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Role of endonucleases XPF and XPG in nucleotide excision repair of platinated DNA and cisplatin/oxaliplatin cytotoxicity

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Abstract

Resistance of tumor cells to platinum anticancer agents poses a major problem in cancer chemotherapy. One of the mechanisms associated with platinum-based drug resistance is the enhanced capacity of the cell to carry out nucleotide excision repair (NER) on platinum-damaged DNA. Endonucleases XPF and XPG are critical components of NER, responsible for excising the damaged DNA strand to remove the DNA lesion. Here we investigate possible consequences of downregulation of XPF and XPG gene expression in osteosarcoma cancer cells (U2OS) and the impact on cellular transcription and DNA repair. We further evaluate the sensitivity of such cells toward the platinum anticancer drugs cisplatin and oxaliplatin.

Keywords

cisplatin; oxaliplatin; nucleotide excision repair; XPF; XPG

Introduction

The preservation of genomic integrity, which is essential for proper cell function, is ensured by multiple DNA repair pathways. One of the most efficient mechanisms for clearing helix-distorting DNA lesions caused by UV irradiation or platinum-based antitumor agents is nucleotide excision repair (NER). Preclinical model studies reveal that most cisplatin cross-links formed on DNA are recognized and repaired by the mammalian NER apparatus.[1, 2]

NER is a multistep process, involving some twenty different genes. Among these genes, ERCC1 and the seven XP* genes, XPA to XPG, play critical roles in damage recognition, demarcation, and strand-incision around the damage site. In the NER pathway, a 24–32 nt incised strand containing the DNA lesion is removed and the resulting gap is subsequently filled by dNTPs, DNA polymerase, and DNA ligase using the complementary strand as template.[3]

The endonuclease XPG cuts the DNA strand 5–6 nucleotides downstream (3′) of the DNA damage, and the second incision of this strand, 20–22 nucleotides upstream (5′) of the DNA

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*Xeroderma pigmentosum (XP) is an autosomal recessive disorder characterized by a genetic predisposition to sunlight-induced skin cancer, and it is commonly due to deficiencies in DNA repair enzymes. Genetic analysis of XP cell lines identified seven complementation groups of XP patients, designated XP-A through XP-G, and a variant form, XP-V.

damage, is performed by the ERCC1-XPF protein complex.[4, 5] Either the presence of XPG or the 3′-incision made by XPG is a prerequisite for the 5′-incision activity of XPF.[6] Although these two endonucleases are independently recruited to pre-incision complexes, the proper assembly of all the factors seems to be required for dual incision at the lesion and progression through the NER pathway.[7, 8]

Osteosarcoma is a cancerous bone tumor, usually presenting in adolescence. Although ideal combinations remain to be determined, combination therapy including cisplatin is typically used for treatment.[9] Oxaliplatin has also been evaluated in clinical trials involving osteosarcoma patients.[10] Platinum chemotherapy can produce significant side-effects and is subject to drug resistance. Previous work has shown platinum-based drug resistance to be complex and multifactorial, with no single factor being able to fully explain the phenomenon. In vitro studies suggest that impaired cellular drug uptake, increased detoxification, increased tolerance of platinum-DNA damage, reduced apoptosis, and increased efflux of platinum from cells to be plausible causes. In addition, platinum adducts may be more rapidly repaired in resistant tumors; increased DNA repair is supposedly one of the major causes of drug resistance.[11]

The major platinum-DNA adducts (~90%) are intrastrand cross-links involving adjacent purines. Interstrand cross-links (ICLs) represent minor lesions (<5%).[12, 13] Intrastrand adducts can be repaired by NER, whereas ICLs are not recognized by this machinery[14] and are instead removed by ICL repair. The latter process is less well understood, but it is known that ERCC1-XPF plays an important role here, too.[15–17]

The clinical benefit from platinum-based chemotherapy can be high if the tumor cells express low levels of NER proteins.[18] For example, testicular cancer, which has low levels of ERCC1-XPF, is generally responsive to cisplatin.[19] Conversely, increased levels of *XPA* mRNA are correlated with resistance in ovarian cancer.[20] Moreover, XPG is a biomarker of ovarian cancer prognosis in platinum chemotherapy.[21]

At the cellular level, sensitivity to cisplatin is inversely correlated with NER capability.[22, 23] Testicular tumor cells, which are highly sensitive to cisplatin, are repair-deficient because of low levels of XPA and ERCC1-XPF.[24] Overexpression of NER factors is associated with cisplatin resistance, and the resistance phenotype of ovarian cancer A2780/C200 cells is due, in part, to enhanced repair as a result of upregulation of ERCC1-XPF endonuclease.[22]

Downregulation of NER factors such as XPA can sensitize cells to cisplatin.[19] The suppression of the ERCC1-XPF complex by RNA interference significantly decreased cellular viability in the presence of cisplatin, which correlates well with the decrease in DNA repair capacity.[25]

Pt-DNA adducts can inhibit transcription by impeding the passage of RNA polymerase II[26–28] and influencing upstream processes such as apoptosis in cancer cells.[12, 29, 30] Understanding how the cell processes these Pt-DNA lesions, particularly how the lesions are repaired, is important for elucidating resistance pathways and for developing new platinum-based anticancer drugs that circumvent resistance mechanisms. Our objective in the current study has been to understand relationships between downregulation of the endonucleases XPF and XPG, the cytotoxicity of cisplatin and oxaliplatin, and repair activity in osteosarcoma cells. To the best of our knowledge, the response of cells to cisplatin in the context of downregulated XPG has thus far not been established. With oxaliplatin, these relationships have not been previously investigated either in XPF- or XPG-deficient cell lines.

Results and Discussion

Approach and methodology

In order to evaluate the role of XPF and XPG NER proteins during the cellular response to platinum drugs, gene knockdown was performed using RNAi. Knockdown cells are expected to display enhanced transcription inhibition by platinum lesions and corresponding greater sensitivity to platinum compounds because of reduced repair capability. Transcription was monitored using a platinated reporter probe, and cellular sensitivity was investigated with the MTT assay. These experiments were carried out using cells that were either deficient in XPF and XPG or had normal levels of these factors. Cisplatin and oxaliplatin were compared, because of their different spectrum of activity against cancer cells.

Knockdown procedure

Long-lasting gene silencing of XPF and XPG in U2OS osteosarcoma cells was achieved by short hairpin RNA (shRNA) expression from a lentivirus-based vector.

The pSicoR-GFP vector was designed according to published methods.[31–33] The shRNA-encoding DNA was prepared from two oligonucleotides (55 and 59 nucleotides long) that were annealed and then ligated into the vector. Because not all rationally designed shRNAs knock down gene expression to the same degree,[32] the silencing capability of three candidates was individually evaluated. Sequences beginning at positions 977, 1128, and 1324 from the origin of the XPF gene and 525, 1936, and 3023 from the origin of the XPG gene were evaluated. After cloning into pSicoR-GFP, positive clones were identified by digestion with XhoI and XbaI, which yielded 400 bp fragments, ~50 bp larger than one from the empty vector. Sequencing was performed to verify the identity of all six plasmids.

The pSicoR-GFP plasmids containing DNA sequences coding for shRNA against XPF/XPG and, as a control, also the empty vector (mock) were transfected into 293T/17 cells (modified human embryonic kidney cells), along with the requisite viral packaging vectors, for generation of lentiviral particles. GFP was expressed by the plasmids, allowing verification of transfection by fluorescence microscopy.

The lentivirus-containing supernatant was added to U2OS cells for infection. After transduction, GFP-expressing U2OS cells were collected by fluorescence-activated cell sorting. These cells comprised 6–7% of cells infected with XPF_977, XPF_1128, and XPF_1324 clones, 10–15% of cells infected with XPG_525, XPG_1936 and XPG_3023 clones, and 11% of cells infected with the U2OS_mock clone.

Validation of XPF and XPG knockdown

Semi-quantitative or relative RT-PCR (reverse transcription and polymerase chain reaction) is commonly used to analyze knockdown at the mRNA level and provides an estimate of the relative changes in the gene expression.[32, 34]

RNA was isolated from U2OS cells (U2OS_normal) and from GFP-sorted XPF/XPG knockdown and mock cells. For analyzing its integrity, RNA was separated on a 1.1% agarose gel to display the 28S and 18S ribosomal RNA (rRNA) bands. The ratio of 28S rRNA and 18S rRNA bands was in the expected range of 1.3:1 – 2:1 (Supporting Material, Figure S1). Yields ranged between 4 and 17 μg per 10^6 cells.

Purified RNA was used as a template for one-step RT-PCR, in which the reverse transcription and amplification reactions took place in the same tube. The following controls were used: no template (data not shown), template from untransfected cells (U2OS_normal),

and template from cells transfected with the empty vector (U2OS_mock). The housekeeping gene β-actin served as an endogenous reference. Amplified products were separated on a 2% agarose gel containing ethidium bromide (EtBr) and visualized by UV light (Figure 1).

The results were quantified after normalization against the β-actin band. The percent knockdown is expressed with reference to the empty vector control (U2OS_mock, Figure 2). Among the three sequences used to knock down XPF, the sequence starting at position 1128 in the gene showed the highest efficiency; expression of *XPF* mRNA was reduced by 82%. Among the three sequences used to knock down XPG, the sequence that started at position 1936 showed the highest efficiency; the expression levels of *XPG* mRNA were reduced by 94%.

Cisplatin/oxaliplatin sensitivity

The response of XPF/XPG knockdown cells to cisplatin and oxaliplatin was evaluated in 3–4 separate MTT assays. The assays were performed after treatment with cisplatin or oxaliplatin of varying concentrations for 24 h followed by an additional 48 h incubation with fresh medium.

Most of the knockdown cells exhibited increased sensitivity to cisplatin compared to the parental cells but comparable sensitivity to oxaliplatin (Figure 3). Overall, cisplatin and oxaliplatin show similar IC_{50} values in all cells, oxaliplatin being somewhat more cytotoxic in normal/mock cells, whereas cisplatin is more or equally cytotoxic in knockdown cells (Table 1).

Decreased *XPF/XPG* mRNA levels were associated with increased sensitivity to cisplatin and oxaliplatin. Cells with the highest knockdown efficiency showed the lowest IC_{50} values, corresponding to the highest sensitivity. For both, cisplatin and oxaliplatin, the lowest IC_{50} values of ~2 and ~3 μM, respectively, were obtained in XPF_1128 cells. Among the XPG knockdown cells, XPG_1936 cells were the most sensitive (~3 and ~5 μM, respectively). With respect to normal/mock cells, cisplatin cytotoxicity increased 4.3-fold in XPF_1128 cells and 2.3-fold in XPG_1936 cells, and oxaliplatin cytotoxicity was increased 2.4-fold in XPF_1128 cells. Only a 1.3-fold difference was observed in XPG_1936 cells. The changes in cisplatin cytoxicity due to XPF knockdown fall within the range of data reported previously for non-small cell lung cancer cells.[25]

An assessment of the effects of mRNA levels on cellular sensitivity to cisplatin and oxaliplatin by a linear regression analysis revealed no correlation. One reason might be that it is not possible under the present conditions to determine IC_{50} values at an accuracy that permits quantitative comparisons. The ability of XPG knockdown to affect cisplatin and oxaliplatin cytotoxicity was not significant by comparison to XPF knockdown.

Transcription assays

Pt-DNA cross-links are efficient inhibitors of transcription in vitro.[29, 35, 36] We recently reported a method to study platinum lesions on plasmid DNAs transfected into live mammalian cells based on Gaussia Luciferase (GLuc) reporter vectors.[37] The expression of the GLuc reporter gene is unimpeded in the absence of the lesion, but is adversely affected if the adduct is an effective transcription inhibitor. GLuc is secreted from the cell, and the levels of expression can be readily determined by sampling the cell culture media and quantifying enzymatic activity by bioluminescence assays using coelenterazine as a substrate.

The effects of platinum adduct formation on transcription using globally platinated pGLuc plasmids were investigated using this assay. Platinated transcription probes were prepared

by reacting pGLuc with varying concentrations of cisplatin[37] or oxaliplatin in HEPES buffer. Platination levels, expressed as the ratio of bound platinum per plasmid, were determined by measuring the Pt content by atomic absorption spectroscopy and the DNA concentration by UV/Vis spectroscopy. By determining GLuc expression from transfected cells, transcription levels as a function of platination were determined.

A set of five plasmids with Pt/plasmid ratios ranging from 0 to ~35 were prepared by incubation with cisplatin or oxaliplatin; the corresponding r_b values (Pt bound per nucleotide) ranged from 0 to ~0.005. Specifically, r_f values (Pt per nucleotide in reaction) of 0, 0.0013, 0.0025, 0.0038, 0.0050, resulted in r_b values 0, 0.0012, 0.0022, 0.0033, 0.0046, corresponding to 0, 9.9, 17.9, 26.5, and 36.4 Pt adducts per plasmid for cisplatin. For oxaliplatin, the r_f values were 0, 0.010, 0.020, 0.040, 0.080, the r_b values were 0, 0.00064, 0.0012, 0.0024, 0.0044, and the corresponding ratios were 0, 5.1, 9.7, 19.2, and 35.1 Pt per plasmid.

Cells were transfected for two hours using these globally platinated plasmids, and cell media were collected at 8, 16, 24, 32 and 44 hours after completion of transfection. GLuc activity in cells was measured over that period using a luminometer and coelenterazine as substrate. The intensity values were normalized against unplatinated controls and summed for the intervals to account for total GLuc expression at a specific time point. Transcription profiles were obtained by plotting normalized GLuc expression levels against platination levels (Pt/plasmid ratio) at five different time points (Supporting Material, Figure S2). U2OS_mock cells were included as a comparison in each experiment, to normalize for possible transfection and growth condition differences from experiment to experiment.

The recovery rates of transcription from probes having low platination levels (Pt/plasmid ≤ 10) were comparable in all cells irrespective of knockdown except for XPG_1936, which showed a strong inhibition, probably due to very efficient XPG knockdown in this cell line (94%). After only 8 h, the expression levels were similar for all cells (mock and knockdown). Upon additional sampling, the difference in transcription inhibition between mock and knockdown cells became clearer: The reporter activity of cisplatin and oxaliplatin treated plasmids was reduced in XPF knockdown cells at similar levels for all three clones, showing the strongest inhibition in XPF_1128 cells. Among the XPG knockdown cells, transcription of platinated reporters was most significantly inhibited in the XPG_1936 cells (Supporting Material, Figure S2).

A globally platinated vector with ~26 Pt atoms per plasmid for cisplatin and ~19 for oxaliplatin displayed the highest discrimination between knockdown and U2OS_mock cells, scoring recovery rates. To provide a better comparison for the two platinum drugs, plasmids containing similar Pt/DNA values (18 vs. 19 Pt/DNA) were selected, and the results are plotted in Figure 4. Transcription recovery as a function of time following transfection is shown for XPF_1128 and XPG_1936, the cells with best knockdown. When comparing cisplatin and oxaliplatin damaged plasmids, oxaliplatin adducts are clearly more effective at inhibiting transcription. After only 8 h the transcription levels were significantly higher in the case of cisplatin (10% of unplatinated control), whereas they were still suppressed in the case of oxaliplatin (0% of unplatinated control). Furthermore, the recovery of transcription of cisplatin treated plasmids is somewhat faster than that of oxaliplatin treated plasmids in U2OS_mock cells.

Linear regression of the transcription levels at different time-points (Figure 4) provided a tool for comparing transcription recovery rates between knockdown and mock cells. The findings are summarized in Table 2 and the ratios of recovery rates are depicted in Figure 5.

For XPF knockdown cells, 50–80% of the transcription capability remained compared to that for U2OS_mock cells. In XPG knockdown cells, however, only 20–30% remained, representing a loss of up to 80% of the transcription capability. These high residual transcription levels in repair deficient cells cannot be explained by incomplete knockdown, because a knockdown of >70% is considered to be significant,[38] and we obtained >80%. Instead, residual transcription levels in repair deficient cells might result from bypass of damage by translesion synthesis or another repair mechanism.

As can be concluded from the ratios of slopes in Figure 5, transcription recovery of cisplatin-treated plasmids in XPF_1128 cells was ~1.2 times slower when compared to U2OS_mock cells. For oxaliplatin, it was ~2 times slower, revealing a greater impact of XPF knockdown for the latter platinum drug. These results are consistent with our previous report that intrastrand cross-links formed by cisplatin and oxaliplatin inhibit transcription by up to two times better in XPF-deficient than-proficient cells, with oxaliplatin being the stronger inhibitor.[37] In XPG knockdown cells, we find that oxaliplatin and cisplatin treated plasmids do not differ within the measured error range. For cisplatin, the recovery of transcription was 3–4 times slower in XPG_1936 cells with respect to U2OS_mock cells; for oxaliplatin it was 4–5 times slower.

Correlations between observations and comparisons with prior findings

The efficiency of removal of DNA adducts of cisplatin and its analogues by NER is a key determinant of cytotoxicity.[39] Inhibition of the activity of endonucleases involved in NER, XPF and XPG, increases cisplatin sensitivity in osteosarcoma cells. This conclusion, however, does not hold true for oxaliplatin. Similarly, XPA levels and Pt-DNA adduct repair are correlated with cisplatin, but not oxaliplatin, cytotoxicity in colon cancer cells.[40] When the two drugs are compared, oxaliplatin is more efficient at inhibiting transcription than cisplatin in normal and knockdown cells. This finding is in accord with previous work showing that, at equal adduct levels, oxaliplatin is more efficient than cisplatin at inhibiting DNA chain elongation.[41] Conversely, the observed cytotoxicity of oxaliplatin is lower than that of cisplatin in osteosarcoma knockdown cells, despite its stronger transcription inhibition. Together these findings indicate that the overall sensitivity of the tested cell lines to platinum drugs is multifactorial, and the contribution of NER to cell sensitivity is difficult to determine quantitatively. Cell death does not necessarily correlate with inhibition of DNA synthesis, because several DNA polymerases can synthesize past platinated-DNA lesions.[42] Details are discussed below.

The expression levels of NER machinery components have previously been compared to cisplatin sensitivity of human cancer cells as measured by mRNA levels.[43–45] The mRNA levels and repair capacity of cells lines lacking genes involved in NER (*ERCC1, XPA, XPB, XPC, XPD, XPF, XPG*) were examined in a panel of different cancer cell lines. The repair activity correlated neither with cisplatin cytotoxicity nor mRNA expression.[43] In A549 cells, however, a correlation of relative of *XPA* mRNA expression, transcription activity, and cisplatin cytotoxicity was noted,[45] indicating that the relationship between expression of NER factors and cisplatin cytotoxicity could be cell-type specific.

Several possible mechanisms have been suggested to explain why oxaliplatin is more cytotoxic despite its kinetically slower DNA binding properties, but none was able to fully explain the findings. Oxaliplatin does not generate a greater proportion of highly lethal lesions, like interstrand or DNA-protein cross-links. It is therefore unable to compensate for its lower inherent DNA reactivity in such a manner.[41] Oxaliplatin-DNA adducts are not more difficult to repair than those of cisplatin, which excludes another possible explanation for the greater cytotoxicity of the former.[46] It is important to note that these latter studies were carried out using HeLa cell-free extracts, which might explain the discrepancy between

their findings and our results, which were obtained using live cancer cells of a different origin and displayed better repair of oxaliplatin lesions. A similar situation was observed in testis cells that were shown to be active in NER in living cells,[47] but have low NER capacity in cell-free extracts.[24]

Considering only transcription, the role of NER factors is more readily analyzed. Several prior studies have addressed the question of whether there is a defined temporal order in which the two DNA strand incisions take place.[39] In our experiments, XPG knockdown had a significant effect on the recovery of transcription from platinated plasmids, whereas the consequences of XPF knockdown were less pronounced. Given that either the presence of XPG or a 3′-incision by XPG is a prerequisite for the 5′-incision by ERCC1-XPF to take place, this result is expected. Moreover, 3′-incision by XPG can occur in the absence of ERCC1-XPF,[6] rendering XPG more important for successful NER.

In order to understand the observed overall repair of Pt lesions and the resulting cytotoxicity, consideration must be given to the nature of individual adducts. The major cisplatin and oxaliplatin lesions are 1,2-intrastrand cross-links involving adjacent d(ApG) and d(GpG) sequences (up to 80%). In addition, there are ~10% 1,3-intrastrand cross-links, and another 2–3% of the products are ICLs or the result of monofunctional binding to guanine.[12, 13] 1,3-Intrastrand cross-links are excellent NER substrates, whereas the major, but less distorting, 1,2-intrastrand cross-links are less well repaired by in part due to shielding by proteins like HMG-domain proteins. These proteins have a higher affinity for cisplatin than oxaliplatin lesions.[48] It therefore is likely that cisplatin adducts are more easily protected from repair and that oxaliplatin-treated plasmids recover their ability to support transcription more rapidly. The ratios of transcription recovery rates in knockdown versus mock cells were lower for oxaliplatin (Table 2) representing better discrimination and a larger impact of knockdown on transcription recovery.

To explain differences in the cytotoxicity of platinum-DNA adducts, processes that bypass intrastrand cross-links might also play a role. Key polymerases involved in translesion synthesis (TLS) are pol ζ and pol ζ [49–51] Oxaliplatin-GG adducts are more efficiently bypassed than cisplatin-GG adducts with the aid of these polymerases.[52] TLS, together with the lower affinity of HMG-domain proteins for oxaliplatin lesions, may help to explain the faster recovery of transcription in oxaliplatin damaged DNA following cell division. Also, the unexpectedly small changes in cytotoxicity due to knockdown of repair factors might arise from the better ability of cells to bypass oxaliplatin lesions.

Cisplatin forms more lethal ICLs when compared to oxaliplatin,[41] but these adducts are not recognized by the NER machinery. In addition to its role in NER, ERCC1-XPF is involved in repairing ICLs by unhooking and homologous recombination.[44] For example, ERCC1-XPF is the rate-limiting factor responsible for both impaired ICL repair and cisplatin hypersensitivity in testis cancer cells.[47] When XPF is knocked down, the repair of not only intrastrand but also interstrand lesions is affected, and higher sensitivity is to be expected. This property could be demonstrated by enhancement of cisplatin cytotoxicity in XPF knockdown cells. The impact of XPF knockdown on oxaliplatin cytotoxicity is low, however, firstly because oxaliplatin forms fewer ICLs and secondly because of the probable involvement of translesion bypass, as described above. Knockdown of XPG, which does not play a role in ICL repair, does not have a great impact on either cisplatin or oxaliplatin cytotoxicity despite its very high knockdown.

Conclusion

Knockdown of two NER factors, the endonucleases XPF and XPG, by RNA interference decreased the repair efficiency of cisplatin- and oxaliplatin-damaged DNA in osteosarcoma cells (U2OS) by up to fivefold. The NER pathway is an important determinant of the cytotoxicity of platinum drugs, as revealed by high sensitivity of those cells lacking XPF and XPG.

We showed that oxaliplatin displays a different pattern of cytotoxicity and transcription inhibition than cisplatin in these repair-deficient cells. The increase in cytotoxicity after knockdown was more prominent for cisplatin. Oxaliplatin displayed greater transcription inhibition after knockdown but faster recovery, possibly due to involvement of translesion bypass.

The NER factor XPG had a larger impact on transcription inhibition, probably because it is the endonuclease that first cuts the damaged DNA. XPF knockdown might also have an effect on ICL repair, which would render this factor more important for controlling overall cytotoxicity.

The clones with highest knockdown efficiencies showed the strongest transcription inhibition and greatest sensitivity, but no quantitative correlation could be established between mRNA levels of different clones, transcription activity, and cytotoxicity. Due to the inherent sensitivity of U2OS cells to platinum anticancer drugs, the observed effects were within the range of only one order of magnitude. In more resistant cell lines, the effects of XPF/XPG knockdown are expected to be more significant and might allow better correlations.

Since repair pathways can enable tumor cells to survive DNA damage that is induced by platinum drugs, small molecule inhibitors of DNA repair are promising therapeutics when used in combination with those drugs. Also, a combination therapy comprising gene knockdown and platinum drugs could potentially enhance efficacy.

Experimental Section

General methods and materials

Platinum stock solutions were prepared in PBS (cisplatin) or water (oxaliplatin) and passed through a 0.22 μm PES (polyethersulfone) filter. Platinum concentrations were determined by flameless atomic absorption spectroscopy on a Perkin Elmer AAnalyst 600 instrument. The solutions were aliquotted for single use and stored at −20 °C for not longer than three months. UV/Vis spectra were measured on a Hewlett Packard 8453 instrument.

Cell culture

Human osteosarcoma cells (U2OS) were obtained from ATCC (Manassas, VA, USA) and maintained in DMEM containing 4.5 g/L glucose and L-glutamine, no sodium pyruvate, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from cellgro, Manassas, VA, USA) at 37 °C, in a humidified atmosphere at 5% CO_2.

pSicoR-GFP plasmid

The pSicoR-GFP plasmid with an shRNA cloning site was designed and generated according to information provided by Prof. Tyler Jacks' Lab at MIT.[31] Target sequences located throughout the mRNA were selected, three different positions each on the *XPF* and *XPG* genes, respectively 977, 1128, 1324 and 525, 1936, 3023, and oligonucleotides to be inserted were designed using the PSICOLIGOMAKER 1.5 software[31] and checked for

uniqueness in BLAST. The oligonucleotides were phosphorylated with T4 polynucleotide kinase (NEB, Ipswich, MA, USA) and phenol extracted. The duplexes were annealed in the presence of 100 mM KOAc, 30 mM HEPES-KOH pH 7.4 and 2 mM $Mg(OAc)_2$ for 4 min at 95 °C, then for 10 min at 70 °C, cooled at a rate of 1 °C/min to 4 °C, and was subsequently stored at −20 °C. The plasmids were digested at HpaI/XhoI restriction sites in NEB buffer 4 with BSA, phenol extracted, and the cut was confirmed on a 0.8% agarose gel. The duplexes were ligated into the plasmids with T4 DNA ligase (NEB) for 3 h at room temperature. The ligated products were then transformed into *E. coli* DH5 α cells and purified by a miniprep column (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Cuts by the restriction enzymes XhoI and XbaI released fragments of 400 vs. 350 bp lengths, confirming the insertion of a 50 bp duplex, as revealed on 2% agarose gels. The DNA sequences of positive clones were verified at the MIT Biopolymers Laboratory. The primer used for sequencing extended upstream into the U6 promoter and the stem loop (5′-TGC AGG GGA AAG AAT AGT TAG AGA C).

Transfection and transduction

Before transfection, human kidney cells (293T/17) were cultured in DMEM including 4 mM glutamine, 10% FBS, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 500 μg/mL Geneticin.

Transfection and transduction were carried out according to the manual for ViraPower Lentiviral Expression Systems (Invitrogen, Carlsbad, CA, USA). Briefly, on the day before transfection, 293T/17 cells were plated in antibiotics-free medium in 10 cm dishes. At 90% confluence, cells were co-transfected with 9 μg ViraPower packaging mix containing the packaging and envelope plasmids (Invitrogen), 3 μg pSicoR-GFP plasmid for XPF and XPG knockdown, respectively, in Opti-MEM medium with Lipofectamine 2000 (Invitrogen) to generate recombinant replication-incompetent lentiviruses.

As a negative control, cells were transfected with the packaging and envelope plasmids but without pSicoR-GFP plasmid and Lipofectamine 2000. As a positive control, the empty vector was used (U2OS_mock).

The medium was replaced with fresh, antibiotics-free medium 24 h after transfection. GFP expression was examined by fluorescence microscopy 48 h after transfection, and the lentivirus-containing supernatant was harvested for the purpose of infecting the target cell line. The culture medium was removed, centrifuged at 3000 rpm and 4 °C for 15 min to pellet debris, and passed through a 0.45 μm PES syringe filter. The medium was aliquotted into 1 ml portions and stored at −80 °C.

Transduction was carried out by adding the supernatant with viral particles containing the different recombinants to U2OS cells at ~50% confluence in 10 cm Petri dishes using polybrene (10 μg/mL, Millipore, Billerica, MA, USA). The virus-containing medium was replaced with fresh medium 24 h after infection. Cells were then grown in two T-175 flasks (~30 million cells) and GFP-expressing cells were sorted by a FACSAria cell sorter (BD, Franklin Lakes, NJ, USA).

RNA isolation

All materials were RNase free. U2OS and knockdown cells were harvested at 90% confluence from T-75 flasks. Total RNA was isolated and purified from $1 - 7 \times 10^6$ cells using the RNeasy Mini Kit (QIAGEN). For homogenization, the cell lysate was passed five times through a 20-gauge needle fitted to a syringe. 2-Mercaptoethanol was added to the lysis buffer (1% v/v) to eliminate RNases released during cell lysis. On-column DNase digestion was performed using the RNase-free DNase set (QIAGEN) to exclude the

possibility of DNA interfering with the subsequent RT-PCR analysis. The RNA was eluted from the column with two times 30 μl of RNase free water, and stored at −20 °C. For gels, fresh water from a Biocel-MilliQ water purification system was used (Millipore).

RT-PCR

Purified RNA was used as a template for one-step RT-PCR (OneStep RT-PCR Kit with Q solution, QIAGEN), in which the reverse transcription reaction and the amplification took place in the same tube. The RT-PCR was carried out in a Minicycler PTC-150 instrument (MJ Research, now Bio-Rad, Hercules, CA, USA).

In a 50 μL sample volume, 5 ng template, 1 μM XPF primers, and 0.1 μM β-actin primers, or 1 μM XPG primers and 0.14 μM β-actin primers, were used. The steps were as follows: reverse transcription for 30 min at 50 °C, activation of DNA polymerase for 15 min at 95 °C, 35 cycles 3-step cycling (denaturing 45 s at 94 °C, annealing 45 s at 55 °C, extension 1 min at 72 °C), final extension for 10 min at 72 °C. Subsequently, the samples were kept at 4 °C.

Primers were designed using Primer-BLAST (NCBI) with the following input sequences: *XPF* mRNA (NM_005236.2), *XPG* mRNA (NM_000123.2), and β-actin mRNA (NM_001101.3) The sequences were: β-actin forward: 5′-AGA GCC TCG CCT TTG CCG AT; β-actin reverse: 5′-TCC CAG TTG GTG ACG ATG CC; XPF forward: 5′-CCA TCG TCG GGG CAT TGA CA; XPF reverse: 5′-TCG TCT TGT GAC AGG GCT GC; XPG forward: 5′-ATG CCC AGC AGA CAC AGC TC; XPG reverse: 5′-AGA TCT GGC GGT CAC GAG GA.

The amplified DNA fragments were designed to yield 548 bp for XPG, 552 bp for XPF, and 292 bp for β-actin.

Cytotoxicity measurements

MTT assays were carried out in at least three independent experiments as previously reported[53] in hexuplicate.

Parental U2OS and knockdown cells were plated in flat-bottomed 96-well plates starting with column 2 and ending with column 11 (1,500 cells/well). The plates were incubated at 37 °C for 2 days such that cells were in the exponential phase of growth for drug addition. A serial four-fold dilution of the cytotoxic drug in growth medium was prepared to give nine concentrations with 8 mL each (6 mL medium + 2 mL drug dilution, 0.0015 – 100 μM). The medium was removed from all wells, and the drug was added in the several dilutions, in column 2 only medium. The plates were incubated for 24 h. At the end of the drug-exposure period the medium was removed from all wells, 200 μL of fresh medium was added, and the plates were incubated for an additional 48 h. The plates were fed with 200 μL of MTT solution (5 mg/mL MTT in PBS) that was filtered through a 0.22 μm PES filter, then diluted 1:5 with medium and incubated for 4 h in a humidified atmosphere at 37 °C and 5% CO_2. The medium was removed from the wells and the purple MTT-formazan crystals were dissolved by addition of 200 μL DMSO/glycine buffer pH 10.5 (8:1). Since the absorption spectrum of MTT-formazan is pH-dependent, and the pH varies with the cell density in the well, the pH in all wells was shifted to 10.5 where the spectrum shows only a single peak at 570 nm.[53] The absorbance was measured at this maximum, and the mean absorbance reading from the wells without drug was used as the control (100% viability). IC_{50} values were determined by interpolation.

Preparation of globally platinated plasmids

pGLuc plasmid was derived from the commercially available pCMV-GLuc vector as previously described.[37, 54]

pGLuc plasmid (125 μg/mL, 46 nM) was treated with cisplatin (0, 0.5, 1.0, 1.5, 2.0 μM) in buffer (50 mM Na-HEPES pH 7.4) or oxaliplatin (0, 3.6, 7.3, 14.5, 29.0 μM) in buffer (25 mM Na-HEPES pH 7.4, 10 mM NaCl) for 16 h at 37 °C. The reaction mixtures were dialyzed (MWCO 3.5 kDa) against TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) overnight at 4 °C with four changes of buffer solution. The r_b values (bound Pt/nucleotide) were determined by UV/Vis and atomic absorption spectroscopy.

Transient transfection of cells for transcription assays

Transcription assays were carried out by transient transfection of the plasmids into U2OS and knockdown cells and subsequent measurement of the levels of luciferase reporter gene expression.[37] Briefly, cells were plated in 96-well plates at 2,500 cells/well and incubated for 48 h. At ~30% confluence, cells were transfected with 50 ng of platinated plasmids using 25 μL Opti-MEM and 0.125 μL Lipofectamine 2000, and subsequently 50 μL of antibiotics-free DMEM including 10% FBS. After 2 h, the cells were washed with medium and 100 μL of fresh medium was added. The experiment was carried out in quadruplicate.

GLuc luminometry assay

GLuc assay solution contained 10 μM colelenterazine (NanoLight Technologies, Pinetop, AZ, USA), made from a 2.5 mM stock in acidified methanol (100 mM HCl), in buffer of the following composition: 10 mM Tris-HCl pH 7.8, 1 mM EDTA, 0.6 M NaCl.

The transcription assay was carried out in a manner similar to that previously described.[37] Briefly, GLuc activity was monitored with a luminescence plate reader (Synergy 2, BioTek, Winooski, VT, USA). Volumes of 10 μL medium were transferred into white 96-well plates at different time points (8, 16, 24, 32, 44 h), and 25 μL GLuc assay solution was added by the automatic injector of the instrument. The fitting of luminescence data in Figure 4 was performed with Origin 8.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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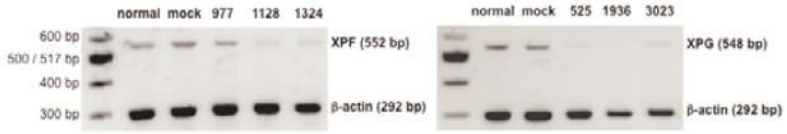

Figure 1.

A 2% agarose gel (0.5 μg/mL EtBr) of amplified DNA from RT-PCR of RNA isolated from cells after XPF (left) and XPG (right) knockdown. 1×TBE was used for the gel and running buffer. The gel was run at 100 V for 120 min.

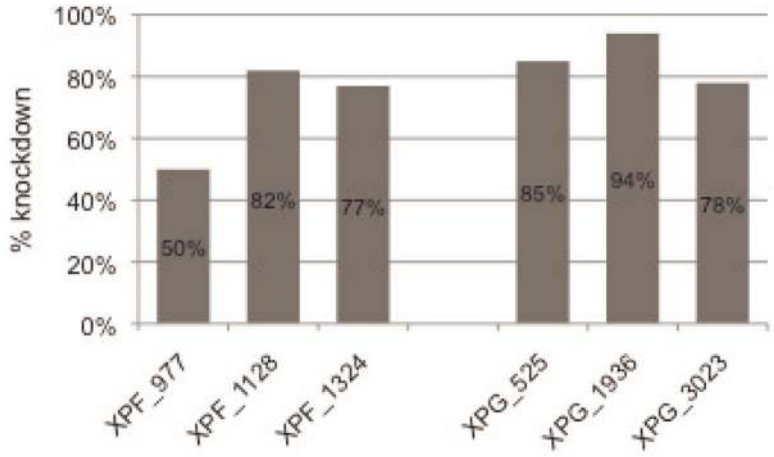

Figure 2.

Semi-quantitative analysis of the agarose gel from Figure 1 showing the knockdown efficiency of *XPF* and *XPG* mRNA, respectively. Relative levels of gene expression inhibition ranged from 50 to 94% when compared to mRNA expression in U2OS_mock cells.

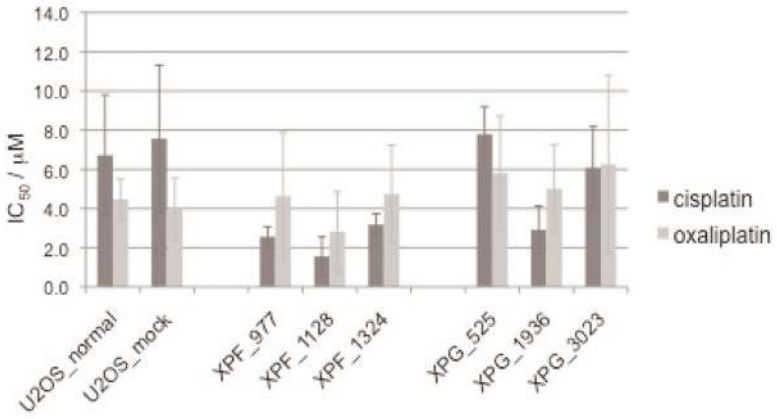

Figure 3.
IC_{50} values for cisplatin and oxaliplatin in U2OS cells and in XPF and XPG knockdown cells.

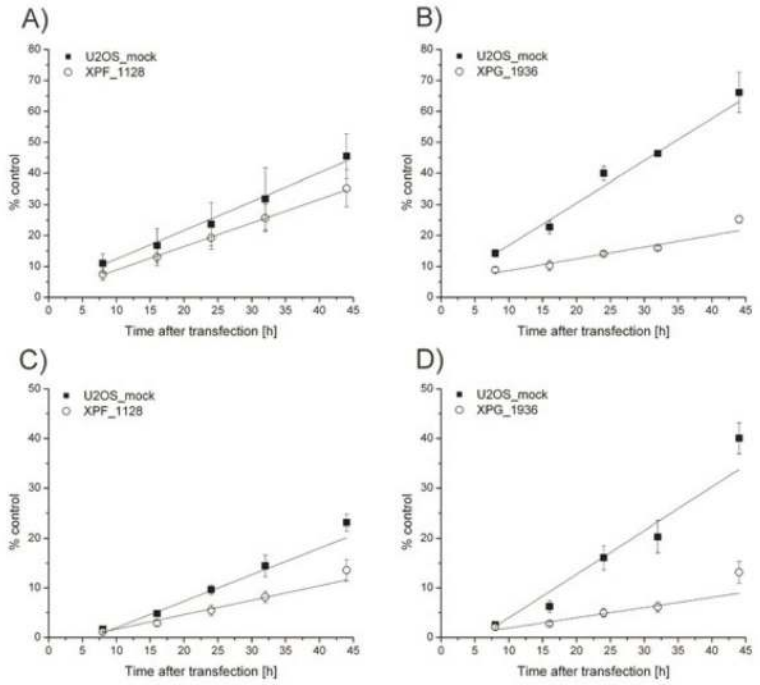

Figure 4.

Time-dependent transcription recovery of pGLuc plasmids containing 18 Pt/DNA (cisplatin – A, B) and 19 Pt/DNA (oxaliplatin – C, D) in U2OS_mock and XPF/XPG knockdown cells.

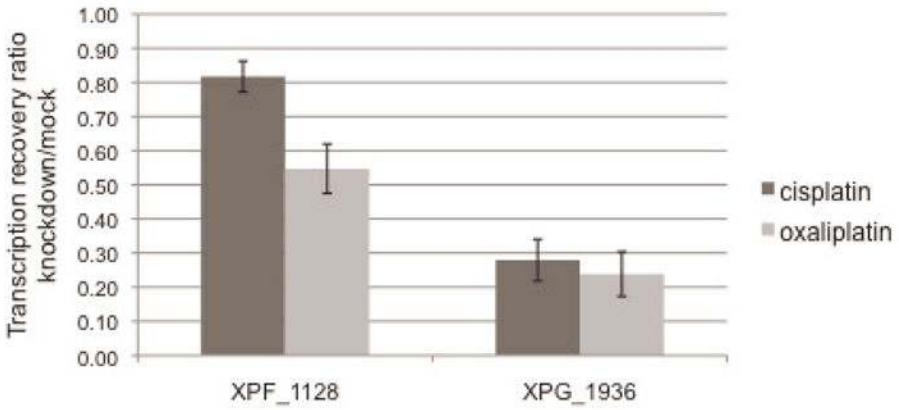

Figure 5.

Ratio of slopes for time-dependent transcription recovery of pGLuc plasmids containing 18 Pt/DNA (cisplatin) and 19 Pt/DNA (oxaliplatin) obtained from U2OS_mock cells vs. knockdown cells, cf. Table 2. Error bars are obtained from linear regression and error propagation by division.

Table 1

IC_{50} values for cisplatin and oxaliplatin in U2OS cells and respective knockdown cells. Errors were obtained from 3–4 replicates for each experiment.

	Knockdown (%)	IC_{50} cisplatin	IC_{50} oxaliplatin	IC_{50} ratio cisplatin/oxaliplatin	Fold change[a] cisplatin	Fold change[a] oxaliplatin
U2OS_normal	0	6.7 ± 3.1	4.5 ± 1.0	~1.5	n/a	n/a
U2OS_mock	0	7.6 ± 3.8	4.0 ± 1.6	~1.9	n/a	n/a
XPF_977	50	2.6 ± 0.5	4.6 ± 3.3	~0.6	~2.6	~1.4
XPF_1128	82	1.6 ± 1.0	2.8 ± 2.1	~0.6	~4.3	~2.4
XPF_1324	77	3.2 ± 0.6	4.7 ± 2.5	~0.7	~2.1	~1.4
XPG_525	85	7.8 ± 1.4	5.8 ± 2.9	~1.3	~0.9	~1.2
XPG_1936	94	2.9 ± 1.2	5.0 ± 2.2	~0.6	~2.3	~1.3
XPG_3023	78	6.1 ± 2.1	6.3 ± 4.5	~1.0	~1.1	~1.1

[a]With respect to normal cells

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Table 2

Rates of transcription recovery and coefficients of determination R^2 for figure 4.

	Cisplatin slope	Cisplatin R^2	Ratio slopes knockdown/mock	Oxaliplatin slope	Oxaliplatin R^2	Ratio slopes knockdown/mock
U2OS_mock	0.93 ± 0.05	0.989	0.82 ± 0.05 (A)	0.53 ± 0.06	0.947	0.55 ± 0.07 (C)
XPF_1128	0.76 ± 0.01	0.999		0.29 ± 0.02	0.974	
U2OS_mock	1.36 ± 0.08	0.987	0.28 ± 0.06 (B)	0.88 ± 0.12	0.925	0.24 ± 0.07 (D)[a]
XPG_1936	0.38 ± 0.08	0.859		0.21 ± 0.07	0.775	

[a]When the 44 h time-point is left out, the slope for XPG_1936 is 0.17 ± 0.04 (R^2 = 0.837) and the ratio is 0.19 ± 0.05.