

Review

Systems biology of cisplatin resistance: past, present and future

L Galluzzi^{*,1,2,3,16}, I Vitale^{4,5}, J Michels^{1,3,6}, C Brenner^{7,8}, G Szabadkai^{9,10}, A Harel-Bellan^{11,12,13}, M Castedo^{1,3,6} and G Kroemer^{*,2,3,6,14,15,16}

The platinum derivative *cis*-diamminedichloroplatinum(II), best known as cisplatin, is currently employed for the clinical management of patients affected by testicular, ovarian, head and neck, colorectal, bladder and lung cancers. For a long time, the antineoplastic effects of cisplatin have been fully ascribed to its ability to generate unreparable DNA lesions, hence inducing either a permanent proliferative arrest known as cellular senescence or the mitochondrial pathway of apoptosis. Accumulating evidence now suggests that the cytostatic and cytotoxic activity of cisplatin involves both a nuclear and a cytoplasmic component. Despite the unresolved issues regarding its mechanism of action, the administration of cisplatin is generally associated with high rates of clinical responses. However, in the vast majority of cases, malignant cells exposed to cisplatin activate a multipronged adaptive response that renders them less susceptible to the antiproliferative and cytotoxic effects of the drug, and eventually resume proliferation. Thus, a large fraction of cisplatin-treated patients is destined to experience therapeutic failure and tumor recurrence. Throughout the last four decades great efforts have been devoted to the characterization of the molecular mechanisms whereby neoplastic cells progressively lose their sensitivity to cisplatin. The advent of high-content and high-throughput screening technologies has accelerated the discovery of cell-intrinsic and cell-extrinsic pathways that may be targeted to prevent or reverse cisplatin resistance in cancer patients. Still, the multifactorial and redundant nature of this phenomenon poses a significant barrier against the identification of effective chemosensitization strategies. Here, we discuss recent systems biology studies aimed at deconvoluting the complex circuitries that underpin cisplatin resistance, and how their findings might drive the development of rational approaches to tackle this clinically relevant problem.

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¹Gustave Roussy, Villejuif, France; ²Université Paris Descartes/Paris V, Sorbonne Paris Cité, Paris, France; ³Equipe 11 labellisée par la Ligue Nationale contre le Cancer, Centre de Recherche des Cordeliers, Paris, France; ⁴Regina Elena National Cancer Institute, Rome, Italy; ⁵National Institute of Health, Rome, Italy; ⁶INSERM, U848, Villejuif, France; ⁷INSERM, UMRS 769; LabEx LERMIT, Châtenay Malabry, France; ⁸Faculté de Pharmacie, Université de Paris Sud/Paris XI, Châtenay Malabry, France; ⁹Department of Cell and Developmental Biology, Consortium for Mitochondrial Research, University College London, London, UK; ¹⁰Department of Biomedical Sciences, Università Degli Studi di Padova, Padova, Italy; ¹¹Laboratoire Epigenétique et Cancer, Université de Paris Sud/Paris XI, Gif-Sur-Yvette, France; ¹²CNRS, FRE3377, Gif-Sur-Yvette, France; ¹³Commissariat à l'Energie Atomique (CEA), Saclay, France; ¹⁴Pôle de Biologie, Hôpital Européen Georges Pompidou, AP-HP, Paris, France and ¹⁵Metabolomics and Cell Biology Platforms, Gustave Roussy, Villejuif, France

*Corresponding authors: L Galluzzi, Université Paris Descartes/Paris V, INSERM, U848, Gustave Roussy, PR1, 39, rue Camille Desmoulins, Villejuif F-94805, France. Tel: +33 1 4211 4516; Fax: 33 1 4211 6665; E-mail: deador@vodafone.it

or G Kroemer, INSERM, U848, Gustave Roussy, PR1, 39, rue Camille Desmoulins, Villejuif F-94805, France. Tel: +33 1 4211 6046; Fax: +33 1 4211 6047; E-mail: kroemer@orange.fr

¹⁶Share senior co-authorship.

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Abbreviations: 5-FU, 5-fluorouracil; ABCC2, ATP-binding cassette, subfamily C (CFTR/MRP), member 2; AKT1, *v-akt* murine thymoma viral oncogene homolog 1; ATP7B, ATPase, Cu²⁺ transporting, β polypeptide; BMP4, bone morphogenetic protein 4; BRCA1, breast cancer 1, early onset; BRCA2, breast cancer 2, early onset; CDDP, *cis*-diamminedichloroplatinum(II); CDK2, cyclin-dependent kinase 2; CTR1, copper transporter 1; DAPK3, death-associated protein kinase 3; DFS, disease-free survival; DSB, double-strand break; DYRK1B, dual-specificity Y-phosphorylation regulated kinase 1B; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; ER, endoplasmic reticulum; ERCC1, excision repair cross-complementing rodent repair deficiency, complementation group 1; FA, Fanconi anemia; FDA, Food and Drug Administration; FGFR2, fibroblast growth factor receptor 2; GSH, reduced glutathione; GST, glutathione S-transferase; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; HR, homologous recombination; HSP, heat-shock protein; HTS, high-throughput screening; IL-7, interleukin-7; LCL, lymphoblastoid cell line; LIPC, lipase, hepatic; MAPK, mitogen-activated protein kinase; MAP3K1, mitogen-activated protein kinase kinase 1; METTL6, methyltransferase like 6; MLH1, *mutL* homolog 1; MMR, mismatch repair; MOMP, mitochondrial outer membrane permeabilization; MSH2, *mutS* homolog 2; mTOR, mammalian target of rapamycin; NER, nucleotide excision repair; NSCLC, non-small cell lung carcinoma; OS, overall survival; PARP1, poly(ADP-ribose) polymerase 1; PDXK, pyridoxal kinase; PPARGC1B, peroxisome proliferator-activated receptor γ , coactivator 1 β ; PTPC, permeability transition pore complex; RAF1, *v-raf-1* murine leukemia viral oncogene homolog 1; ROS, reactive oxygen species; SNP, single-nucleotide polymorphism; TLS, translesion synthesis; TSCC, tongue squamous cell carcinoma; USP1, ubiquitin specific peptidase 1; VDAC1, voltage-dependent anion channel 1

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Facts

- A large fraction of human malignancies rapidly becomes (or intrinsically is) insensitive to the cytostatic/cytotoxic effects of cisplatin.
- Cisplatin resistance is generally multifactorial, that is, it relies on the activation of multiple, non-redundant molecular or cellular circuitries.
- Throughout the past two decades, a number of systems biology studies has been performed to obtain additional insights into cisplatin resistance.
- The results of such an intense wave of investigation may promote the development of strategies to circumvent this clinically relevant hurdle.

Open Questions

- The precise molecular mechanisms whereby cisplatin exerts antineoplastic effects have not yet been elucidated.
- In particular, it remains unclear to which extent the cytoplasmic and nuclear events elicited by cisplatin contribute to its cytostatic/cytotoxic activity.
- Moreover, the possibility that cisplatin resistance may originate (at least in part) from cell-extrinsic (stromal or immune system-related) mechanisms has not yet been investigated in detail.

First described by the Italian chemist Michele Peyrone as early as in 1845, *cis*-diamminedichloroplatinum(II) (CDDP, best known as cisplatin or cisplatinum), is the founding member of a class of antineoplastic agents born in the second half of the 19th century around the peculiar atomic configuration of platinum.^{1,2} More than 120 years had indeed to pass from Peyrone's observations for the American chemist Barnett Rosenberg to characterize the robust antiproliferative effects of CDDP, first in *Escherichia coli* cultures, then in solid and hematopoietic tumor xenografts.^{3,4} Rosenberg's findings initiated an intense wave of preclinical and clinical investigation aimed at elucidating not only the molecular mechanisms that underpin the cytostatic/cytotoxic effects of CDDP, but also its safety and therapeutic profile.⁵⁻⁷ Such an experimental effort culminated in 1978 with the approval of CDDP by the US Food and Drug Administration (FDA) for use in testicular and bladder cancer patients.⁷ Since then, CDDP has been licensed worldwide for the treatment of multiple other solid neoplasms, including head and neck, lung, colorectal and ovarian cancers.^{5,8,9} Thus, during the last 35 years, several millions of cancer patients have received CDDP-based antineoplastic regimens, either as part of consolidated therapeutic procedures or in the context of clinical studies. Unfortunately, CDDP-based chemo(radio)therapy was often destined to fail.

As a matter of fact, CDDP is highly efficient only against testicular germ cell cancer, leading to a durable complete remission in >80% of the patients.^{10,11} Conversely, the clinical responses elicited by CDDP-based chemo(radio)therapeutic regimens in patients affected by other solid tumors (e.g., ovarian carcinoma) are temporary and vanish

as malignant cells become chemoresistant. Moreover, a significant fraction of lung, prostate and colorectal cancer patients bears neoplastic lesions that are intrinsically resistant to the cytostatic/cytotoxic activity of CDDP.¹²⁻¹⁴ Thus, although the use of CDDP (which is generally administered i.v. as a short-term infusion in physiological saline) has been associated with mild-to-moderate nephrotoxic, neurotoxic, cardiotoxic and ototoxic side effects,^{7,15} chemoresistance (be it intrinsic or acquired) constitutes the most prominent obstacle against the use of this drug. The biological reasons underlying the exquisite sensitivity of testicular germ cell cancers to CDDP have not been completely elucidated. Indeed, although defects in several DNA repair pathways (see below) have been associated with improved disease outcome in CDDP-treated testicular germ cell cancer patients,¹⁶⁻¹⁹ additional hitherto unidentified factors are likely to influence this phenomenon.

In the early 1980s, investigators and clinicians began to refocus their attention on the development of CDDP derivatives that would elicit robust therapeutic responses accompanied by clinically manageable side effects. Such an effort led to the discovery of two additional platinum derivatives that are nowadays approved by FDA for use in cancer patients: *cis*-diammine (cyclobutane-1,1-dicarboxylate-*O,O'*) platinum(II) (carboplatin) and [(1*R*,2*R*)-cyclohexane-1,2-diamine] (ethanedioato-*O,O'*)platinum(II) (oxaliplatin).^{20,21} The former has been licensed in 1989 for the treatment of ovarian cancer,²⁰ whereas the latter has entered clinical practice in 2002, as part of a 5-fluorouracil (5-FU)- and folinic acid-containing cocktail (the so-called FOLFOX regimen) that is routinely employed as a neo-adjuvant or adjuvant intervention in colorectal cancer patients.²¹ Carboplatin and CDDP exert antineoplastic effects via very similar, if not undistinguishable, mechanisms, reflecting the fact that the active forms of these drugs are identical. Still, carboplatin appears to be less nephro- and neurotoxic than CDDP, perhaps reflecting its reduced biological potency.²² Conversely, oxaliplatin and CDDP constitute two chemically distinct entities and as such exhibit distinct pharmacological and immunological properties.²³⁻²⁵ Nonetheless, a significant proportion of CDDP-insensitive tumors is resistant not only to carboplatin but also to oxaliplatin as well as to a large array of antineoplastic agents with a completely unrelated mechanism of action.²⁶⁻³⁶ These observations, which reflect the multilevel and multifactorial nature of CDDP resistance (see below), have fostered the development of additional platinum derivatives, including amminedichloro(2-methylpyridine)platinum (picoplatin) and (OC-6-43)-bis(acetato)amminedichloro(cyclohexylamine)platinum (satraplatin). As it stands, however, neither picoplatin- nor satraplatin-containing regimens provide consistent advantages over CDDP-, oxaliplatin- and carboplatin-based chemotherapy.³⁷⁻⁴⁰ Thus, the interest in developing novel platinum derivatives as well as clinically applicable strategies to increase the sensitivity of human neoplasms to CDDP remains high.

In this review, we summarize the mechanisms whereby CDDP exerts antineoplastic effects as well as the major determinants of CDDP resistance, laying special emphasis on recent systems biology studies aimed at avoiding or overcoming this crucial clinical issue.

Mode of Action

CDDP is chemically inert until one or both of its *cis* chloro groups are replaced by water molecules.^{41,42} Such an 'aquation' occurs spontaneously in the cytoplasm, presumably due to the relatively low concentration of chloride ions (~2–10 mM, as compared with ~100 mM in the extracellular space).^{43,44} Mono- and bi-aquated forms of CDDP are highly electrophilic and hence prone to form covalent bonds with methionine as well as with a large panel of cysteine-containing peptides and polypeptides, including reduced glutathione (GSH) and metallothioneins.⁴⁵ The interaction between aquated CDDP and endogenous nucleophiles has dual consequences. On one hand, it depletes the cytosol of reducing equivalents, hence promoting the establishment of oxidative stress, which may have direct cytotoxic effects or provoke DNA damage (see below).^{46,47} On the other hand, it results in the inactivation of (at least a fraction of) chemically reactive CDDP, hence functioning as a cytoprotective buffer.^{12,48}

Upon aquation, CDDP also binds with high affinity to mitochondrial and nuclear DNA, in particular to nucleophilic N7 sites on purines, thus favoring the generation of heterotypic protein–DNA complexes as well as homotypic inter- and intra-strand DNA adducts.^{49–51} If limited in amount, the DNA lesions provoked by CDDP can be recognized and safely removed by several repair systems that normally operate in the context of a temporary cell cycle arrest.^{52–56} Conversely, when the CDDP-induced DNA damage is irreparable, either such a cell cycle arrest becomes permanent (an oncosuppressive response known as cellular senescence)^{57,58} or cells become committed to die, most often via mitochondrial apoptosis.^{59–64} Both these processes can be initiated by the sequential activation of ATM- and RAD3-related protein (ATR, a sensor of DNA damage) and checkpoint kinase 1 (CHEK1, its major downstream effector), eventually resulting in the stabilizing phosphorylation of the oncosuppressor protein p53.^{65–69} A precise description of the molecular mechanisms whereby an excess of DNA lesions promotes widespread mitochondrial outer membrane permeabilization (MOMP), and hence cell death, exceeds the scope of this review and can be found in other publications.^{65–67,70,71}

Importantly, it is now clear that the cytostatic/cytotoxic effects of CDDP do not represent a mere consequence of its genotoxic activity, but originate from both nuclear and cytoplasmic signaling pathways.^{48,72} In line with this notion, (i) only ~1% of intracellular CDDP forms covalent bonds with nuclear DNA⁷³ and (ii) CDDP (as well as oxaliplatin) exerts prominent cytotoxic effects in enucleated cells (cytoplasts).^{51,74–78} The molecular mechanisms that underlie the cytotoxic potential of cytoplasmic CDDP are poorly understood, yet may involve: (i) the accumulation of reactive oxygen species (ROS) and nitric oxide (NO), which not only exacerbate CDDP genotoxicity but also exert direct cytotoxic effects by favoring the opening of the so-called permeability transition pore complex (PTPC);^{70,79,80} (ii) the transduction of a MOMP-stimulatory signal via the pro-apoptotic BCL-2 family member BAK1, the PTPC component voltage-dependent anion channel 1 (VDAC1) and the BAK1 homolog BAX;^{81,82} (iii) the activation of a cytoplasmic pool of p53 that is capable

of promoting MOMP via various mechanisms^{83–86} and (iv) in specific cellular models (see below), the establishment of an endoplasmic reticulum (ER) stress response.^{77,87}

The relative contribution of all these nuclear and cytoplasmic effects to the cytostatic/cytotoxic activity of CDDP remains to be deciphered and may exhibit a consistent degree of context dependency (Figure 1).

Mechanisms of Resistance

We have recently discussed the molecular mechanisms that reduce the sensitivity of malignant cells to CDDP, and classified them based on functional and hierarchical parameters. In particular, we proposed that CDDP resistance can surge from alterations (i) in processes that precede the binding of CDDP to its actual targets, including DNA and cytoplasmic structures (pre-target resistance), (ii) directly related to the molecular damage provoked by CDDP (on-target resistance), (iii) in the lethal signaling pathways triggered by such molecular lesions (post-target resistance) and (iv) influencing molecular circuitries that are not (or at least have not yet been) intimately associated with CDDP-elicited signals (off-target resistance).⁴⁸

Pre-target resistance. CDDP resistance can develop along with alterations that reduce the amount of aquated CDDP forms in the cytoplasm. Although for a long time CDDP has been believed to get access to the cytoplasmic compartment by passively diffusing across the plasma membrane,^{42,88} it is now clear that the majority of CDDP is actively moved in and out of the cell by copper transporters. In particular, copper transporter 1 (CTR1) has turned out to mediate a significant fraction of CDDP intake,^{89–93} whereas ATPase, Cu²⁺-transporting, β polypeptide (ATP7B), a Cu²⁺-extruding P-type ATPase involved in the pathogenesis of Wilson's disease,⁹⁴ plays a significant role in CDDP export.^{90,95} In line with this notion, alterations in the expression level, subcellular localization and/or functionality of CTR1 and ATP7B have been associated with CDDP resistance, both in preclinical models and in cancer patients.^{90,91,93,96–102} Promising (though very preliminary) results from a pilot clinical trial investigating the safety and chemosensitizing potential of trientine (a copper chelator) plus platinum-based chemotherapy in advanced cancer patients (NCT01178112) have recently been disseminated.^{102–104}

Other plasma membrane transporters have been suggested to contribute to the extrusion of CDDP (hence promoting CDDP resistance in preclinical as well as clinical settings), notably ATP-binding cassette, subfamily C (CFTR/MRP), member 2 (ABCC2), best known as multidrug resistance-associated protein 2 (MRP2)^{105–109} and ATPase, class VI, type 11B (ATP11B).¹¹⁰ Moreover, CDDP-resistant cancer cells may exhibit increased levels of GSH, the enzyme that catalyzes GSH synthesis (i.e., γ -glutamylcysteine synthetase), the enzyme that conjugates CDDP with GSH (i.e., glutathione S-transferase), or metallothioneins.^{111–114} Although none of these alterations has been shown to be clinically relevant, all of them may contribute to pre-target resistance by incrementing the capacity of the cytoplasmic CDDP buffer.

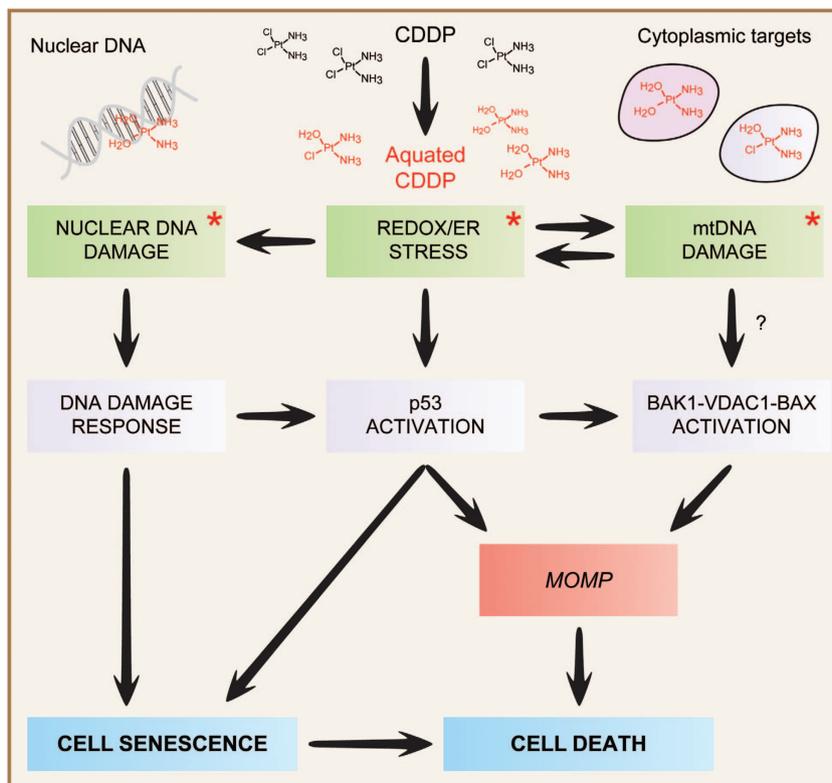


Figure 1 Mode of action of cisplatin. As a result of the reduced cytoplasmic concentration of chloride ions, intracellular cisplatin (CDDP) is rapidly 'aquated', hence acquiring a pronounced electrophilic reactivity. Aquated CDDP binds with high affinity to nuclear DNA, in particular to nucleophilic N7 sites on purines, thereby promoting the activation of the DNA damage response. In addition, CDDP can physically interact with several cytoplasmic nucleophiles, including mitochondrial DNA (mtDNA) as well as multiple mitochondrial and extramitochondrial proteins, hence (i) favoring the establishment of oxidative and reticular stress; (ii) eliciting a signal transduction cascade that involves the pro-apoptotic BCL-2 family members BAK1 and BAX, as well as voltage-dependent anion channel 1 (VDAC1) and (iii) activating the cytoplasmic pool of p53. The relative contribution of these nuclear and cytoplasmic modules to the cytostatic/cytotoxic activity of CDDP remains to be precisely elucidated and may exhibit an elevated degree of context dependency. Asterisks tag the primary consequences of CDDP reactivity. ER, endoplasmic reticulum; MOMP, mitochondrial outer membrane permeabilization

On-target resistance. The sensitivity of cancer cells to the cytostatic/cytotoxic effects of CDDP is limited in the presence of a proficient DNA repair apparatus. In particular, the nucleotide excision repair (NER) system is believed to resolve the majority of DNA lesions provoked by CDDP,^{53,54} although components of the mismatch repair (MMR) machinery have also been implicated in this process (at least in its detection, as opposed to resolution, phase).⁵⁵ In line with this notion, an increased NER proficiency has been associated with CDDP resistance *in vitro*, in murine models as well as in cohorts of cancer patients.^{115–123} Convenient biomarkers for assessing NER proficiency in human tumors are still missing, as the actual predictive value of excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1) expression levels (first proposed in 2006),¹¹⁸ has recently been reconsidered.¹²⁴ The MMR system is believed to detect DNA adducts caused by CDDP, engage in their repair and ultimately fail, thus transmitting a pro-apoptotic signal.¹²⁵ Accordingly, genes encoding MMR components such as *mutS* homolog 2 (MSH2) and *mutL* homolog 1 (MLH1) are frequently mutated or downregulated in the context-acquired CDDP resistance.^{126–129} Moreover, non-small cell lung carcinoma (NSCLC) lesions expressing high MSH2 levels exhibit an improved prognosis upon surgical resection (in the absence of adjuvant chemotherapy).¹³⁰ Thus, high

expression levels of MMR components may exert a positive influence both on the propensity of chemotherapy-naïve tumors to relapse (perhaps because MMR limits the accumulation of additional genetic alterations) and on the probability that neoplasms exposed to CDDP become chemoresistant. *MLH1* mutations are also associated with increased rates of translesion synthesis (TLS), the process whereby DNA is replicated (by a peculiar class of polymerases) in spite of unrepaired lesions.¹³¹ In line with this notion, defects in TLS polymerases including polymerase (DNA-directed), η (POLH) and REV3-like, polymerase (DNA directed), ζ , catalytic subunit (REV3L) have been linked to increased CDDP sensitivity *in vitro*.^{132–135} However, compelling clinical evidence in support of the translational relevance of these findings is elusive.

CDDP-elicited DNA adducts can engender double-strand breaks (DSBs), which are normally repaired along with DNA synthesis (or shortly after) via homologous recombination (HR).¹³⁶ Accordingly, HR-deficient neoplasms, such as those bearing loss-of-function mutations in the genes encoding breast cancer 1, early onset (BRCA1) or breast cancer 2, early onset (BRCA2),^{137,138} are generally more susceptible to the genotoxic effects of CDDP than HR-proficient cancers of the same type.^{139–141} Moreover, the appearance of compensatory mutations in *BRCA1* and *BRCA2* that restore the functionality of HR has been shown to favor CDDP resistance

in breast carcinoma cells.¹⁴² Mutations of this type not only have been associated with the resistance of ovarian carcinoma to platinum-based chemotherapy^{142,143} but also appear to influence the sensitivity of pancreatic and ovarian neoplasms to inhibitors of poly(ADP-ribose) polymerase 1 (PARP1), another important regulator of DNA repair.¹⁴⁴ Of note, while the innate resistance of pancreatic carcinoma cells to CDDP and PARP1 inhibitors resolves simultaneously in response to HR-restoring *BRCA2* mutations,¹⁴³ we have recently reported that NSCLC cells cultured for prolonged periods in the presence of subtoxic amounts of CDDP often (but not always) manifest a constitutive hyperactivation of PARP1, hence becoming susceptible to pharmacological or genetic PARP inhibition.¹⁴⁵ In line with these notions, the concomitant administration of CDDP and PARP inhibitors appears to elicit at least additive antineoplastic effects, *in vitro* and *in vivo*.^{146–148}

Although the identity of the cytoplasmic components that account for the extranuclear toxicity of CDDP has only recently begun to emerge, these molecules as well as the enzymatic systems that regulate their preservation/turnover may also be involved in the development of on-target CDDP resistance.⁷² So far, only a few extranuclear CDDP-binding partners have been identified, including near-to-ubiquitous (e.g., valosin-containing protein), as well as mostly cytosolic (e.g., myosin IIa, HSP90), ribosomal (e.g., ribosomal protein L5), reticular (e.g., calreticulin) and mitochondrial components (e.g., mitochondrial DNA, VDAC1).^{51,149} Remarkably, human NSCLC cells developing CDDP resistance as they are exposed for long periods to sublethal doses of the drug appear to be positively selected for specific mitochondrial DNA mutations, resulting in partial defects of the respiratory chain.^{150,151} Two mechanisms that may account for this effect are the compensatory stimulation of mitochondrial biogenesis by retrograde mitochondrio-nuclear signaling and the activation of a peroxisome proliferator-activated receptor γ , coactivator 1 β (PPARGC1B)-dependent (but mitochondrion-independent) signal transduction cascade.^{150,152} Moreover, both rho⁰ cells and cells depleted of VDAC1 by RNA interference are less susceptible to CDDP cytotoxicity than their wild-type counterparts.^{51,81} In line with this notion, the transfection-enforced overexpression of VDAC1 reportedly sensitizes carcinoma cells to cell death induction by CDDP.⁸² However, elevated expression levels of VDAC1 may exert broad chemosensitization effects.¹⁵³ Thus, whether these observations constitute examples of *bona fide* on-target resistance or rather reflect the critical role of mitochondria (and in particular of the PTPC) in CDDP-elicited cell death, hence constituting examples of post-target resistance, remains an open conundrum. Along similar lines, although physical interactions between CDDP and cyclin-dependent kinase 2 (CDK2) have not yet been documented, CDK2 has been shown to contribute to extranuclear CDDP toxicity (by inducing ER stress, at least in some cellular models), and CDK2-deficient cells exhibit some extent of CDDP resistance.^{77,87}

Post-target resistance. In line with the notion that regulated cell death (be it apoptotic or necrotic) is under the control of a plethora of checkpoints and safeguard mechanisms,

post-target CDDP resistance may develop along with a wide panel of alterations in the systems that detect the molecular damage caused by CDDP and convert it into a lethal signal, as well as in the machinery that *de facto* executes cell death.^{12,48,154} Malignant cells are (i) intrinsically more resistant to adverse microenvironmental and intracellular cues than their non-transformed counterparts^{155,156} and (ii) highly prone to acquire additional genetic and epigenetic alterations.^{157,158} Thus, (at least some degree of) post-target resistance can be documented in most, if not all, CDDP-exposed tumors. Importantly, post-target CDDP resistance is rarely (if ever) specific, rather extending to other DNA-damaging agents (including radiation),^{27,33,159} as well as to cytotoxic stimuli that engage a wide panel of primary targets (e.g., death receptor agonists).¹⁶⁰

As a general rule, intracellular stress conditions (such as those elicited by CDDP) promote the rapid activation of an integrated adaptive response aimed at the re-establishment of cellular homeostasis.¹⁶¹ This is generally accompanied by the emission of robust anti-apoptotic cues and only when homeostasis cannot be restored (for instance, when stress conditions are excessive in intensity or duration) lethal signals are transmitted, *de facto* representing a mechanism for the preservation of organismal homeostasis.¹⁶² In the case of CDDP, these signals consist in (i) the switch of the DNA damage response from a cytoprotective to a cytotoxic mode, followed by the (often, but not always, p53-dependent) activation of BAX and BAK1¹⁶³ and (ii) the accumulation of ROS and consequent PTPC opening.^{81,164} Both these processes eventually promote MOMP, in turn resulting in the functional and physical breakdown of mitochondria followed by the activation of caspase-dependent and independent mechanisms of cell death.^{70,165} Thus, post-target CDDP resistance has been associated not only with genetic and epigenetic alterations that impair p53 signaling (*in vitro*, in murine tumor models as well as in several distinct clinical scenarios)^{11,166–170} but also with defects in several other pro-apoptotic signal transducers, including mitogen-activated protein kinase 14 (MAPK14, best known as p38^{MAPK}) and c-Jun N-terminal kinase 1 (JNK1).^{171,172} Along similar lines, post-target CDDP resistance appears to be significantly influenced by the expression levels and functional status of BCL-2 family members and caspases, a class of cysteine proteases that has a major role in the execution of apoptotic cell death.^{81,173,174} This said, only a few of these factors have been formally correlated with CDDP resistance in clinical studies, including various BCL-2-like proteins such as BCL-2 itself, BCL-X_L and MCL-1,^{175–178} survivin, a caspase inhibitor that is often upregulated in response to CDDP,^{179–182} as well as other members of the baculoviral IAP repeat-containing (BIRC) protein family.¹⁸³ In line with this notion, small molecules that antagonize the anti-apoptotic effects of BCL-2-like proteins (e.g., ABT-263) as well as IAP inhibitors (e.g., YM155, LY2181308, LBW242) have been intensively investigated in clinical trials, either as standalone anticancer interventions or in combination with various chemotherapeutics including CDDP.^{184–188} In spite of an acceptable safety profile and promising antineoplastic activity, the clinical development of all these agents appears to stand at an impasse, at least as judged by the low rate of

ongoing *versus* completed, terminated or withdrawn trials (source www.clinicaltrials.gov).

Off-target resistance. The susceptibility of cancer cells to CDDP can also be limited by off-target mechanisms, that is, molecular circuitries that deliver compensatory survival signals even though they are not directly activated by CDDP.⁴⁸ For instance, the overexpression of *v-erb-b2* avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2), which is common in breast and ovarian carcinomas,^{189,190} has been suggested to promote CDDP resistance not only by delivering robust pro-survival signals via the *v-akt* murine thymoma viral oncogene homolog 1 (AKT1) signaling axis, but also by finely regulating the transitory cell cycle arrest that is required for the repair of CDDP-induced DNA lesions.^{191,192} Along similar lines, dual-specificity Y-phosphorylation-regulated kinase 1B (DYRK1B, also known as MIRK) appears to sustain CDDP resistance as it favors the expression of various antioxidant enzymes.¹⁹³ By augmenting the intracellular pool of antioxidants, DYRK1B may actually reduce the susceptibility of cancer cells to CDDP via on-target, post-target as well as off-target mechanisms.⁴⁸ Recently, a poorly characterized transmembrane protein (TMEM205) has been shown to favor CDDP resistance via a molecular cascade that involves the small RAS-like GTPase RAB8A.^{194,195} Whether this constitutes an off-target mechanism of resistance or rather reflects the inhibition of caspase-9 by AKT1-transduced signals¹⁹⁶ (hence representing a post-target effect) remains to be clarified. Finally, several relatively unspecific adaptive responses to stress have been implicated in CDDP resistance. Such general responses include macroautophagy and the so-called heat-shock response, that is, the adaptation of cells to increased temperatures and other conditions that promote protein unfolding.¹⁶¹ Thus, various components of the autophagic machinery and several chaperones of the heat-shock protein (HSP) family reportedly impair the cytostatic/cytotoxic response of cultured cancer cells to CDDP.^{149,197–201} Moreover, the expression levels of HSP27 may constitute a predictive biomarkers of clinical responses to CDDP in esophageal squamous cell carcinoma patients.²⁰²

In conclusion, malignant cells can lose their sensitivity to CDDP owing to a wide panel of molecular and functional alterations, many of which are *a priori* connected to oncogenesis and tumor progression. CDDP resistance is often multifactorial, as it relies on the simultaneous activation of multiple, non-redundant molecular circuitries (Figure 2).^{48,203} This obviously poses a major hurdle against the development of therapeutically useful chemosensitization strategies. As summarized below, several systems biology studies have recently been undertaken to address this clinically relevant issue.

Systems Biology and Cisplatin Resistance

Throughout the last decade, a huge number of high-content and/or high-throughput studies have been initiated to tackle – by means of an empirical approach – the complex and clinically relevant problem of CDDP resistance. For illustrative purposes, these studies can be classified into

three large groups: (i) genomic, transcriptomic, methylomic and proteomic approaches, investigating the correlation between CDDP resistance and gene expression/regulation or the activation of specific signal transduction pathways (in pre-clinical models or patient material); (ii) large-scale silencing approaches and functional screenings, determining whether the genetic or pharmacological inhibition of specific proteins alters CDDP sensitivity (in preclinical models only) and (iii) multiplex genotyping studies, assessing in a high-throughput manner whether particular (combinations of) single-nucleotide polymorphisms (SNPs) are associated with increased or reduced CDDP sensitivity (in clinical settings). Each of these approaches obviously brings about specific advantages and disadvantages, which we will not systematically discuss here (for further details please consult a recent study by He *et al.*²⁰⁴). Still, it is important to note that, with a few exceptions (including some genotyping studies), the majority of these strategies have relied on relatively reductionistic models (e.g., cultured cancer cells) or readouts (e.g., mRNA levels in whole-tumor lysates). Considering tumors as relatively homogeneous entities exclusively composed of malignant cells and attributing CDDP resistance only to cancer cell-intrinsic mechanisms (hence neglecting the prominent structural and functional impact of stromal and immune cells) appears indeed as an excessive oversimplification (see below), and may in part explain why clinically applicable strategies for restoring CDDP sensitivity are still missing. Nonetheless, these studies have provided us with ever more precise insights into the molecular mechanisms that account for CDDP resistance, at least at the cell-intrinsic level. Here, we provide an overview of recent systems biology studies (for the most part published in the last 3 years) that have significantly added to our understanding of CDDP resistance (Supplementary Table 1).

Genomic, methylomic, transcriptomic and proteomic approaches. For nearly one decade, it has been possible – and cost-efficient – to employ microarrays for quantifying (i) variations in gene copy number, by comparative genomic hybridization (CGH), (ii) the abundance of mRNAs and (iii) the expression levels of regulatory RNA species (including microRNAs and long non-coding RNAs) in both human and murine samples. Thus, several research groups worldwide, including us, have adopted one of these approaches to get further insights into CDDP resistance. Nonetheless, the potential of these methods is intrinsically limited by the frequent lack of correlation between copy number or mRNA/microRNA expression levels and phenotype, calling for robust rounds of functional validation.²⁰⁵ Recently, along with the evolution of mass spectrometry and the development of high-throughput techniques for the study of post-translational protein modifications (including, but not limited to, phosphorylation and acetylation),^{206–209} attention has been refocused to proteomic/interactomic approaches as well as to the identification of novel signal transduction cascades that underpin CDDP resistance. In addition, great enthusiasm has been generated by the possibility to sequence the whole genome of neoplastic lesions, as first achieved for acute myeloid leukemia and quickly thereafter for various other malignancies.^{210–214} Further increasing the

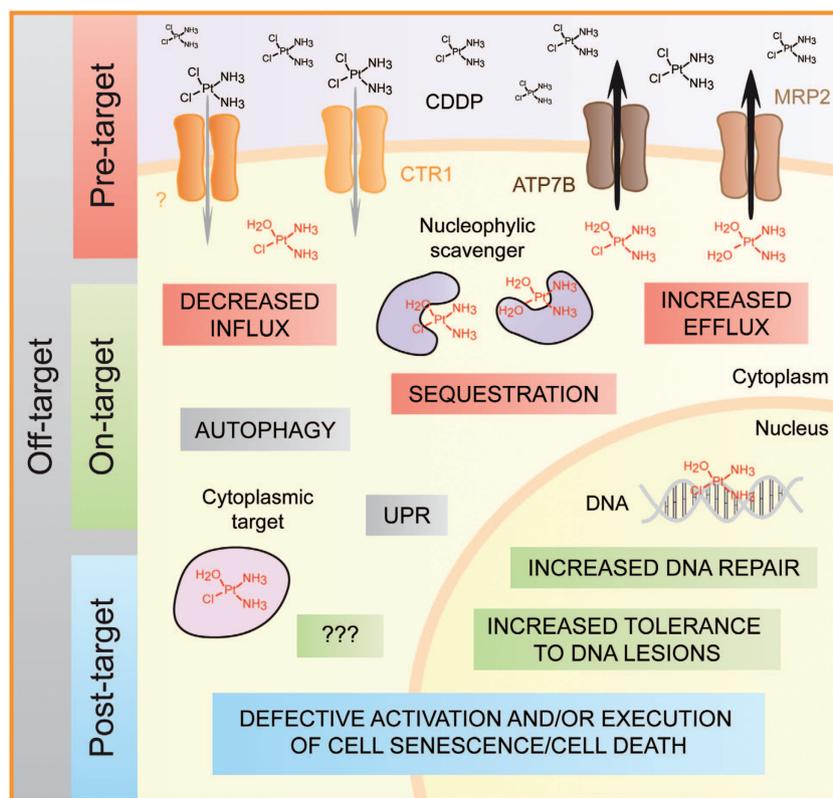


Figure 2 Molecular mechanisms of cisplatin resistance. Malignant cells can lose their sensitivity to the cytostatic/cytotoxic activity of cisplatin (CDDP) as a result of a wide panel of genetic or epigenetic defects. These alterations can (i) affect processes that precede the actual binding of CDDP to its targets (pre-target resistance); (ii) potentiate the ability of cells to repair the molecular damage caused by CDDP (on-target resistance); (iii) impair the transmission of signals that normally relay such a CDDP-induced damage to cell senescence or apoptosis (post-target resistance) or (iv) stimulate the delivery of pro-survival signals that antagonize CDDP cytotoxicity although they are normally not elicited by this drug (off-target resistance). Of note, CDDP resistance is generally multifactorial, that is, it relies on the activation of several, non-overlapping mechanisms that concur to limit the cytostatic/cytotoxic effects of CDDP at multiple levels. At least in part, this explains why efficient strategies to increase the sensitivity of human neoplasms to CDDP are still lacking in spite of a prolonged and intense wave of investigation. ATP7B, ATPase, Cu^{2+} transporting, β polypeptide; CTR1, copper transporter 1; MRP2, multidrug resistance-associated protein 2; UPR, unfolded protein response. CDDP aquation is depicted in red

potential of this approach, Murtaza *et al.*²¹⁵ have just demonstrated that advanced solid tumors can be sequenced starting from DNA molecules released by malignant cells into the plasma, *de facto* constituting a non-invasive liquid biopsy. Again, although these approaches can undoubtedly improve our understanding of CDDP resistance, caution should be taken while interpreting sequencing data in the absence of functional validation.

Several factors have recently been revealed or confirmed to contribute to CDDP resistance as a result of genomic, methylomic, transcriptomic and proteomic studies (Supplementary Table 1). Using CGH arrays, Cui *et al.*²¹⁶ identified an interleukin-7 (IL-7)-dependent autocrine loop specifically activated in glioma cells that have lost their sensitivity to CDDP. By means of an integrated approach involving CGH arrays and transcriptomic studies, Jiffar *et al.*²¹⁸ demonstrated that head and neck squamous cell carcinomas (HNSCCs) can become resistant to CDDP along with the loss of KiSS-1 metastasis-suppressor (KISS1), a G protein-coupled receptor ligand that also mediates CDDP-independent oncosuppressive functions.^{217,218} By comparing the methylome of CDDP-sensitive and CDDP-resistant isogenic ovarian cancer cells, Yu *et al.*²¹⁹ found that the latter exhibit not only a global decrease in the methylation of CpG

islands but also specific alterations including the hypomethylation (which is generally associated with increased gene expression) of protein tyrosine kinase 6 (*PTK6*), coding for a non-receptor tyrosine kinase with prominent pro-survival functions; protein kinase $\text{C}\epsilon$ (*PRKCE*), encoding a Ca^{2+} - and diacylglycerol-activated serine/threonine kinase, and BCL-2-like 1 (*BCL2L1*), coding for BCL-X_L.²¹⁹ By means of a systems biology approach comprising a methylomic and a transcriptomic component, Zeller *et al.*²²⁰ identified a set of nine genes that are hypermethylated (resulting in decreased transcription) both in several, independent CDDP-resistant derivatives of an ovarian carcinoma cell line (i.e., A2780 cells), and in clinical samples from ovarian carcinoma patients relapsing upon platinum-based chemotherapy. Besides *MLH1*, which is often mutated or downregulated in the context of CDDP resistance,^{126–129} these genes include Rho GDP dissociation inhibitor β beta (*ARHGDI3*), armadillo repeat containing, X-linked 2 (*ARMCX2*), collagen, type I, $\alpha 1$ (*COL1A1*), filamin A α (*FLNA*) filamin C γ (*FLNC*), mesoderm-specific transcript (*MEST*), neurotensin (*NTS*) and proteasome (prosome, macropain) subunit, β type, 9 (*PSMB9*), pointing to the existence of hitherto unexplored mechanisms whereby malignant cells can lose their sensitivity to CDDP.²²⁰ Ivanova *et al.*²²¹ applied a very similar experimental strategy

to study gastric cancer, identifying bone morphogenetic protein 4 (BMP4) as an epigenetically regulated factor that underpins CDDP (but not oxaliplatin) resistance and exerts oncogenic functions (at least in this setting) by promoting the epithelial-to-mesenchymal transition (EMT). It remains unclear whether the oncogenic activity of BMP4, similar to that of IL-7, relies on autocrine signaling circuitries.

The profound implication of the DNA repair machinery and the BCL-2 protein family in CDDP resistance has been confirmed by a large number of recent transcriptomic studies.^{118,124,222–226} However, none of these factors has yet been validated as a reliable clinical predictor of CDDP sensitivity.¹²⁴ Alongside, the abundance of transcripts coding for several proteins that have no obvious links with CDDP-elicited signaling pathway has been correlated with clinical CDDP resistance. These factors include AKT1, eukaryotic translation initiation factor 4B (EIF4B) and ribosomal protein S6 (RPS6), defining a metabolic signature linked to the activity of mammalian target of rapamycin (mTOR),²²⁷ cytochrome P450, family 4, subfamily B, polypeptide 1 (CYP4B1), choline/ethanolamine phosphotransferase 1 (CEPT1) and charged multivesicular body protein 4A (CHMP4A),²²⁸ as well as several proteins implicated in the EMT.²²⁹ However, whether the EMT triggers a specific pathway of CDDP resistance or these observations rather reflect the high number of genetic and epigenetic alterations that characterize advanced neoplastic lesions (underpinning their aggressiveness and chemoresistance) remains to be determined. Furthermore, by analyzing HNSCC biopsies with whole-genome microarrays and quantitative reverse transcriptase PCR cards, Tomkiewicz *et al.*²³⁰ delineated a 10-gene signature that predicts the response of HNSCC patients to CDDP/5-FU-based induction chemotherapy. As it stands, most of these genes have not been previously implicated in CDDP resistance, although some of them code for factors that have a role in the cytoprotective response to redox stress, such as DnaJ (Hsp40) homolog, subfamily A, member 1 (*DNAJA1*) and thioredoxin domain containing 9 (*TXNDC9*).²³⁰

By comparing the transcriptomic profile of NSCLC A549 cells exposed to cytotoxic doses of CDDP, cadmium dichloride (CdCl_2 , an environmental pollutant that triggers oxidative stress-dependent apoptosis) and C_2 -ceramide (a plasma membrane permeant ceramide with mitochondriotoxic effects), we have recently demonstrated that CDDP elicits signaling cascades (encompassing both pro-survival and pro-death pathways) that are for the most part 'private', that is, they manifest limited overlap with the molecular mechanisms set in motion by other inducers of mitochondrial apoptosis.²³¹ Along similar lines, by studying the microRNAome of A549 cells succumbing to CDDP, CdCl_2 or C_2 -ceramide, we identified miR-181a and miR-630 as functional components of the molecular mechanisms that underpin CDDP toxicity and the general cytoprotective response of A549 cells to stress, respectively.²³² On a similar note, several research groups have recently investigated the microRNAomic profile of CDDP-sensitive *versus* CDDP-resistant cancer cell lines of various origin, including glioblastoma,²³³ tongue squamous cell carcinoma (TSCC),²³⁴ NSCLC,^{235–238} hepatocellular carcinoma (HCC)²³⁹ and ovarian carcinoma.^{240,241} Several of these studies identified the

downregulation of microRNAs that inhibit the expression of anti-apoptotic BCL-2-like proteins (e.g., miR-135 and miR-497) as a prominent mechanism of CDDP resistance.^{235,236} In addition, a decrease in the sensitivity of cancer cells to the cytostatic/cytotoxic effects of CDDP has been associated with the upregulation of microRNAs that target the major onco-suppressive factor phosphatase and tensin homolog (PTEN), such as miR-130a and miR-93,^{240,241} and ERCC1, like miR-138,²³⁷ as well as with the downregulation of microRNAs that normally limit the expression of cell cycle-associated proteins such as let-7b.²³³ Similarly, CDDP resistance in cultured A549 cells has been correlated with the downregulation of a long non-coding RNA (AK126698) that inhibits WNT/ β -catenin signaling.²³⁸

At odds with our observations, elevated expression levels of miR-181a have been associated with a decreased (rather than increased) sensitivity of cultured HCC cells to multiple antineoplastic agents, including CDDP.²³⁹ Such an apparent discrepancy may originate from the experimental systems employed (i.e., acute response to CDDP in NSCLC cells *versus* stably acquired resistance in HCC cells) or reflect a context-dependent role of miR-181a. This said, elevated expression levels of miR-181 species (in the context of a 58-component microRNA signature) have been shown to predict the response of gastric carcinoma patients to CDDP/5-FU-based chemotherapy.²⁴² The precise elucidation of the molecular targets of miR-181 may provide important insights this issue. In another clinical study, the upregulation of miR-135b and miR-145 in response to trimodal therapy (concurrent CDDP, irinotecan and irradiation, followed by surgery) has been associated with poor disease-free survival (DFS) among esophageal carcinoma patients,²⁴³ but the molecular mechanisms underpinning these observations have not been further investigated. Interestingly, microRNAomic studies also implicate the EMT in CDDP resistance. In particular, miR-200b and miR-15b (which are downregulated in CDDP-resistant TSCC cells) turned out to actively inhibit the EMT (hence exerting oncosuppressive effects) by targeting the polycomb ring finger oncogenic factor BMI1.²³⁴ In line with this notion, reduced intratumoral levels of miR-200b or miR-15b have been associated with clinical CDDP resistance and poor survival in a cohort of TSCC patients.²³⁴ Of note, the expression of several microRNAs and proteins that contribute to CDDP resistance is under the control of epigenetic mechanisms, including DNA methylation and histone acetylation.^{244–247} This raises the intriguing possibility that pharmacological inhibitors of DNA methyltransferases and histone deacetylases, some of which are currently approved by international regulatory agencies for use in cancer patients, may limit CDDP resistance at least in part by modulating the abundance of specific microRNAs.^{247–252} Robust experimental evidence in support of this hypothesis is lacking.

Various aspects of CDDP resistance have been uncovered by proteomic approaches. By combining quantitative proteomics with interaction network analyses, Chavez *et al.*²⁵³ demonstrated that the levels of no less than 374 proteins are altered in CDDP-resistant (as compared with wild-type, CDDP-sensitive) cervical carcinoma HeLa cells, including various components of energy-producing metabolic pathways as well as proteins involved in DNA repair and other stress

response mechanisms. Zeng *et al.*²⁵⁴ found CDDP-insensitive NSCLC A549 cells to differ from their CDDP-susceptible counterparts in the expression levels of 9 proteins, including parkinson protein 7 (PARK7, best known as DJ-1), a transcription-modulatory and antioxidant factor implicated in the development of familiar Parkinson disease. These findings had an important clinical correlate, as elevated amounts of DJ-1 turned out to predict poor disease outcome in a cohort of 67 locally advanced NSCLC patients.²⁵⁴ Along similar lines, Roesch *et al.*²⁵⁵ demonstrated that melanoma cells exposed for long periods to suboptimal CDDP concentrations not only express increased levels of the histone demethylase Jumonji, AT-rich interactive domain 1 (JARID1) but also contain increased amounts of several enzymes implicated in mitochondrial respiration. Importantly, inhibition of the mitochondrial respiratory chain appears to prevent the accumulation of these slowly proliferating CDDP-insensitive cells, delineating a strategy for the selective elimination of malignant cell clones that initially resist CDDP-based chemotherapy.²⁵⁵ Accordingly, the response of esophageal adenocarcinoma patients to neo-adjuvant CDDP-based chemotherapy correlates with mitochondrial defects provoked by the loss of specific cytochrome c oxidase (COX) subunits.²⁵⁶ On a slightly different note, Vasko *et al.*²⁵⁷ investigated the acute proteomic alterations caused by therapeutic concentrations of CDDP in renal cell carcinoma cells. In this context, CDDP-treated cells were found to express increased levels of multiple cytoskeletal proteins, molecular chaperones of the HSP family and glycolytic enzymes.²⁵⁷ Taken together, these observations suggest that cancer cells acutely exposed to CDDP might temporarily rely on glycolysis as a source of energy (presumably reflecting the mitochondriotoxic effects of cytoplasmic CDDP), whereas the acquisition of stable CDDP resistance might require a robust respiratory activity.^{255–257} At least in part, this is at odds with our recent findings, indicating that CDDP resistance can be mediated by the activation of a PPARGC1B-dependent signaling pathway that is elicited by respiratory chain defects but does not depend on mitochondrial biogenesis.¹⁵⁰ Thus, the precise contribution of glycolysis and mitochondrial respiration to the development of CDDP resistance remains to be clarified.

As an alternative to conventional whole-cell approaches, proteomic studies to elucidate CDDP resistance have been performed on enriched subcellular compartments (e.g., nuclei, mitochondria),^{258–260} purified components known to participate in CDDP-elicited signaling pathways (e.g., immobilized CDDP, Y-box binding protein 1),^{149,261} as well as on serum samples from cancer patients.^{262,263} The latter approach allowed for the identification of α 1-antitrypsin (a circulating protease inhibitor with anti-inflammatory functions) and S100A9 (a Ca²⁺-binding protein that exerts immunomodulatory effects) as possible biomarkers of clinical CDDP resistance.^{262,263} Moreover, CDDP resistance has been associated with immunopeptidomic alterations, that is, changes in the composition and abundance of peptides presented on the cell surface in complex with MHC molecules.²⁶⁰ This is particularly important in view of the facts that (i) several anticancer regimens operate by eliciting or boosting anticancer immunity, some of them by altering the immunopeptidome of malignant cells^{264,265} and that (ii) CDDP

is generally considered as a non-immunogenic anticancer agent.^{24,25} Thus, it is tempting to speculate that CDDP resistance might involve specific changes in the immunopeptidome of neoplastic cells as well as in the tumor microenvironment that prevent the activation of anticancer immune responses. Formal evidence in support of this hypothesis is missing.

Large-scale silencing approaches and functional screenings.

In the mid 2000s, key technological advances have allowed researchers to tackle CDDP resistance with large-scale functional/empirical approaches, as opposed to high-throughput observational studies followed by low-throughput functional validation (such as the genomic, methylomic, transcriptomic and proteomic approaches described above). These developments include (but perhaps are not limited to): (i) the availability of the first genome-wide siRNA sets, at least theoretically permitting the downregulation of each mRNA encoded by the human or murine genome;^{266,267} (ii) the explosion of combinatorial chemistry, allowing for the generation of huge libraries of compounds for investigational purposes^{268,269} and (iii) the rapid diffusion of fully robotized platforms compatible with the sterile handling of 96- and 384-well plates.^{270,271} Thus, along with the establishment of reproducible and cost-efficient workflows, large-scale silencing approaches and ample chemical screenings have been undertaken by several groups worldwide, providing profound insights into the phenomenon of CDDP resistance.^{231,272–277} It should be noted that these functional/empirical approaches are not exempted from validation. Rather, primary hits must be confirmed upon (i) the exclusion of putative off-target effects and the establishment of a robust cause–effect relationship between the downregulation of a specific protein and CDDP resistance (possibly coupled to the elucidation of the underlying mechanisms) or (ii) the precise identification of the molecular target(s) through which a given chemical alters the sensitivity of cancer cells to CDDP (and again the clarification of mechanistic aspects of this phenomenon). Similar to the genomic, transcriptomic, methylomic and proteomic approaches described above, these functional/empirical approaches rely on homotypic experimental settings, mainly cultured cancer cells, *de facto* overlooking the notion that CDDP resistance (at least in some settings) might originate from alterations of the tumor microenvironment or systemic defects (see below). Approximately in the same period, investigators have attempted to elucidate the mechanisms that limit the sensitivity of malignant cells to CDDP by taking advantage of well-established, genetically tractable models that would be compatible with ample knockout strategies, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Dictyostelium discoideum*.^{89,231,278–281} Although these genetic approaches are cost-efficient and straightforward, hits must be subjected to multiple, intense rounds of cross-species functional validation.

We have been working intensively along these lines to obtain clinically relevant insights into CDDP resistance. As a result of a genome-wide siRNA screening approach, we identified the metabolism of vitamin B6 as a central regulator of the response of cancer cells to a wide array of nutritional,

physical and chemical stress conditions, including the administration of CDDP.^{272,282} In particular, we found that the cytotoxic response of a large panel of cancer cells to stress is significantly exacerbated in the presence of a cell permeant B6 vitamers, pyridoxine. Such an effect was only observed when cells expressed pyridoxal kinase (PDXK), the enzyme that generates the bioactive form of vitamin B6 (pyridoxal-5-phosphate). In line with this notion, the downregulation of PDXK conferred a significant degree of protection from stress to cancer cells, while that of pyridoxal phosphatase (PDXP), the enzyme that degrades bioactive vitamin B6, caused a robust increase in the cytotoxicity of several stimuli.²⁷² Importantly, NSCLC patients (from two distinct cohorts, one from Europe and one from North America) bearing lesions that expressed high levels of PDXK had an improved disease-free and overall survival (OS) than individuals affected by tumors with low PDXK levels, irrespective of therapy.²⁷² Of note, the prognostic value of the vitamin B6 metabolism among NSCLC patients was clear when PDXK levels were quantified by immunohistochemistry (IHC) in malignant cells, but neither when the adjacent, non-malignant lung tissue was analyzed (implying that the effect of vitamin B6 on disease progression originates in the tumor) nor when the amount of the PDXK-coding mRNA was assessed in tumor biopsies (perhaps suggesting that PDXK is subjected to a relevant degree of post-transcriptional regulation).²⁷² Although the precise molecular mechanisms whereby vitamin B6 sensitizes malignant cells to adverse conditions remain unclear, we found that the administration of pyridoxine (i) facilitates the establishment of oxidative stress (presumably explaining the influence of vitamin B6 on tumor progression)²⁷² and (ii) promotes the intracellular accumulation of CDDP (at least in part accounting for the ability of vitamin B6 to promote the cytotoxicity of CDDP, *in vitro* and *in vivo*).²⁷³ Both these effects were strictly dependent on the presence of PDXK, implying that they both are mediated by pyridoxal-5-phosphate.^{272,273,282} Of note, the same study led us to identify 84 additional factors that influence the response of NSCLC to CDDP, including lipase, hepatic (LIPC).²⁸³ Similar to those of PDXK, the intratumoral levels of LIPC turned out to positively correlate with disease outcome (independent of several other prognostic factors, including PDXK expression) in two distinct cohorts of NSCLC patients.²⁸³ Moreover, in one of these relatively small cohorts, LIPC expression levels tended to have a predictive (as opposed to merely prognostic) value.²⁸³ Thus, although validation in large patient cohorts is still pending, LIPC expression levels may constitute a biomarker that allows for the identification of patients who obtain a true clinical benefit from the administration of platinum-based chemotherapy. This said, the molecular mechanisms through which LIPC influences tumor progression (and perhaps response to chemotherapy) remain obscure.

A few other groups have undertaken global silencing approaches to discover novel factors involved in CDDP resistance. For instance, Ho *et al.*²⁷⁷ found that the GTPase RAS homolog family member J (RHOJ) and p21 protein (CDC42/RAC)-activated kinase 1 (PAK1) render melanoma cells resistant to DNA-damaging agents (including dacarbazine and CDDP) by uncoupling ATR from its downstream effectors, resulting in the expression of the survival gene SRY

(sex determining region Y)-box 10 (*SOX10*). By focusing this strategy on lipid and protein kinases, Guerreiro *et al.*²⁸⁴ identified a set of six factors involved in the decreased sensitivity of medulloblastoma cells to CDDP, including ATR, membrane protein, palmitoylated 2 (MPP2), phosphatidylinositol 4-kinase, catalytic, α (PI4KA), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit γ (PIK3CG), STE20-related kinase adaptor α (STRADA, a pseudokinase also known as LYK5) and WNK lysine-deficient protein kinase 4 (WNK4). A kinome-wide siRNA screen allowed Salm *et al.*²⁷⁶ to identify fibroblast growth factor receptor 2 (FGFR2) as an important determinant of CDDP resistance in neuroblastoma. FGFR2 turned out to decrease the sensitivity of neuroblastoma cells to CDDP via an autocrine loop promoting the expression of BCL-2 and BCL-X_L. Moreover, the expression levels of FGFR2 were shown to have a clinical relevance, as they correlated with the amplification of *v-myc* avian myelocytomatosis viral oncogene neuroblastoma-derived homolog (*MYCN*) and advanced disease stage.²⁷⁶ Of note, silencing approaches can also be implemented by means of microRNA mimics. By screening a panel of these reagents, Pouliot *et al.*²⁸⁵ demonstrated that several microRNAs of the miR-15 family, including miR-15a, miR-15b, miR-16, miR-16-1* and miR-424*, alter the sensitivity of epidermoid carcinoma cells to CDDP as they regulate the expression of cell cycle-associated kinases.

Large chemical libraries have been screened for the presence of compounds that may increase the susceptibility of malignant cells to the cytostatic/cytotoxic effects of CDDP. Using this approach, Chen *et al.*²⁷⁵ identified pimozone and GW7647, two distinct inhibitors of the deubiquitinase complex assembled by ubiquitin specific peptidase 1 (USP1) and WD repeat domain 48 (WDR48, also known as UAF1), as putative chemosensitizers in NSCLC cells. As the USP1/UAF1 complex is involved in TLS, pimozone and GW7647 may operate by limiting the ability of cancer cells to bypass CDDP-induced DNA lesions. By screening a validated library of kinase inhibitors, Wong *et al.*²⁸⁶ demonstrated that the inhibition of several signal transducers including AKT1, mTOR, NF- κ B, transforming growth factor β receptor 1 (TGFB β 1), phosphoinositide-3-kinase (PI3K) and various MAPKs sensitizes basal-like breast carcinoma cells to the toxic effects of CDDP. Specifically, the mTOR inhibitor rapamycin was shown to synergize with CDDP in the killing of basal-like MDA-MB-468, MDA-MB-231 and HCC1937 cells, but not luminal-like T47D or MCF-7 cells, owing to the induction of the p53-related protein p73.²⁸⁶ Jacquemont *et al.*²⁷⁴ tested > 16 000 compounds for their ability to inhibit the formation of DNA damage foci triggered by irradiation or CDDP, identifying a dozen molecules that sensitize ovarian cancer cells to the cytotoxic effects of the latter. These molecules include several inhibitors of the Fanconi anemia (FA) DNA repair pathway (which coordinates HR and TLS for the repair of interstrand crosslinks) and indeed were confirmed to exacerbate the cytostatic/cytotoxic potential of CDDP in FA-proficient but not in FA-deficient ovarian cancer cells. Finally, Garnett *et al.*²⁸⁷ recently undertook a large screen to identify genes associated with the cellular responses to most anticancer agents available to date. To this aim, 130 drugs currently approved by regulatory agencies for use in cancer patients or under clinical development were

tested on several hundred cancer cell lines, representing much of the tissue-type and genetic diversity of human tumors. This systematic screening approach generated several unexpected findings, including the extremely high sensitivity to both PARP inhibitors and CDDP of Ewing's sarcoma cells harboring a translocation that involves EWS RNA-binding protein 1 (*EWSR1*) and Fli-1 proto-oncogene, ETS transcription factor (*FLI1*).²⁸⁷

Functional screenings recently performed in genetically tractable organisms have also unveiled various aspects of CDDP resistance. For instance, by screening the clonogenic potential of a collection of *S. cerevisiae* mutants exposed to an overtly toxic amount of CDDP, we demonstrated that the molecular cascades elicited by CDDP, CdCl₂ and C₂-ceramide in yeast exhibit very limited overlaps.²³¹ Indeed, none of the *S. cerevisiae* mutants tested was protected from the cytostatic/cytotoxic potential of all these compounds. Of note, $\Delta por2$ cells, lacking one isoform of the yeast ortholog of human VDAC1, turned out to be specifically protected against CDDP cytotoxicity, indicating that the molecular mechanisms that underlie CDDP resistance are phylogenetically conserved.²³¹ Upon the development of a high-resolution high-throughput screening (HTS) platform, Sletta *et al.*²⁸¹ tested the response of >1400 *S. cerevisiae* deletion mutants to three distinct DNA-damaging agents, namely, methyl methanesulphonate, 5-FU and CDDP, identifying MAPK7 (also known as ERK5) as an evolutionarily ancient regulator of the DNA damage response that also operates in human cells. Using 2359 non-essential haploid deletion mutants of the fission yeast *S. pombe*, Gatti *et al.*²⁸⁰ identified no less than 13 distinct gene products involved in the ubiquitin-proteasome system whose absence increases (3 proteins) or decreases (10 proteins) CDDP sensitivity. Although these authors did not validate their findings in mammalian systems, we and others confirmed the implication of the proteasome in the response of human cells to CDDP.^{220,272}

Multiplex genotyping studies. During the last decade, dozens of studies have been carried out to determine whether SNPs affecting one or more genetic loci would influence the natural course of multiple neoplasms (leading to the identification of prognostic biomarkers) or the propensity of neoplastic lesions to respond to specific therapeutic regimens, including CDDP-based chemotherapy (resulting in the characterization of predictive biomarkers).^{288–291} This approach has obvious clinical implications because it may lead to the identification of patient subgroups that will benefit from treatment, and hence allow for the implementation of personalized medicine.²⁹² Moreover, the identification of genetic backgrounds that influence the antineoplastic activity of CDDP would have one major advantage as compared with most strategies for the elucidation of resistance discussed above: it would intrinsically consider all possible cancer cell-extrinsic factors that may impact on this phenomenon, irrespective of whether they have previously been described or not. However, until recently, genotyping studies could only focus on SNPs affecting one (or a few) protein(s) at the same time, owing to technical limitations. Thus, near-to-invariably, these approaches would study SNPs known or expected to

influence cellular or organismal processes linked to CDDP sensitivity, such as CDDP extrusion (e.g., SNPs affecting ABC2), DNA damage-elicited signal transduction (e.g., SNPs affecting ERCC1), apoptosis (e.g., SNPs affecting caspases) or immune responses (e.g., SNPs affecting cytokines or their receptors).^{293–298} This limitation has nowadays been circumvented by the introduction of technical platforms that allow for the simultaneous determination of up to 384 different SNPs in one single assay.^{299–301}

A few genotyping studies of this type have already been performed to obtain insights into the organismal aspects of CDDP resistance. Tan *et al.*³⁰² reported that two SNPs affecting the expression levels of death-associated protein kinase 3 (DAPK3) and methyltransferase like 6 (METTL6) influenced disease outcome in 222 small cell lung carcinoma and 961 NSCLC patients treated with platinum-based therapy. Accordingly, the knockdown of DAPK3 and METTL6 was shown to significantly decrease CDDP sensitivity in lung carcinoma cells.³⁰² By adopting a similar approach, Wheeler *et al.*³⁰³ tested the association between 2 million SNPs and the response of lymphoblastoid cell lines (LCLs) derived from 83 individuals of African American descent to cytarabine, 5'-deoxyfluorouridine, carboplatin and CDDP. This study led to the identification of 41 genes that putatively influence the response of African Americans to CDDP-based chemotherapy. Notably, the set of SNPs associated with the sensitivity of LCLs to CDDP exhibited some degree of overlap with that linked to carboplatin susceptibility, reinforcing the notion that platinum derivatives activate similar, but not identical, signal transduction cascades.³⁰³ More recently, the same authors expanded their analysis to a total of 608 LCLs and >3 million SNPs, reporting a robust association between CDDP susceptibility and a SNP upstream of keratin 16 pseudogene 2 (*KRT16P2*) as well as a link between the sensitivity of LCLs to CDDP and SNPs affecting *BCL2*, *ERCC2*, *ERCC6*, glutathione S-transferase $\mu 1$ (*GSTM1*) and glutathione S-transferase $\theta 1$ (*GSTT1*).³⁰⁴ Li *et al.*³⁰⁵ analyzed the association between 894 SNPs affecting 70 genes involved in glutathione metabolism, DNA repair, cell cycle and epidermal growth factor receptor (EGFR) signaling and disease outcome among 1076 NSCLC patients treated with various platinum-based chemotherapeutic regimens. Globally, SNPs affecting glutathione synthetase (*GSS*) and mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*) were linked to poor OS. Moreover, SNPs affecting *MAP3K1* turned out to negatively influence the OS of NSCLC patients treated with platinum derivatives plus a taxane, whereas SNPs in the *v-raf-1* murine leukemia viral oncogene homolog 1 (*RAF1*) had an opposite effect on the OS of this patient subgroup. Along similar lines, SNPs involving *RAF1* (as well as glutathione peroxidase 5, *GPX5*) were associated with improved OS among patients receiving platinum derivatives plus gemcitabine. Conversely, SNPs affecting *GPX7* and neuroblastoma RAS viral (*v-ras*) oncogene homolog (*NRAS*) had a negative influence on OS in these individuals.³⁰⁵ Wu *et al.*³⁰⁶ studied the influence of 307 260 distinct SNPs on the survival of three distinct cohorts of (327, 315 and 420) advanced-stage NSCLC patients who received platinum-based chemotherapy with or without irradiation, identifying a relatively reproducible (though not absolute) association between SNPs in chemokine-like

receptor 1 (*CMKLR1*) and sortilin-related VPS10 domain containing receptor 2 (*SORCS2*) and poor OS. Along similar lines, Hu *et al.*³⁰⁷ performed a fast-track genotyping study on three distinct cohorts of (307, 228 and 340) NSCLC patients receiving first-line platinum-based chemotherapy, finding an association between three SNPs (rs7629386 at 3p22.1, rs969088 at 5p14.1 and rs3850370 at 14q24.3) and poor OS as well as a link between two SNPs (rs41997 at 7q31.31 and rs12000445 at 9p21.3) and improved OS. These studies and others of a similar type to come may provide significant insights into the organismal aspect of CDDP resistance.

Concluding Remarks

Throughout the last 4 decades, several millions of cancer patients have been treated with CDDP-based chemo (radio)therapeutic regimens. Exception made for individuals bearing testicular germ cell tumors, most of these patients only obtained temporary clinical benefits, which were destined to vanish along with the progressive transition of malignant cells from a chemosensitive to a chemoresistant state. Thus, even though CDDP provokes moderate-to-severe adverse effects in a fraction of patients, the most prominent hurdle related to the use of CDDP in the clinical practice is posed by the elevated incidence of chemoresistance, be it innate or acquired. An intense wave of preclinical and clinical investigation aimed at developing strategies to restore the sensitivity of human neoplasms to the cytostatic/cytotoxic effects of CDDP has been initiated worldwide in the late 1970s. Reflecting the huge clinical and socioeconomic impact of CDDP resistance, such an investigational effort has not yet come to an end. Indeed, although several strategies have been devised to circumvent CDDP resistance in cancer patients, including (but not limited to) the use of CDDP in combination with targeted anticancer agents such as erlotinib (a pharmacological inhibitor of the tyrosine kinase activity of EGFR), trastuzumab (an EGFR-specific antibody) and bevacizumab (a vascular endothelial growth factor-blocking antibody), a majority of these combinatorial regimens failed to improve the therapeutic profile of CDDP in randomized clinical trials.^{308–310}

Past. Before the advent of high-content, high-throughput technologies, the elucidation of the molecular mechanisms underpinning the cytostatic/cytotoxic effects of CDDP has long been under the limelight. It was indeed tempting to accept the relatively straightforward assumptions that (i) CDDP would exert antineoplastic effects by binding one (or a few) molecular target(s), hence activating relatively simple signaling pathways, and (ii) that CDDP resistance would originate from the activation of mutually exclusive mechanisms. Starting with the early 1990s, the attention of molecular oncologists focused on the cellular factors that are responsible for the detection and repair of CDDP-elicited DNA lesions, and on the mechanisms whereby these systems transduce a lethal signal when damage is irreparable (on-target and post-target resistance). The increasingly more precise characterization of plasma membrane transporters that actively extrude various xenobiotics, including several members of the ATP-binding cassette (ABC) protein family,³¹¹ led to the hypothesis that CDDP resistance would

mainly involve pre-target mechanisms. Such a hypothesis, however, was soon disconfirmed by findings from many groups indicating that the inhibition of ABC transporters does not suffice to reinstate CDDP sensitivity. In the early 2000s, the role of copper transporters in the uptake and extrusion of CDDP became clear, yet the experimental modulation of these systems also failed to completely abolish the insensitivity of some cancer cells to CDDP. Thus, the redundant and multifactorial nature of CDDP resistance has been established even before the advent of high-content, high-throughput technologies.

Present. The automation of functional screenings and the introduction of high-content experimental platforms have literally revolutionized the study of several biological phenomena, including CDDP resistance. The first high-content, high-throughput technologies (such as mRNA and CGH microarrays and mass spectrometry) have been available for more than a decade, but the true explosion of this area has occurred in the late 2000s, along with the commercialization of platforms such as automated fluorescence microscopes and the introduction of innovative protocols like deep sequencing. Several attempts have recently been made to harness these technologies for empirically resolving the clinical hurdle posed by CDDP resistance, with very promising results. In addition, increasingly more detailed insights into the complexity of the signal transduction pathways triggered by CDDP in cancer and normal cells have been obtained. Nonetheless, no chemosensitization strategies have yet been introduced into the clinical practice (though a few of them are currently being investigated in clinical settings).

Future. Accumulating evidence indicates that, contrarily to long-standing beliefs, the limited sensitivity of human neoplasms to CDDP may reflect not only cell-intrinsic but also cell-extrinsic alterations.^{24,25,312,313} On one hand, CDDP appears to be much less effective than other chemotherapeutics, including oxaliplatin (in spite of their chemical resemblance), at inducing the immunogenic demise of cancer cells.^{314,315} On the other hand, it has recently been shown that CDDP resistance is significantly influenced by non-transformed mesenchymal stem cells and other components of the tumor stroma, including proteins of the extracellular matrix and immunosuppressive cell populations.^{312,313,316} Until now, such a cell-extrinsic dimension of CDDP resistance was largely underestimated. We surmise that the application of high-content, high-throughput technologies to heterotypic experimental settings that involve not only malignant cells but also various other components of the tumor microenvironment (including endothelial, mesenchymal and immune cells) is the key for the development of efficient strategies to restore the sensitivity of multiple human neoplasms to CDDP. Along similar lines, we believe that the untargeted metabolomic profiling of CDDP-sensitive *versus* CDDP-resistant tumor models, an approach that only recently has begun to be applied to the study of chemoresistance,^{313,317,318} may yield important insights into this considerable clinical impediment.

Conflict of Interest

The authors declare no conflict of interest.

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