Cisplatin Resistance Associated with PARP Hyperactivation

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Abstract

Non–small cell lung carcinoma patients are frequently treated with cisplatin (CDDP), most often yielding temporary clinical responses. Here, we show that PARP1 is highly expressed and constitutively hyperactivated in a majority of human CDDP-resistant cancer cells of distinct histologic origin. Cells manifesting elevated intracellular levels of poly(ADP-ribosyl)ated proteins (PARhigh) responded to pharmacologic PARP inhibitors as well as to PARP1-targeting siRNAs by initiating a DNA damage response that translated into cell death following the activation of the intrinsic pathway of apoptosis. Moreover, PARP1-overexpressing tumor cells and xenografts displayed elevated levels of PAR, which predicted the response to PARP inhibitors in vitro and in vivo more accurately than PARP1 expression itself. Thus, a majority of CDDP-resistant cancer cells appear to develop a dependency to PARP1, becoming susceptible to PARP inhibitor–induced apoptosis. Cancer Res; 73(7); 2271–80. ©2013 AACR.

Introduction

Several among the 18 PARP proteins described so far constitute prospective targets for anticancer therapy (1, 2). PARP1 and 2 are the most abundant and best-characterized members of this family and are involved in transcriptional regulation, DNA repair, as well as in the maintenance of genomic stability (3–5). PARP1, which is mostly localized to the nucleus, accounts for approximately 75% of overall PARP enzymatic activity, fixing PAR polymers on multiple nuclear, cytoplasmic, and mitochondrial substrates, including proteins that modulate chromatin structure (e.g., histones, topoisomerases I and II), factors that stimulate DNA synthesis and repair (e.g., XRCC1, DNA polymerases α and β, DNA ligases I and II), and transcriptional regulators (e.g., p53; refs. 6–8).

PARP1 has been implicated in DNA repair via the base excision repair (BER) pathway (9). Upon DNA damage, PARP1 is recruited to single-strand breaks (SSB), where it becomes activated (10) and catalyzes the poly(ADP-ribosyl)ation of local substrates, including histones. As poly(ADP-ribosyl)ation advances and PAR polymers extend, histones progressively acquire negative charges, causing their electrostatic repulsion from interacting proteins and DNA (11). As a result, nuclear poly(ADP-ribosyl)ation facilitates the relaxation of supercoiled DNA structures, improving the accessibility of DNA to repair enzymes (12).

PARP1 is characterized by 3 functionally distinct domains: (i) an N-terminal DNA-binding domain that includes 2 zinc fingers for the detection of DNA SSBs (13, 14); (ii) an intermediate domain with autoinhibitory poly(ADP-ribosyl)ation sites, and (iii) a C-terminal catalytic domain that fixes NAD and transfers its ADP-ribose moiety (which bears one negative charge) to proteins in an ATP-consuming reaction (15). Several chemically distinct inhibitors of PARP have been synthesized and explored for their therapeutic profile against cancer, either as single agents or within combination regimens (16, 17). Current clinical trials are evaluating the safety and antineoplastic profile of at least 9 distinct PARP inhibitors (http://www.clinicaltrials.gov/).
PARP inhibitors are currently being evaluated for the treatment of tumors bearing loss-of-function mutations of *BRCA1* and *BRCA2* (18, 19), in particular breast, ovarian, and prostate cancers. *BRCA1* mutations compromise DNA repair via homologous recombination (HR), obliging cells to correct double-strand breaks via the less accurate non-homologous end-joining (20). By interfering with BER, PARP inhibitors indirectly promote the accumulation of DNA double-strand breaks (20). In line with this notion, PARP inhibition and loss-of-function *BRCA1* mutations display a robust synthetic lethality (21). Although initial phase I clinical trials tended to support this concept (22, 23), less promising results have been obtained in phase II studies (18, 19), raising an urgent need for predictive biomarkers. In addition, PARP inhibitors have been used to sensitize cancer cells to DNA-damaging agents including (but not limited to) ionizing irradiation, the alkylating agent temozolomide, the topoisomerase inhibitor camptothecin as well as platinum compounds such as cis-diammineplatinum(II) dichloride, also known as cisplatin (CDDP), and carboplatin (24–26). Some of these combinatorial chemotherapeutic regimens are currently under clinical evaluation (27, 28).

Driven by the facts that non–small cell lung carcinoma (NSCLC) is the leading cause of cancer-related morbidity and mortality worldwide (29) and that NSCLC patients often develop resistance against CDDP-based therapies, we addressed the question as to whether NSCLC cells may respond to PARP inhibitors. Here, we report the unexpected findings that NSCLC cells that have become resistant to CDDP often overexpress PARP1, constitutively exhibit high levels of PARP enzymatic activity and apparently rely on it for their survival, as PARP inhibition kills NSCLC cells that harbor hyperactivated PARP1.

Materials and Methods

**Cell lines, culture conditions, and chemicals**

The following culture media were used for both wild-type (WT) cells and their CDDP-resistant counterparts (generated as depicted in Supplementary Fig. S1A): Glutamax-containing Dulbecco’s Modified Eagle’s Medium/F12 medium for human NSCLC A549 cells; RPMI-1640 medium for human NSCLC H460 and H1150 cells; MCDB105/M199 medium for human ovarian cancer TOV-112D cells, and Eagle’s Minimal Essential culture medium with Earl’s salts for human mesothelioma P31, NSCLC H1299, and cervical carcinoma HeLa cells. Media for cell culture were invariably supplemented with 10% fetal bovine serum, 10 mmol/L HEPES buffer, 100 U/mL penicillin G sodium salt, and 100 μg/mL streptomycin sulfate. Cell lines were routinely maintained at 37°C under 5% CO2 in T175 flasks and seeded in appropriate supports (6, 12, or 96 wells plates) 24 hours before experimental determinations. Authenticated WT cells were obtained from American Type Culture Collection, immediately amplified to constitute liquid nitrogen stocks and (upon thawing) never passaged for more than 1 month before use in experimental determinations. Once generated, CDDP-resistant cells were treated similarly. CEP 9893-07 (CEP) and Z-Val-Ala-Asp (OMe)-fluoromethylketone (Z-VAD-fmk) were purchased from Cephalon and Bachem, respectively.

**RNA interference**

siRNA heteroduplexes specific for PARP1 (PARP1.a, sense 5’-CAACUGGAACAGAUCCGdTdT-3’, PARP1.b, sense 5’-GCCUCGGCUCCUGAAACAUdTdT-3’, PARP1.c, sense 5’-GA-UAGAGGUGAAGCCGAdTdT-3’, ref. 30), as well as 2 non-targeting siRNAs (UNR, sense 5’-GCCGGUAUCCGGUUAA-GUdTdT-3’, EMR, sense 5’-CCUGUCUCCUGGGCGGdTdT-3’, ref. 31), were purchased from Sigma-Aldrich. A549 cells preseeded in 12-well plates were transfected with siRNAs at 30% to 40% confluence by means of the HiPerFect transfection reagent (Qiagen), as previously described (32, 33). Cells were used for experiments no earlier than 24 hours after transfection.

**Cytofluorimetric studies**

For the simultaneous quantification of plasma membrane integrity and mitochondrial transmembrane potential (ΔΨm), both adherent and nonadherent cells were collected, washed, and costained with 1 μg/mL propidium iodide (PI, which only incorporates into dead cells) and 40 nmol/L 3,3’-dihexyloxacarbocyanine iodide DiOC6(3), a mitochondrial transmembrane potential (ΔΨm)-sensitive dye, (Molecular Probes–Invitrogen), following standard protocols (34, 35). Cytofluorimetric acquisitions were conducted on a FACScalibur (BD Biosciences), FACScan (BD Biosciences), or Gallios cytometer (Beckman Coulter). First-line statistical analyses were performed by means of the CellQuest (BD Biosciences) or Kaluza software (Beckman Coulter), upon gating on events exhibiting normal forward scatter and side scatter parameters.

**Immunoblotting**

Cells were collected, washed with cold PBS, and lysed as previously described (32, 36). Thereafter, protein extracts (30 μg/lane) were separated on precast 4% to 12% SDS-PAGE gels (Invitrogen), followed by electrotransfer to Immobilon membranes (Sigma-Aldrich) and immunoblotting with antibodies specific for PAR (10H; Calbiochem, Merck KGaA), PARP1 (Cell Signaling Technology Inc.), POLβ (Abcam), and XRCC1 (Abcam). An antibody that recognizes glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Millipore-Chemicon International) was used to monitor equal lane loading. Finally, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Southern Biotech), followed by chemiluminescence detection with the SuperSignal West Pico reagent and either CL-XPosure X-ray films (both from Thermo Scientific-Pierce) or the ImageQuant LAS 4000 Biomolecular Imager (GE Healthcare Life Sciences).

**Clonogenic survival assays**

To evaluate clonogenic survival, cells were seeded at different concentrations (from 0.2 to 4 × 103 per well) into 6-well plates, let adhere overnight, treated for 48 hours with CDDP and PARP inhibitors, and then cultured in drug-free medium for up to 10 days. Eventually, colonies were stained with 0.25% (w/v) crystal violet, 70% (v/v) methanol, and 3% (v/v) formaldehyde in water and quantified as previously described (37). Only colonies made of 30 cells and more were included in the quantification. The surviving fraction (SF) was
PARP Inhibitors and Cisplatin-Resistant Cells

In vivo experiments
Mice were maintained in specific pathogen-free conditions and all animal experiments were approved by the local Ethics Committee (CEEA IRICV/IGR n°26, registered with the French Ministry of Research), were in compliance with Directive EU 63/2010 and followed the Federation for Laboratory Animal Science Associations (FELASA) guidelines. Athymic nu/nu 8 weeks old female mice (Charles River Laboratories) were subcutaneously xenografted with $1 \times 10^6$ WT or CDDP-resistant R6 A549 cells (suspended in 200 µL PBS). Mice were treated starting from day 8 postinoculation with 5 mg/Kg PJ34 or an equivalent volume of vehicle (PBS) intraperitoneally 3 times a week for 3 consecutive weeks. Tumor growth was routinely monitored with a common caliper, and tumor volume was calculated according to the formula $V = \text{maximal diameter} \times \text{perpendicular diameter}^2$.

Statistical procedures
Unless otherwise specified, all experiments were conducted in triplicates and independently repeated at least 2 times, yielding comparable results. Data were analyzed with Microsoft Excel (Microsoft Co.) and statistical significance was assessed by means of one-tailed Student $t$ tests. $P$-values were considered significant when lower than 0.05. Minimum effective doses (MED) were defined as the smallest dose to achieve a statistically significant effect over control conditions. MEDs were computed on the basis of a 4-parameter logistic or 3-parameter Emax dose-response models (38). When MEDs could not be determined within the dose range, maximum doses are reported. Paired Wilcoxon tests were used to assess the one-sided hypothesis that MEDs would not differ between WT cells and their CDDP-resistant counterparts. See also Supplementary Information.

Results
PARP inhibitors interact with CDDP to induce NSCLC cell death
To identify pharmacologic agents that may influence the activity of the commercial PARP inhibitor PJ34 hydrochloride hydrate (PJ), we assessed nuclear shrinkage (pyknosis), which is indicative for the induction of apoptosis (35, 39), in NSCLC A549 cells exposed to 1,040 U.S. Food and Drug Administration (FDA)-approved drugs alone or combined with PJ for 48 hours. This screen led to the identification of 10 compounds that induce significant apoptosis in the presence, but not in the absence, of PJ, including alkylating agents such as CDDP, thiotepa, dacarbazine, and mitomycin C (Fig. 1A and B). The apparent interaction between PJ and CDDP was confirmed in a set of independent experiments (Fig. 1C), and this observation extended to another PARP inhibitor, CEP, whose precursor (CEP-9722) is currently being tested in phase I clinical trials (http://www.clinicaltrials.gov; Fig. 1D). On the basis of these results, we decided to investigate the possibility that PARP inhibitors might be useful for the treatment of tumors that become insensitive to CDDP-based chemotherapy.

Selective killing of CDDP-resistant cell clones by PARP inhibitors
CDDP-resistant NSCLC cell clones were isolated from A549 cells that had been cultured in the continuous presence of CDDP (2 µmol/L for 6 months followed by 5 µmol/L for 1 year). This procedure generated nine A549 cell clones (R1–R9) that—1 month after the withdrawal of CDDP—exhibited variable extents of CDDP resistance. Thus, as compared with parental, WT A549 cells, all resistant clones exhibited a reduced frequency of dying (DiOC6(3)lowPI−) and dead (PI+) cells upon exposure to CDDP concentrations ranging from 10 to 50 µmol/L (Fig. 2A and B and Supplementary Fig. S1). To get further insights into the cytotoxic activity of PARP inhibitors in
Figure 2. CDDP-resistant human NSCLC cell clones are susceptible to cell death induced by PARP inhibition. A–D, WT human NSCLC A549 cells and 3 CDDP-resistant derivatives (R) were maintained in control conditions or treated with increasing concentrations of CDDP (10, 15, 20, 30, and 50 μmol/L; A and B), CEP (1.25, 2.5, 5, and 10 μmol/L; C), or PJ (10, 20, 30, and 40 μmol/L; D) for 48 hours. Eventually, cells were subjected to the cytofluorometric assessment of apoptosis-related parameters. Representative dot plots and quantitative data are shown in A and B–D, respectively. In A, numbers refer to the percentage of cells in the corresponding quadrant. In B–D, white and black columns illustrate the percentage of dying [DiOC6(3)lowPI\(^+\)] and dead (PI\(^+\)) cells, respectively (means \(\pm\) SEM, \(n = 3\)). E and F, WT and CDDP-resistant R1 A549 cells were exposed to 1 μmol/L CEP, 5 μmol/L PJ, 1 μmol/L CDDP (WT cells), or 5 μmol/L CDDP (R1 cells) for 48 hours and then allowed to generate colonies in drug-free medium for 10 days. In E, representative pictures of colonies as observed upon crystal violet staining are shown (scale bar, 1 cm). In F, columns depict the normalized surviving fraction (means \(\pm\) SEM, \(n = 3\) parallel wells). G and H, WT and CDDP-resistant R1 A549 cells were left untransfected (–) or transfected with two distinct control siRNAs (UNR, EMR) or with PARP-specific siRNAs for 24 hours, then subjected to the cytofluorometric assessment of apoptosis-related variables. In G, white and black columns depict the percentage of DiOC6(3)\(^-\)PI\(^-\) and PI\(^+\) cells, respectively (means \(\pm\) SEM, \(n = 3\)). In H, representative immunoblots confirming PARP1 downregulation are reported. GAPDH levels were monitored to ensure equal loading of lanes. \(*P < 0.05; **P < 0.01; ***P < 0.001\) (Student t test), compared with equally treated WT cells (B–D) or UNR-transfected cells of the same type (G). See also Supplementary Fig. S1.
the context of CDDP resistance, WT A549 cells and all their CDDP-resistant derivatives were exposed to increasing doses of CEP or PJ for 48 hours, followed by the cytofluorometric evaluation of cell death. Unexpectedly, we found that some CDDP-resistant clones (R1, R2, R3, R6, R7) were more sensitive than parental A549 cells to both PARP inhibitors used in this study (CEP and PJ), although a few others were not (R4, R5) or exhibited intermediate responses (R8, R9). Thus, although 10 μmol/L CEP and 30 μmol/L PJ exerted negligible cytotoxic effects against WT A549 cells, they did induce significant extents of cell death in several of their CDDP-resistant clonal counterparts (Fig. 2C and D and Supplementary Fig. S1).

Similar results were obtained in clonogenic assays. Thus, although the concentration of CDDP that halved the clonogenic potential of parental A549 cells (IC50) was approximately 1 μmol/L, the IC50 of the resistant clone R1 was approximately 5 μmol/L. Conversely, the clonogenic potential of WT A549 cells was barely affected by exposure to 1 μmol/L CEP or 5 μmol/L PJ, although that of CDDP-resistant R1 cells was significantly compromised (Fig. 2E and F). In line with these observations, CDDP-resistant A549 cell clones that were sensitive to the cytotoxic activity of PARP inhibitors (such as R1) also died in response to PARP1-targeting siRNAs (Fig. 2G and H).

Altogether, these results indicate that CDDP resistance often, but not always, entails a hypersensitivity to PARP inhibition.

**PARP hyperactivation predicts the sensitivity of cancer cells to PARP inhibitors**

To characterize the molecular cascades through which PARP inhibitors kill CDDP-resistant NSCLC cells, we first determined the expression level and activation status of PARP1. This was achieved by the immunoblotting-assisted detection of PARP1 and of the products of its enzymatic activity, that is, poly(ADP-ribose) (PAR)-containing proteins. As compared with their CDDP-resistant counterparts, WT A549 cells in steady-state conditions exhibited low levels of both PARP1 and PAR-containing proteins. The amount of PAR-containing proteins increased (at least to some extent) in WT cells upon addition of CDDP, a phenomenon that could be entirely suppressed by the administration of CEP or PJ. Con- versely, the constitutively high levels of PAR-containing proteins observed in CDDP-resistant clones could not be further elevated by CDDP, yet were fully suppressed by CEP or PJ (Supplementary Fig. S2). Of note, CDDP-resistant clones that were particularly sensitive to the cytotoxic effects of PARP inhibitors were characterized by higher levels of PAR-containing proteins than clones that responded less efficiently to CEP and PJ (Fig. 3A). These results indicate a correlation between PARP1 enzymatic activity and the lethal effects of PARP inhibitors in CDDP-insensitive NSCLC clones.

To assess whether the intracellular levels of PAR-containing proteins might predict the susceptibility of cancer cells of different histologic origin to PARP inhibitors, we explored this parameter in 3 different NSCLC (H160, H460, H1299), 1 mesothelioma (P31), 1 ovarian (TOV-112D), and 1 cervical cancer (HeLa) cell lines, invariably comparing parental cells with their CDDP-resistant derivatives. In 5 out of 6 such pairwise comparisons, CDDP-resistant cells exhibited increased PAR levels. Only in H1299 cells the amount of PAR-containing proteins was reduced along with the development of CDDP resistance (Fig. 3B). Importantly, all cell lines characterized by elevated levels of PAR proteins (PARhigh), were more sensitive to CEP and PJ than their parental PARlow counterparts (Fig. 3C and Supplementary Table S1). These results underscore a tight link between high intracellular levels of PAR-containing proteins (and hence an elevated enzymatic activity of PARPs) and the susceptibility of cancer cells to succumb to PARP inhibitors. Of note, although PARhigh cells tend to express high levels of PARP1 as well, we observed no absolute correlation between these 2 variables (for instance, see P31 and TOV-112D cells, Fig. 3B), indicating that PAR levels predict the sensitivity of cells to PARP inhibition more accurately than the expression levels of PARP1.

**Mechanism of cell death induced by PARP inhibitors**

CDDP-resistant A549 cells succumbed to PARP inhibitors via the intrinsic pathway of apoptosis, as indicated by the mitochondrial release of cytochrome c (assessed by immunofluo-

rescence as the redistribution of cytochrome c from a compartmentalized to a diffuse cytoplasmic localization) and caspase-3 activation (detected by immunostaining with an antibody specific for the cleaved, proteolytically active form of caspase-3; ref. 40; Fig. 4A). In line with this notion, the co-administration of the broad-spectrum caspase inhibitor Z-VAD-fmk blunted the cytotoxic effects of CEP or PJ (Fig. 4B). Moreover, CDDP-resistant, but not WT, A549 cells responding to CEP or PJ manifested (at least to some extent) the apoptosis-associated cleavage of PARP1 (41; Supplementary Fig. S2). In response to PARP inhibitors, CDDP-resistant cells also mounted a DNA damage response characterized by an accumulation of nuclear foci containing histone 2AX phosphorylated on serine 139 (γH2AX) that was far more pronounced than that of CDDP-sensitive cells (Fig. 4C). In addition, the percentage of cells exhibiting γH2AX+ foci in steady-state conditions was significantly higher among CDDP-resistant cells than among their parental counterparts (Fig. 4C). The DNA damage response elicited by PARP inhibitors was detectable as early as 6 hours after exposure and could not be suppressed by the co-administration of Z-VAD-fmk (Supplementary Fig. S3). Of note, DNA repair via HR appeared to be functional in both parental and their CDDP-resistant counterparts, as reflected by the formation of BRCA1− and RAD51− nuclear foci formation in response to γ irradiation (Fig. 4D) or treatment with PARP inhibitors (Supplementary Fig. S4).

As PARP1 is crucially involved in the BER pathway, we comparatively examined the proficiency of this DNA repair system in parental and CDDP-resistant A549 cells. To this aim, we tested the capacity of cell-free extracts to catalyze the cleavage of synthetic DNA substrates in assays that reflect the first steps in the BER cascade, as catalyzed by apurinic/apyrimidinic endonuclease 1 (APE1), alkyl-N-purine DNA glycosylase, human uracil-DNA glycosylase, human endonuclease III, and human 8-oxoguanine glycosylase 1 (42). We found that WT and CDDP-resistant A549 cells possess similar repair activities relative to specific DNA substrates (Supplementary...
Figs. S5, S6, and S7), suggesting that the upstream steps of BER are not impaired in these cellular models. SSBs as generated by combined action of DNA glycosylases and APE1 are normally repaired by proteins that operate in downstream steps of BER such as X-ray repair cross-complementing protein 1 (XRCC1), PARP1, and polymerase β (POLβ; ref. 42). Remarkably, various (but not all) CDDP-resistant cells characterized by PARP1 overexpression and high levels of PAR-containing proteins exhibited reduced expression levels of XRCC1 and POLβ (i.e., A549 R6 to R9, H1650, H460, P31, HeLa). Accordingly, several among these clones were more sensitive to temozolomide (a DNA damaging agent reputed to selectively kill...
BER-deficient cells; ref. 43) than their CDDP-sensitive counterparts (Supplementary Fig. S8).

Taken together, these results indicate that CDDP-resistant cells that are characterized by high PARP1 expression levels and high amounts of PAR-containing proteins execute the early steps of BER as proficiently as their WT counterparts, but exhibit some degree of deficiency in downstream reactions such as those catalyzed by XRCC1 and POLβ. In addition, our results indicate that PARP activity may increase in CDDP-resistant cells in the absence of obvious HR defects.

**Effects of PARP inhibition on CDDP-resistant tumor xenografts**

To validate the hypothesis that PARP-hyperactivating, CDDP-resistant NSCLCs might respond to PARP inhibitors in vivo, athymic nu/nu mice carrying parental (WT) or CDDP-resistant cell-derived xenografts were treated with PJ. In contrast to tumors developing from WT A549 cells, which failed to respond to PJ monotherapy, the growth of cancers generated by CDDP-resistant cells, which exhibit high levels of PAR-containing proteins (Fig. 3A), was significantly delayed by the administration of PJ as a standalone intervention (Fig. 5A).

Thus, the levels of PAR-containing proteins may be used as a biomarker to estimate the effects of PARP inhibitors in vivo. To get further insights into this issue, we developed an immunohistochemical-staining method that specifically detects PAR-containing proteins on paraffin-embedded cell pellets and tissue sections (as shown by the fact that the siRNA-mediated depletion of PARP1 as well as the treatment with PARP inhibitors result in a complete loss of the signal, Fig. 5B). In the absence of chemotherapy, tumors derived from CDDP-resistant R6 cells were characterized by higher levels of PAR-containing proteins than WT A549 cell-derived xenografts (Fig. 5C), indicating that the levels of PAR-containing proteins are preserved during tumor formation in vivo. Moreover, xenografts featuring high amounts of PAR-containing proteins and exposed to PJ in vivo underwent a consistent reduction of poly (ADP-ribosyl)ation-dependent immunoreactivity (Fig. 5C).

Hence, PARP inhibitors appear to exert antineoplastic effects in vivo along with a significant decrease in PARP enzymatic activity.
Discussion

Here, we show that NSCLC cell clones that have been selected for CDDP resistance by prolonged exposure to CDDP can upregulate PARP1 and hence accumulate elevated levels of PAR-containing proteins. Both high levels of PAR-containing proteins and CDDP resistance were maintained upon the withdrawal of CDDP, suggesting the existence of yet elusive, genetic or epigenetic mechanisms that stabilize the upregulation of PARP1 and its hyperactivation. Irrespective of these unresolved issues, the hyperactivation of PARP appears as a relatively frequent event in the context of acquired CDDP resistance, as it could be observed in more than half of CDDP-resistant cells used in this study. As a caveat, the hyperactivation of PARP is not a universal characteristic of CDDP resistance but rather constitutes an optional mechanism. Indeed, one of the CDDP-resistant cell lines (H1299) characterized in this study exhibited a combined reduction in PARP1 expression levels and in the abundance of intracellular PAR-containing proteins.

The most striking observation reported in the present study concerns the ability of PARP inhibitors to kill CDDP-resistant cells that exhibit constitutive PARP hyperactivation. It is therefore tempting to speculate that this trait—which can be monitored by the abundance of PAR-containing proteins—may reflect a situation of PARP-dependent survival. Inhibition of PARP in PARP-hyperactivating cells led indeed to the activation of a DNA damage response (as indicated by the appearance of γH2AX+ foci) and ignited the intrinsic pathway of apoptosis. Thus, not only oncogenesis but also the development of chemoresistance appears to be coupled to the constitutive activation of stress response mechanisms that may constitute preferential targets for chemotherapy or chemosensitization (44).

The mechanisms through which CDDP-resistant cells upregulate PARP1 expression and its enzymatic activity remain largely obscure. Actually, we found that CDDP-resistant cells can be categorized into different classes, those that overexpress PARP1 and contain high levels of PAR-containing proteins and those that do not. This classification can be correlated with other parameters, including (but perhaps not limited to) XRCC1 and POLβ expression levels as well as the cytotoxic response to the DNA-damaging agent temozolomide. Although these results are insufficient to establish direct cause–effect...
relationships, they support a functional connection between defects in the downstream steps of the DNA repair via the BER pathway and upregulation/hyperactivation of PARP1.

CDDP remains one of the first-line agents for the therapy for NSCLC, in particular when such tumors lack activating mutations of the EGFR receptor (45). However, NSCLC patients treated with CDDP often develop chemoresistance, near-to-invariably leading to relapse and therapeutic failure (46). Here we show that CDDP-resistant cells respond more near-to-invariably leading to relapse and therapeutic failure (46). Here we show that CDDP-resistant cells respond more efficiently than their WT counterparts to PARP inhibitors. If the results of in vitro studies could be extrapolated to the clinics, PARP inhibitors would be most beneficial for (at least a fraction of) NSCLC patients relapsing after CDDP-based chemotherapy. In this context, it will be crucial to understand not only to which extent and under which specific circumstances PARP inhibitors may be effective as stand-alone interventions, but also whether the levels of PAR proteins may be used in clinical settings to predict the responsiveness of cancer patients to PARP inhibitor-based chemotherapeutic regimens.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Michels, L. Galluzzi, K.A. Olaussen, J.-C. Soria, M. Castedo, G. Kroemer

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