindahl, T. & Wood, R.D. (1990). Complementation of the xeroderma pigmentosum DNA repair synthesis defect with *Escherichia coli* UvrABC proteins in a cell-free system. *Nucleic Acids Res.* **18**, 35-40.
49. Glaab, W.E., Risinger, J.I., Umar, A., Barrett, J.C., Kunkel, T.A. & Tindall, K.R. (1998). Cellular resistance and hypermutability in mismatch repair-deficient human cancer cell lines following treatment with methyl methanesulfonate. *Mutat. Res.* **398**, 197-207.
50. Brown, S.J., Kellett, P.J. & Lippard, S.J. (1993). Ixr1, a yeast protein that binds to platinated DNA and confers sensitivity to cisplatin. *Science* **261**, 603-605.
51. Huang, J.-C., Zamble, D.B., Reardon, J.T., Lippard, S.J. & Sancar, A. (1994). HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. *Proc. Natl Acad. Sci. USA* **91**, 10394-10398.
52. McA'Nulty, M.M. & Lippard, S.J. (1996). The HMG-domain protein Ixr1 blocks excision repair of cisplatin-DNA adducts in yeast. *Mutat. Res.* **362**, 75-86.
53. Donahue, B.A., *et al.*, & Essigmann, J.M. (1990). Characterization of a DNA damage-recognition protein from mammalian cells that binds specifically to intrastrand d(GpG) and d(ApG) DNA adducts of the anticancer drug cisplatin. *Biochemistry* **29**, 5872-5880.
54. Treiber, D.K., Zhai, X., Jantzen, H.-M. & Essigmann, J.M. (1994). Cisplatin-DNA adducts are molecular decoys for the ribosomal RNA transcription factor hUBF (human upstream binding factor). *Proc. Natl Acad. Sci. USA* **91**, 5672-5676.
55. Vichi, P., *et al.*, & Egly, J.-M. (1997). Cisplatin- and UV-damaged DNA lure the basal transcription factor TFIID/TBP. *EMBO J.* **16**, 7444-7456.
56. Kingston, R.E., Chen, C.A., Okayama, H. & Rose, J.K. (1997). Calcium phosphate transfection. In *Current Protocols in Molecular Biology*. (Ausubel, F.M., ed.), pp. 9.1.4-9.1.11, John Wiley & Sons, Inc., New York.
57. Shackleford, G.M. & Varmus, H.E. (1987). Expression of the proto-oncogene *int-1* is restricted to postmeiotic male germ cells and the neural tube of mid-gestational embryos. *Cell* **50**, 89-95.
58. Greenberg, M.E. & Bender, T.P. (1997). Identification of newly transcribed RNA. In *Current Protocols in Molecular Biology.* (Ausubel, F.M., ed.), pp. 4.10.1-4.10.11, John Wiley & Sons, Inc., New York.

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