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TOX4 and its binding partners recognize DNA adducts generated by platinum anticancer drugs

Christophe Bounaix Morand du Puch [a], Ewa Barbier [a], Alexandra Kraut [b], Yohann Couté [b], Julia Fuchs [c], Arnaud Buhot [c], Thierry Livache [c], Michel Sève [d], Alain Favier [a], Thierry Douki [a], Didier Gasparutto [a], Sylvie Sauvaigo [a], Jean Breton [a,*]

[a] CEA Grenoble, INAC, SCIB (UMR E_3 CEA-Université Joseph Fourier, CNRS FRE3200) – Laboratoire Lésions des Acides Nucléiques, 17 rue des Martyrs, 38054 Grenoble cedex 09, France
[b] CEA Grenoble, iRTSV, Laboratoire d'Étude de la Dynamique des Protéomes (INSERM U880/Université Joseph Fourier), 17 rue des Martyrs, 38054 Grenoble cedex 09, France
[c] CEA Grenoble, INAC, SPrAM (UMR 5819 CNRS-CEA-UJF), 17 rue des Martyrs, 38054 Grenoble cedex 09, France
[d] INSERM U823, Institut A. Bonniot, BP 170, 38 042 Grenoble cedex 9, France

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ABSTRACT

Platinating agents are commonly prescribed anticancer drugs damaging DNA. Induced lesions are recognized by a wide range of proteins. These are involved in cellular mechanisms such as DNA repair, mediation of cytotoxicity or chromatin remodeling. They therefore constitute crucial actors to understand pharmacology of these drugs. To expand our knowledge about this subproteome, we developed a ligand fishing trap coupled to high throughput proteomic tools. This trap is made of damaged plasmids attached to magnetic beads, and was exposed to cell nuclear extracts. Retained proteins were identified by nano-HPLC coupled to tandem mass spectrometry. This approach allowed us to establish a list of 38 proteins interacting with DNA adducts generated by cisplatin, oxaliplatin and satraplatin. Some of them were already known interactome members like high mobility group protein 1 (HMGB1) or the human upstream binding factor (hUBF), but we also succeeded in identifying unexpected proteins such as TOX HMG box family member 4 (TOX4), phosphatase 1 nuclear targeting subunit (PNUTS), and WD repeat-containing protein 82 (WDR82), members of a recently discovered complex. Interaction between TOX4 and platinated DNA was subsequently validated by surface plasmon resonance imaging (SPRi). These interactions highlight new cellular responses to DNA damage induced by chemotherapeutic agents.

Introduction

Cisplatin was the first member of platinum-based compounds approved as an anticancer drug in 1978 [1]. This molecule is still widely used to treat patients suffering from tumoral diseases such as testicular, upper aero-digestive tract, lung or ovarian cancers. In order to circumvent adverse side effects or to enlarge the panel of targeted tumors, further research led to the development and approbation of carboplatin and oxaliplatin, the latter being often used in chemotherapeutic regimen to treat colorectal tumors. Hundreds of other platinating agents are still in the pipelines at research or clinical trial stages, including compounds such as satraplatin or picoplatin [2]. Following intracellular activation, the main mechanism of action of these molecules consists in forming DNA lesions such as intra- and interstrand crosslinks (ICL)[1], the most common being 1,2-d(GpG) and 1,2-d(ApG) intrastrand adducts [3]. These damages impair transcription and replication, and generally cause a cell-cycle arrest to provide a time frame for DNA repair. When the damage extent exceeds repair capacities, apoptosis is induced. These cellular and molecular events involve a wide range of proteins that interact with DNA lesions or with subsequent DNA distortions and constitute the "platinated DNA interactome". These include damage-response proteins (DRP) but also other factors whose ordinary targets are not DNA lesions.

Xeroderma pigmentosum complementation group C (XPC) is an important member of this subproteome as it binds to platinum adducts during the recognition step of the global genome repair

[1] *Abbreviations used:* CPD, cyclobutane pyrimidine dimers; DRP, damage-response protein; DSB, double-strand breaks; HMG, high mobility group; HMGB1, high mobility group protein B1; ICL, interstrand crosslinks; LCP1, epidermal Langerhans cell protein 1; NER, nucleotide excision repair; PNUTS, phosphatase 1 nuclear targeting subunit; SPRi, surface plasmon resonance imaging; TOX4, TOX HMG box family member 4; TRF2, telomeric repeat factor 2; hUBF, human upstream binding factor; WDR82, WD repeat-containing protein 82; (6-4)-PP, (6-4)-photoproducts.

* Corresponding author. Fax: +33 438785090.
E-mail address: jean.breton@cea.fr (J. Breton).

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subpathway of nucleotide excision repair (NER). Among the other well-studied members also stand the high mobility group (HMG) proteins, whose affinity for platinum adducts has been known for years [4]. Indeed, HMGB1 was one of the first proteins identified as being able to interact with platinated DNA [5]. The biological repercussions of HMGB1 binding are still controversial, as some published works demonstrated a subsequent facilitation of DNA repair whereas others suggest a shielding effect towards NER factors [6]. A second example is hUBF. This ribosomal RNA transcription factor is hijacked from its natural binding site by cisplatin lesions, for which it demonstrates a strong affinity [7]. Lists of other proteins already characterized as capable of recognizing DNA platinum adducts are accessible in dedicated reviews [8]. Discovery of these protein–DNA interactions already brought crucial data for the understanding of platinating agents pharmacology. In order to establish the cartography of damaged DNA-interacting proteins, protocols using proteomic tools were recently designed for DNA lesions such as double-strand breaks [9], abasic sites [10], oxidative damage [11] and DNA crosslinks generated by platinating agents [12,13]. The aim of our work was to optimize one of these approaches combining a ligand fishing system and high throughput proteomics, to allow us to trap and identify new proteins interacting with DNA alterations. Our tool is made of plasmids damaged by one of three different platinum drugs (cisplatin, oxaliplatin or the satraplatin metabolite JM118) and immobilized onto magnetic beads. This system was exposed to HeLa (cervical cancer cell line) and MDA-MB231 (breast cancer cell line) nuclear extracts, and retained proteins were identified by proteomics. This strategy allowed selection of relevant candidates that were validated by immunoassays and SPRi. Identified proteins may improve our understanding of molecular and cellular responses to this particular type of anticancer drugs.

Materials and methods

Protein sources

Two different protein sources were used in our study. HeLa nuclear extracts were purchased from CIL Biotech (Mons, Belgium). MDA-MB231 extracts were prepared in our laboratory. This cell line was a gift from Prof. Philippe Becuwe (University of Nancy, France). Cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) fetal calf serum and 800 μg/ml geneticin (Invitrogen) at 37 °C under 5% CO_2. Cells were passaged or harvested at ~80% confluence. Nuclear extracts were prepared as previously described [14].

Ligand fishing

Probe synthesis

Principle for this step was adapted from [15]. Plasmid pBlueScript II SK (−) (Stratagene) was linearized using restriction enzymes ClaI and BamHI (NEB). The larger fragment obtained was purified with Quick Spin G-50 Sephadex columns (Roche), precipitated and resuspended in water.

Cohesive ends obtained through ClaI and BamHI restrictions allowed 3′ labeling of the plasmids by biotinylated dATP using Klenow polymerase (NEB), biotin-14-dATP (Invitrogen). Alpha-thio 2′dNTPs (Trilink biotechnologies) or normal dNTPs (GE Healthcare) were added to fill in the remaining part of the overhangs. Biotinylated DNA was purified by isopropanol/sodium acetate precipitation.

Biotinylated plasmids (100 μg) were exposed at 37 °C in the dark to either 67 μM cisplatin (Bayer Pharma) for 2 h, 200 μM oxaliplatin (Sanofi Aventis) for 4 h, or 67 μM JM118 (kindly provided by Agennix) for 2 h. Probes were then precipitated, resuspended in water and stored at −20 °C. These conditions generated homogenous levels of adducts: 7 1,2-d(ApG) and 35 1,2-d(GpG) crosslinks/plasmid for cisplatin, 2 1,2-d(ApG) and 22 1,2-d(GpG) crosslinks/plasmid for oxaliplatin, 5 1,2-d(ApG) and 31 1,2-d(GpG) crosslinks/plasmid for JM118. UVC-damaged plasmid probes were also employed in our study. These probes were prepared with biotinylated plasmids following a method previously described [16], which allowed the generation of 12 (6–4)-photoproducts ((6–4)-PP) and 26 cyclobutane pyrimide dimers (CPD) per plasmid. Adduct levels were measured by HPLC-MS/MS (1100 Agilent chromatographic system coupled to SCIEX API 3000 electrospray triple quadrupolar spectrometer, multiple reaction monitoring analysis) after plasmid digestion to obtain a mix of normal deoxynucleosides and monophosphate adducts. The amount of each lesion was determined by using an external calibration obtained by the injection of known amounts of the authentic compound. Normal nucleosides were quantified by HPLC-UV with a detector set at 260 nm placed at the outlet of the column. Representative spectra for both UV and mass detections are available in Supplementary Fig. 1.

Probes were finally attached to polystyrene magnetic beads (Dynabeads kilobaseBINDER, Invitrogen) according to the manufacturer instructions, using 500 μg beads for 10 μg damaged plasmids. Functionalised beads were finally rinsed twice with water, and then stored in water at 4 °C.

Ligand fishing

Buffer A (final concentrations: 40 mM Hepes-KOH pH7.8, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT) was used to equilibrate functionalised beads. In a 100 μL final volume, 1.2 μg of DNA probes, 120 μg of fish sperm DNA (Roche), and 100 μg of nuclear extracts were mixed in buffer A. Samples were incubated under shaking for 45 min at 37 °C. Supernatant was eliminated using a magnetic tray, and beads were rinsed twice with 100 μL of rinsing buffer: 1 M (high stringency), 150 mM (intermediate stringency) or 50 mM (low stringency) NaCl, 20 mM Hepes-KOH pH 7.8, 5 mM $MgCl_2$, 0.025% Nonidet-P-40. Proteins were finally released in 20 μL of Laemmli buffer (Tris–HCl pH 6.8 25 mM, SDS 2%, glycerol 4%, β-mercaptoethanol 5%, bromophenol blue 0.0008%) by heating at 90 °C for 10 min.

Proteomic analyses

Proteins contained in ligand fishing eluates were stacked by SDS–PAGE in a 2 mm-high band and stained with Coomassie blue. Protein bands were manually excised from the gels, proteins digested in-gel with trypsin, and resulting peptides extracted using a sample preparation robot (EVO150, Tecan) as previously described [17]. Extracted peptides were analyzed by nano-liquid chromatography coupled to tandem mass spectrometry (Ultimate 3000, Dionex and LTQ-Orbitrap Discovery, Thermo Fischer) and identified using Mascot software (Matrix Science) and the Uniprot database (*Homo sapiens* taxonomy) as described in [18]. Proteins identified with a minimum score of 35 were automatically validated using IRMa software [19]. Proteins showing a score $\geqslant 70$ and a number of identified peptides $\geqslant 2$ are provided in Supplementary Table 1. Functions of identified proteins were assessed using Generic GO Term Mapper (http://go.princeton.edu).

Western blot

Proteins obtained by ligand fishing were separated by SDS–PAGE, transferred to nitrocellulose and analyzed by Western blot using the following monoclonal antibodies: rabbit anti-HMGB1 (Abcam), rabbit anti-PNUTS (Abcam), rabbit anti-TOX4 (Abcam) and mouse anti-β actin (Sigma). Secondary antibodies were monkey anti-rabbit and anti-mouse coupled to horseradish-peroxydase

Table 1
Sequences of oligonucleotides utilized to construct the SPRi probes.

Target		5′-CCT CTC TGG ACC TTC C-3′
Zip probes	Zip4	5′-Py-(5T)-TGC GGG TAC AGC ACC TAC CTT GCG-3′
	Zip6	5′-Py-(5T)-GAC CGG TAT GCG ACC TGG TAT GCG-3′
	Zip7	5′-Py-(5T)-TGC GAT CGC AGC GGT AAC CTG ACC-3′
	Zip9	5′-Py-(5T)-GAC CAT CGT GCG GGT AGG TAG ACC-3′
Complementary sequences	Zip4c	5′-GGA AGG TCC AGA GAG GCG CAA GGT AGG TGC TGT ACC CGC A-3′
	Zip6c	5′-GGA AGG TCC AGA GAG GCG CAT ACC AGG TCG CAT ACC GGT C-3′
	Zip7c	5′-GGA AGG TCC AGA GAG GGG TCA GGT TAC CGC TGC GAT CGC A-3′
	Zip9c	5′-GGA AGG TCC AGA GAG GGG TCT ACC TAC CCG CAC GAT GGT C-3′

(GE Healthcare). ECL Plus and Hyperfilm ECL (GE Healthcare) were used to detect the bands.

SPRi

DNA probe synthesis and control

The design of SPRi ODN probes is illustrated on Fig. 3. Sequences employed are detailed in Table 1. Four Zip ODNs were grafted on the chip, allowing localization of each type of short damaged or control ODNs on specific areas of the gold layer (DNA damaged with cisplatin, oxaliplatin and JM118, and undamaged DNA). Long 40-base sequences complementary to Zip and damaged probes were used to obtain the double-stranded structure. The short sequences bearing DNA crosslinks were adapted from [20]. Short damaged ODNs were synthesized using the phosphoramidite chemistry carried out on an Applied Biosystems 392 DNA synthesizer. Target ODNs (20 nmol) were site-specifically damaged by overnight incubation at 37 °C with cisplatin, oxaliplatin or JM118 (150 nmol), then purified by RP-HPLC. Correct masses were measured by MALDI-TOF mass spectrometry (Bruker) measurements. 5′-pyrrole-modified-Zip and long complementary sequences were obtained from Eurogentec.

Grafting of DNA probes

SPRi biochips were made of glass prisms covered with a 50 nm gold layer (GenOptics), onto which DNA probes were grafted. Electro-copolymerization of these probes using pyrrole chemistry was performed according to [21], except that the electric pulse voltage was 2.4 V. This voltage was applied by a potentiostat galvanostat PG 263 (Princeton Applied Research). Quality of spotting was assessed by controlling electrochemical charges of each spot. All sequences were grafted in triplicate.

Target/probe hybridization and interaction analysis

Duplex of damaged targets ODNs (275 nM) was obtained by mixing damaged/undamaged short ODN (275 nM) with long complementary ODNs (250 nM) in buffer A already described in the ligand fishing section. Solution was heated for 5 min at 95 °C, and cooled at room temperature to ensure hybridization. SPRi experiments were performed on a homemade system running under LabView software (GenOptics) for real-time data collection. Prism was placed in a heated cell allowing temperature control for target hybridization (37 °C) and protein interaction (25 °C). All steps were performed at a constant flow rate of 2.5 mL/h in buffer A. SPRi chip surface was first blocked with 1% BSA in buffer A. Damaged ODNs were subsequently injected for final hybridization with zip sequences. Tested proteins were HMGB1 and TOX4 full length recombinant proteins (Abnova) and ultrapure BSA (USB), injected at 20 nM in buffer A. Proteins were separated from the probes with a 1.5 M NaCl solution. At the end of each experiment the biochip was regenerated with a 0.1 M NaOH solution, rinsed with water, dried with argon and stored at 4 °C.

Results

Ligand fishing

The list of proteins isolated from HeLa nuclear extracts by our ligand fishing approach was obtained after proper optimizations of crucial parameters including (i) levels of platinum DNA damage within grafted probes (1,2-(d(GpG) and 1,2-d(ApG) crosslinks levels were respectively of ≈22–35 and ≈2–7 lesions/plasmid depending on the platinating agent), (ii) plasmidic probes concentration/beads quantity ratio (iii) beads blocking and (iv) methods and buffers used to wash the trap and to release proteins before identification. The sharpest criterion was the releasing protocol. Methods aiming at reducing the proportion of non specific proteins binding (mainly those adsorbed on magnetic beads), including the use of endonucleases to digest the DNA fishing probes, led to poorly reproducible results (data not shown). Actually, the best results were obtained by releasing the proteins directly within Laemmli buffer. Beads without DNA and beads grafted with undamaged plasmids were used as controls to discriminate between interesting candidates and non specific proteins identified in the proteomic experiments. After optimizations, each experiment was performed twice, both using low and intermediate stringency rinsing buffers to extend the range of potentially retained proteins (we observed that the highest salt concentration was a too stringent condition and caused loss of proteins of interest). Four different analyses per plasmid probe were therefore performed. The extensive list of identified proteins obtained by proteomics for each experiment and condition (including controls) is available as Supplementary Table 1. A summary of these results is described in Fig. 1 and Table 2. Taking only into account reliably identified proteins (i.e. with sufficient proteomic scores and peptide numbers in two independent experiments), 38 of them were specifically retained by platinated probes, 47% of which possess a known DNA binding activity according to the Gene Ontology

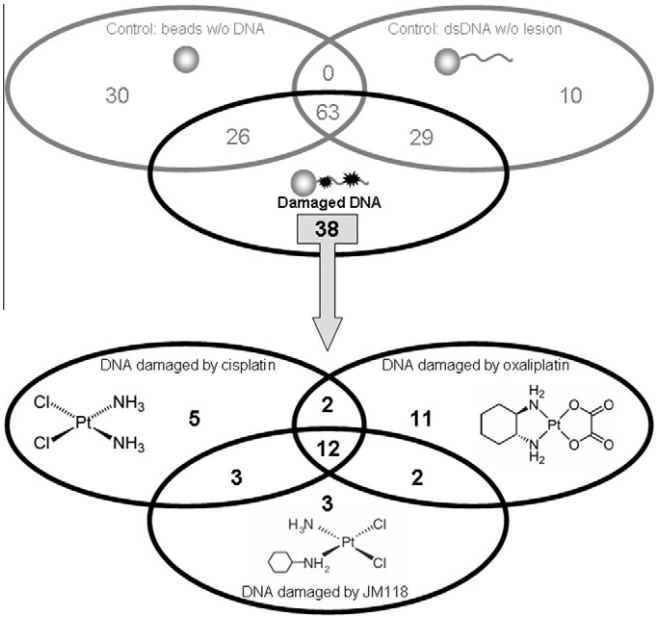

Fig. 1. Proteomics results: numbers of proteins reliably identified in the different ligand fishing assays. Indicated numbers are limited to proteins identified in two distinct experiments with Mascot scores ⩾70 and numbers of identified peptides ⩾2. Results obtained with low and intermediate stringency buffers are mixed.

Table 2
Proteins specifically identified with plasmidic probes damaged with cisplatin (CDDP), oxaliplatin (OxaliPt) and JM118. The list is restricted to proteins reliably identified in two distinct ligand-fishing/proteomics experiments (for more detailed lists, see Supplementary Table 1). Candidates whose affinity for platinum adducts had not been shown previously are highlighted in bold. S-: low stringency rinsing, S+: intermediate stringency rinsing.

Protein name (Uniprot)	Entry name (Uniprot)	Number of identified peptides (min–max)	Mascot scores (min–max)	Cisplatin S+	Cisplatin S–	Oxaliplatin S+	Oxaliplatin S–	JM118 S+	JM118 S–	Protein functions
Apoptotic chromatin condensation inducer in the nucleus ACIN1	**ACINU_HUMAN**	**2–3**	**103–156**	**X**	**–**	**–**	**–**	**–**	**–**	**Component of a complex responsible for exon junction during RNA maturation**
ADP-ribosylation factor 6 ARF6	**ARF6_HUMAN**	**2–3**	**97–181**	**X**	–	–	–	–	–	**Involved in cell motility; essential for cytokinesis**
60 kDa heat shock protein, mitochondrial HSPD1	**CH60_HUMAN**	**3–4**	**144–264**	–	–	**X**	–	–	–	**Involved in protein folding and import inside the mitochondrion**
Probable ATP-dependent RNA helicase DDX46	**DDX46_HUMAN**	**2**	**77–121**	**X**	**X**	**–**	**–**	**–**	**–**	**Responsible for mRNA processing (splicing)**
DNA ligase 3	DNLI3_HUMAN	2–7	96–436	–	–	X	-	X	X	DNA repair (base excision repair and double-strand breaks repair)
HEATR1	**HEAT1_HUMAN**	**2–12**	**75–710**	**X**	**–**	**–**	**–**	**–**	**X**	**–**
High mobility group box 1 HMGB1	HMGB1_HUMAN	11–16	472–1168	X	X	X	X	X	X	Regulation of chromatin structure and transcription, inflammation mediator
HMGB2	HMGB2_HUMAN	3–11	137–611	X	X	X	X	X	X	Regulation of chromatin structure and transcription
HMGB3	HMGB3_HUMAN	2–6	92–389	X	X	X	–	–	–	DNA binding and unwinding
Eukaryotic translation initiation factor 2 subunit 1 IF2a	**IF2A_HUMAN**	**2–3**	**83–120**	**–**	**–**	**–**	**X**	**X**	**-**	**Translation initiation**
Eukaryotic translation initiation factor 2 subunit 2 IF2b	**IF2B_HUMAN**	**2–3**	**96–178**	**–**	**–**	**X**	**–**	**–**	**–**	
Ras GTPase-activating-like protein IQGA1	**IQGA1_HUMAN**	**2–4**	**95–246**	**X**	**–**	**–**	**–**	**–**	**–**	**Potentially involved in cytoskeleton reorganization**
Leucine-rich repeat-containing protein 40 LRRC40	**LRC40_HUMAN**	**2**	**87–95**	**–**	**–**	**X**	**–**	**–**	**–**	**-**
Mediator of DNA damage checkpoint protein 1 MDC1	**MDC1_HUMAN**	**2–4**	**99–208**	**–**	**–**	**X**	**–**	**–**	**–**	**Recruited at site of double-strand breaks (DSB); required for DNA damage checkpoint (S phase and G_2/M transition)**
Metastasis-associated protein MTA2	**MTA2_HUMAN**	**2**	**80–136**	**–**	**–**	**X**	**–**	**–**	**–**	**Transcription repression via chromatin remodeling and histone deacetylation**
Nucleolar protein 11 NOL11	**NOL11_HUMAN**	**2–4**	**99–176**	**-**	**-**	**X**	**–**	**–**	**–**	**–**
Putative ribosomal RNA methyltransferase NOP2/NOL1	**NOP2_HUMAN**	**2–3**	**90–226**	**–**	**–**	**–**	**–**	**X**	**–**	**–**
Phosphatidylinositol-binding clathrin assembly protein PICALM	**PICAL_HUMAN**	**2–5**	**93–253**	**X**	**–**	**X**	**–**	**X**	**–**	**Mediates clathrin-vesicle assembly**
Pinin	**PININ_HUMAN**	**2**	**80–92**	**–**	**–**	**X**	**–**	**–**	**–**	**Transcriptional activator of the E-cadherin gene**
Serine/threonine-protein phosphatase 1 subunit 10 PNUTS	**PP1RA_HUMAN**	**2–12**	**90–653**	**X**	**X**	**X**	**X**	**X**	**X**	**Phosphatases inhibition, regulation of cell proliferation and apoptosis**
PC4 and SFRS1-interacting protein PSIP1	**PSIP1_HUMAN**	**2–3**	**120–162**	**–**	**–**	**X**	**–**	**–**	**–**	**Transcriptional coactivator involved in neuroepithelial stem cell differentiation and neurogenesis**
Telomere-associated protein RIF1	**RIF1_HUMAN**	**2–8**	**103–583**	**X**	**–**	**–**	**–**	**X**	**X**	**Negative regulator of telomere elongation; prevents replication in presence of DSB**
U2 small nuclear ribonucleoprotein A′ SNRPA1	**RU2A_HUMAN**	**2**	**89–96**	**–**	**–**	**X**	**–**	**–**	**–**	**–**

(*continued on next page*)

Table 2 (*continued*)

Protein name (Uniprot)	Entry name (Uniprot)	Number of identified peptides (min–max)	Mascot scores (min–max)	Cisplatin S+	Cisplatin S−	Oxaliplatin S+	Oxaliplatin S−	JM118 S+	JM118 S−	Protein functions
RuvB-like 2 RUVBL2 (reptin)	**RUVB2_HUMAN**	**2–4**	**83–260**	**–**	**–**	**–**	**–**	**X**	**–**	**Activator of ATM-mediated DNA damage checkpoint; chromatin remodeling (member of the TIP60/NuA4 complex)**
Septin-2	**SEPT2_HUMAN**	**2–5**	**98–281**	**–**	**X**	**–**	**X**	**–**	**X**	**Cytoskeleton organization**
Structural maintenance of chromosomes protein 3 SMC3	**SMC3_HUMAN**	**2–7**	**92–397**	**X**	**–**	**–**	**X**	**X**	**X**	**Involved in chromosome cohesion during cell cycle and in DNA repair**
Transcription activator BRG1	SMCA4_HUMAN	2	76–157	X	–	–	–	X	–	Transcriptional activator
SMARCA5	**SMCA5_HUMAN**	**2–3**	**106–140**	**–**	**–**	**–**	**–**	**X**	**–**	**Helicase with nucleosome-remodeling activity**
Chromatin-specific transcription elongation factor SPT16	SP16H_HUMAN	6–18	403–1292	X	X	X	X	X	X	Nucleosome reorganization
Signal recognition particle 68 kDa protein SRP68	**SRP68_HUMAN**	**2–5**	**85–278**	**X**	**–**	**X**	**–**	**–**	**–**	**Targets secretory proteins to the rough endoplasmic reticulum membrane**
Structure-specific recognition protein 1 SSRP1	SSRP1_HUMAN	5–12	254–625	X	X	X	X	X	X	Nucleosome reorganization
Telomeric repeat-binding factor 2 TRF2	**TERF2_HUMAN**	**2–5**	**129–254**	**X**	**X**	**X**	**X**	**X**	**–**	**Responsible for protection of telomeres**
Serine/threonine-protein kinase tousled-like 1 TLK1	**TLK1_HUMAN**	**2**	**92–113**	**–**	**–**	**X**	**–**	**–**	**–**	**Involved in nucleosome sliding, chromatin remodeling and DNA repair**
TOX high mobility group box family member 4 TOX4	**TOX4_HUMAN**	**2–6**	**111–381**	**X**	**X**	**X**	**X**	**X**	**–**	**Transcription activator; involved in thymocytes differentiation**
Human upstream binding factor hUBF	UBF1_HUMAN	6–22	340–1264	X	X	X	X	X	X	Ribosomal RNA transcription factor
WD repeat-containing protein 82 WDR82	**WDR82_HUMAN**	**2–7**	**140–430**	**X**	**–**	**X**	**–**	**–**	**X**	**Histone methylation**
X-ray repair cross-complementing protein 6/Ku70	XRCC6_HUMAN	2	73–92	X	–	–	–	–	–	Helicase activity ; involved in chromosome translocation and double-strand break repair
Zinc finger protein 638 ZN638	**ZN638_HUMAN**	**2–3**	**104–153**	**–**	**–**	**X**	**–**	**–**	**–**	**Binds to cytidine clusters in double-stranded DNA**

annotation. Nine out of the 38 proteins are already known members of the platinated DNA interactome (namely HMGB1, HMGB2, HMGB3, hUBF, SSRP1, SPT16, Ku70, SMCA4 and DNA ligase III). To our knowledge, the 29 other candidates had never been described before as associated with platinum-damaged DNA. Twelve out of the 38 proteins were recovered from ligand fishing reactions with the 3 different damaging agents, while 26 proteins were retained by only one or two types of damaged probes. Oxaliplatin adducts were those that retained the greatest number of unique protein (11), as opposed to cisplatin (5) and JM118 (3).

Our proteomics results were tested and confirmed by Western blot analyses of the ligand fishing eluate for 3 identified candidates (Fig. 2): HMGB1 as a well-known platinum adduct-interacting protein, but also TOX4 and PNUTS as original candidates linked to each others in recently published works [22,23]. Also, capture of TOX4 (6 peptides, score 273), PNUTS (7 peptides, score 319) and WDR82 (7 peptides, score 269) onto the cisplatin-damaged probes was observed using MDA-MB231 nuclear extracts as a second source of proteins. No additional original candidate was identified with these extracts. However, we noticed the presence of TFAM (18 peptides, score 543), another well-known platinum adduct-recognizing factor [4] that was absent from the protein list obtained with HeLa extracts.

The affinity of HMGB1 for another type of bulky adducts, namely UV-induced pyrimidine dimers, has already been reported [24]. We thus evaluated by proteomics the binding of TOX4 and PNUTS from HeLa nuclear extracts onto a UVC-damaged plasmid probe. As a positive control, we also monitored the binding of DNA Damage Binding protein 2 (DDB2) on these probes. DDB2 is a member of the UV-DDB complex responsible for UV-damage recognition and triggers the recruitment of NER machinery. Neither TOX4 nor PNUTS were captured with this particular trap, while DDB2 was (9 peptides, score 345). The MDA-MB231 nuclear extracts (in which DDB2 is absent) were also employed with the UVC-damaged probe. Once again, TOX4 and PNUTS were absent, demonstrating that their interaction with these particular lesions is not impaired by a shielding mechanism of DDB2.

We introduced thio-dNTPs at the extremities of our probes to avoid their degradation by nucleases present in the nuclear extracts [25]. In order to check that the identification of TOX4 and PNUTS was not an artifact originating from the formation of adducts on the internucleotide thiophosphate groups, we carried out an experiment with normal dNTPs to fill in the 5′ and 3′ ends of the plasmids. Western blot analyses confirmed the trapping of TOX4 and PNUTS (Supplementary Fig. 2A).

Also, high stringency washes (salt concentration $\geqslant 1$ M) are generally employed when using probes that capture proteins thanks to the formation of a covalent bond. It is not the case for the protocol we developed, and high stringency buffers revealed less efficient: HMGB1 signal was reduced (Supplementary

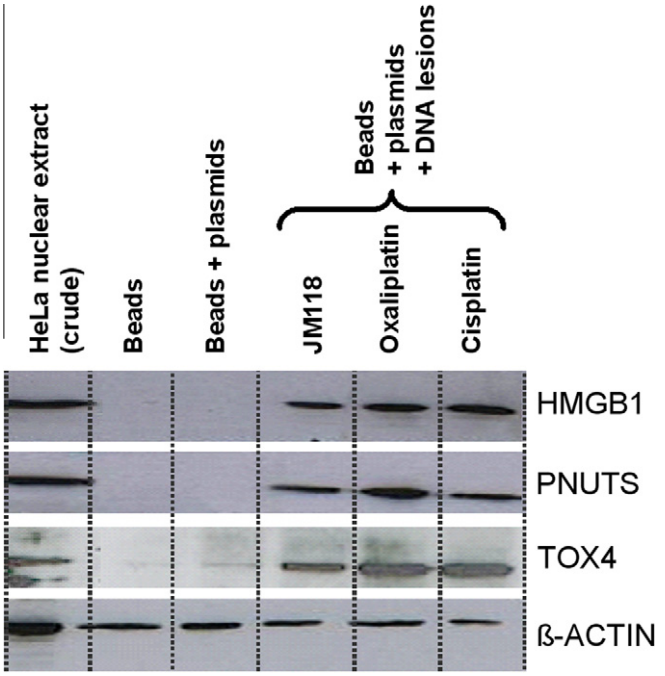

Fig. 2. Confirmation of proteomics identification by Western-blotting performed on ligand fishing eluates obtained from HeLa nuclear extracts. β-actin is used as a loading control. Nuclear extracts contain β-actin originating from actin nuclear pool and/or cytoplasmic contamination. This highly abundant protein is known to adsorb onto beads presenting different surface chemistries including polystyrene [44]. It is one of the proteins non-specifically adsorbed on magnetic beads and is therefore released in all eluates (Table S1).

Fig. 2B), while that of TOX4 and PNUTS was abolished. This is the reason why we relied on intermediate and low stringencies, thereby preserving these interactions, but also the presence of non-specific proteins. This explains the large number of total proteins found in each sample (between 92 and 194, Supplementary Table 1). Appropriate controls (undamaged DNA grafted onto the beads, and beads alone) are therefore indispensable to sort relevant candidates from all the identified proteins.

SPRi

SPRi is a versatile tool allowing the simultaneous, label-free and real-time study of biomolecular interactions [26]. We used this approach to assess in parallel interactions between recombinant purified proteins and four different double-stranded DNA probes: oligodeoxynucleotides (ODNs) bearing 1,2-d(GpG) cisplatin, oxaliplatin and JM118 crosslinks and ODNs without lesion. These damaged ODNs were synthesized, purified, controlled and grafted onto SPRi biochips. The tested proteins were successively HMGB1 (positive control), TOX4 (original candidate selected from proteomic results) and BSA (negative control). These experiments were performed at least twice with two different SPRi biochips for each protein. TOX4 showed a significant but lower capacity to bind platinated probes in comparison with HMGB1 (Fig. 3). It also showed a stronger affinity for all 1,2-d(GpG) crosslinks tested in comparison with non-damaged ODNs. Our repeated experiments did not demonstrate clear differences in binding affinity for TOX4 among the 3 platinated lesions tested. We nonetheless noticed that in our experimental conditions, HMGB1 recognizes cisplatin and JM118 crosslinks with a stronger affinity than oxaliplatin lesions.

Discussion

Proteomics methods applied to the identification of proteins recognizing DNA lesions constitute a recent research area. Various approaches have been developed to study these groups of proteins. Regarding lesions induced by platinating agents, Stansfield et al. [27] used protein microarrays incubated with cisplatin-modified fluorescent ODNs. Using this tool, the authors identified new interactions such as an association between cisplatin-modified DNA and Aurora kinase A. The most common strategy consists in trapping proteins using site-specifically damaged DNA immobilized on a solid phase such as magnetic beads. Extensive lists of proteins interacting with 1,2-instrastrand crosslinks, 1,3-instrastrand crosslinks and ICL were thus recently obtained by photocrosslinking proteins onto the photoactive cisplatin analogue Pt-BP6 inserted in duplex or dumbbell probes [12,13,28]. These original methods allowed the highlighting of new proteins interacting with platinum adducts, such as PARP-1. To carry out our experiments, we chose a different type of DNA probe consisting in 3 kb linearized plasmids exposed to three platinating agents: cisplatin, oxaliplatin or JM118. This strategy offers the following advantages: (i) a longer DNA sequence potentially allowing association with large protein complexes (ii) presence of several lesions/plasmid enhancing the quantity of trapped proteins and therefore the sensitivity of the method (iii) presence of the whole spectrum of lesions generated by the real pharmacological agent. Dealing with this last point, it must be emphasized that our probes contain a mixture of different crosslinks distributed all

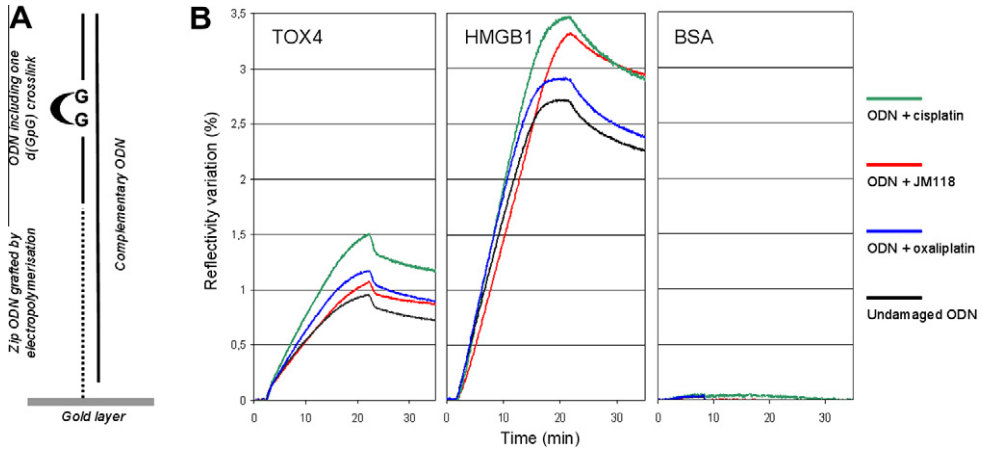

Fig. 3. SPR imaging. (A) Probes design (B) SPRi sensorgrams obtained with 20 nM TOX4 (tested candidate), HMGB1 (protein already known to interact with platinated DNA) and BSA (negative control).

along the sequence rather than one well characterized alteration at a specific site. These differences with other ligand fishing probes may explain some discrepancies observed with the above-mentioned studies [12,13]. Indeed, when comparing the lists of proteins identified in our study with previously published works, we can observe the presence of common elements (HMGB1, HMGB2, HMGB3, hUBF, DNA ligase III, Ku70) but also of proteins specific to our study. For instance, we succeeded in trapping both SSRP1 (not found by other global methods but already known to interact with platinated DNA) and SPT16, but also more original candidates such as WDR82, PNUTS or TOX4. In addition to the identification of novel proteins involved in platinum adducts recognition, we have also demonstrated the binding of already known candidates to satraplatin lesions. Limited clinical development work is currently being done with satraplatin, which may be ultimately used as an oral chemotherapeutic drug [29]. Binding of HMGB1 to JM118-related DNA lesions was already demonstrated [30]. In this work, we showed that DNA ligase III, hUBF, SSRP1, SPT16 and HMGB2 also interact with JM118 lesions.

The 3 platinating compounds that were tested present different carrier ligands (those which remain bound to platinum after DNA adduction). They comprise a small ammonia group for cisplatin, a bulky 1,2-diaminocyclohexane for oxaliplatin, and asymmetrical ammonia and cyclohexylamine groups for JM118. These nonleaving groups are the cause of structural differences between adducts and have distinct repercussions on DNA bending and unwinding [8,31]. It has been demonstrated that these differences are affecting the affinity of proteins for DNA lesions. As an example, HMGB1 has a less important affinity for DNA crosslinks generated by oxaliplatin than for cisplatin adducts [32]. These structural differences may also explain the different panels of proteins that have been trapped with the 3 agents.

Among proteins listed in Table 2, we must underline the absence of known repair factors from the NER pathway. Nevertheless, we noticed the presence of Ku70 and MDC1, proteins recruited to DSB [33], and ARF6, a factor implicated in cell motility but also capable of interacting with Ku70 [34]. In addition, a large portion of our list comprises chromatin remodeling factors, including HMGB factors but also other proteins involved in this process: RuvBL2, SMARCA5, TLK1, MTA2, SMC3. Interactions between these candidates and platinum DNA damage require further validation using complementary methods.

For our study, we selected TOX4, also called epidermal Langerhans Cell Protein 1 (LCP1). Indeed, this protein has never been directly linked to processes associated with damaged DNA. TOX4 belongs to the HMG domain proteins family [35]. Several HMG-box proteins have an established capacity to bind DNA damaged by platinating agents through their HMG domain, a DNA binding motif made of 3 alpha helices [3,36]. Depending on the HMG protein, this binding has been associated with a wide range of molecular mechanisms such as shielding toward DNA repair factors, recruitment of DNA repair factors or hijacking of transcription factors. The TOX subfamily of HMG-box proteins has been recently discovered and the biological role of TOX4 is far from being elucidated. Its N-terminus domain was characterized as a strong transcription activator [23]. Its identification as a platinated-DNA interacting protein suggests it may be hijacked from its main role by adducts as it is the case for hUBF. Additionally, we identified TOX4 using the same ligand fishing strategy in nuclear extracts of another cell line (MDA-MB231, breast cancer). To bring complementary clues of TOX4/damaged DNA interaction, we confirmed proteomic data using Western blot and took advantage of the SPRi method. This approach had already been used in our team to analyse interactions between oxidative DNA lesions and base excision repair proteins [37]. Here, it allowed the assessment of recombinant TOX4 binding on ODNs bearing 1,2-d(GpG) crosslinks, and showed that platinated drugs induced lesions are recognized with a higher affinity than ODNs without DNA damage. These parallel interactions studies also showed that binding of TOX4 was weaker than that of HMGB1 for the 3 lesions and non-damaged DNA.

Using our ligand fishing strategy on HeLa nuclear extracts but also on MDA-MB231 nuclear extracts, we identified PNUTS (also called PPP1R10) and WDR82. PNUTS inhibits protein phosphatases PPP1CA and PPP1CC, binds to nucleic acids and plays a role in mechanisms such as cell proliferation and apoptosis by regulating key actors such as pRb [38] and p53 [39]. Our bait also trapped the WDR82 protein, involved in histone methylation and initiation of transcription. Interestingly, an association between PNUTS and TOX4 has recently been demonstrated [23], followed by another study showing the formation of a PNUTS/TOX4/WDR82 complex possibly involved in chromatin structuration during the mitosis/interphase transition [22]. PNUTS/TOX4 interaction suppresses the transcriptional activity of TOX4. It would be of great interest to find out if the interaction between damaged DNA and PNUTS is affecting its biological properties, and if the biological role of the PNUTS/TOX4/WDR82 complex is modified by platinating agents. Moreover, it would be interesting to test if PNUTS and WDR82 directly bind platinated crosslinks or only through their interaction with TOX4.

In addition, two telomeric proteins were identified by our assay: telomeric repeat-binding factor 2 (TRF2) and RIF1. A recent publication by Kim et al. [40] suggests that TRF2 directly interacts with PNUTS, this association playing a role in telomere length regulation. TRF2 is a member of the shelterin complex in charge of telomere protection from end-to-end fusion process or DNA damage sensors. Using telomere mimicking ODNs, Ourliac-Garnier et al. [41] found a decreased affinity of TRF2 towards platinated sequences in comparison with intact ODNs. In addition, telomere chromatin immunoprecipitation indicated a 60% decrease of TRF2 binding when cells were treated by cisplatin. This discrepancy with our results may be explained by differences of DNA probes sequences or DNA adducts patterns. The decrease in telomere/TRF2 association in cisplatin-treated cells may also be explained by a reduction of the available pool of TRF2 being once again hijacked by cisplatin adducts in non-telomeric DNA. Demonstration of these assumptions and a better understanding of relationships between DNA lesions and TRF2 have to be further assessed.

Our study brought results that partially overlap those of other teams, but also comprise several new candidates. This emphasizes the usefulness of carrying out complementary methods rather than one single approach to tend toward a complete cartography of the factors capable of interacting with damaged DNA. Following validation, these new members of the protein network surrounding platinated DNA lesions may be of great interest to go further in the understanding of the pharmacology of these therapeutic drugs and improve it in terms of efficiency, resistance overcoming and side effects limitations. These proteins might also constitute future targets for their potentialisation. These interactions could even find therapeutic applications in non cancerous diseases: low dose cisplatin regimens are for example tested as HMGB1 intracellular sequestration methods to prevent the release of this pro-inflammatory mediator [42]. Future directions include the adaptation of the presented tools to allow the screening of platinated DNA-interacting proteins within a chromatin context [43], which would permit to take into account the complex environment that might interfere with other proteins/damaged DNA interactions. More generally, both the ligand fishing system and the SPRi biochip developed for this study may be applied to screen interactomes associated with other types of DNA lesions generated by other anticancer drugs, radiations or environmental mutagenic compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2010.12.021.

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