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MINI REVIEW

Mitochondria as a critical target of the chemotheraputic agent cisplatin in head and neck cancer

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Abstract Cisplatin is among the most important chemotherapeutic agents ever developed. It is a critical component of therapeutic regimens in a broad range of malignancies. However, more than a generation after its clinical introduction, the exact mechanism of cisplatin action on tumor cells is not fully defined. The preponderance of research over the last three decades has focused on cisplatin interactions with nuclear DNA which are felt to lead to apoptotic cell death in sensitive cells.

However, recent data have shown that cisplatin may have important direct interactions with mitochondria which can induce apoptosis and may account for a significant portion of the clinical activity associated with this drug. These direct interactions between cisplatin and mitochondria may have critical implications for our understanding of this class of drugs and the development of new therapeutic agents.

Keywords cisplatin · mitochondria · head and neck cancer · DNA · voltage dependent anion channel

Introduction

Cisplatin (cis-diamminedichloroplatinum II) has been in widespread clinical use for more than a generation and is one of the most important chemotherapeutic agents ever introduced. Despite the importance of cisplatin in the treatment of head and neck cancer and a broad range of other malignancies, there are many uncertainties about its molecular pharmacology and ultimate mechanism of action. This has been an area of very active investigation for more than two decades.

Upon entering the low chloride intracellular environment, cisplatin is hydrated to form a positively charged species which can react with nuclear DNA and other nucleophilic species within the cell (Andrews and Howell, 1990).

Cisplatin has been most extensively characterized as a DNA damaging agent and the cytotoxicity of cisplatin has generally been attributed to the ability to form inter- and intra-strand nuclear DNA crosslinks. Formation and repair of these cisplatin/nuclear DNA adducts have been widely studied for the last two decades. For some time after the introduction of cisplatin, its cytotoxicity was felt to result from inhibition of DNA synthesis by cisplatin/DNA adducts, but several lines of evidence demonstrated that this was not the case (Sorenson and Eastman, 1988).More recently, it was demonstrated that tumor cell exposure to cisplatin ultimately results in apoptosis (Eastman, 1990; Eastman and Barry, 1992). However, the mechanism or mechanisms by which nuclear cisplatin/DNA adducts generate the cytoplasmic cascade of events leading to apoptosis have not been defined.

While most investigations of the cellular and molecular pharmacology of cisplatin have focused on interactions between cisplatin and nuclear DNA, only approximately 1% of intracellular platinum is bound to nuclear DNA, with the great majority of the intracellular drug available to interact with nucleophilic sites on other molecules, including but not limited to phospholipids, cytosolic, cytoskeletal and membrane proteins, RNA, and mitochondrial DNA (Fuertes et al., 2003; Gonzalez et al., 2001).

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Cisplatin based chemotherapy in the management of head and neck cancer

Squamous carcinoma of the head and neck is a major public health problem world wide. In the US in 2006, these cancers will afflict more than 40,000 individuals, leading to approximately 15,000 deaths. Until recently, chemotherapy has had relatively little role in the curative therapy of head and neck cancers. Methotrexate has been used in head and neck cancer for over 30 years but has primarily been reserved for palliative treatment of relapsed patients. However, since roughly 20,000 patients per year will present with recurrent or metastatic disease, which is generally treatable only with chemotherapy, this is a large and important subset of patients (Jacobs et al., 1992). Response rates for methotrexate and most other single agents are in the 30% range (Wheeler, 1990). In the 1970's, cisplatin was introduced into the chemotherapeutic armamentarium. While the efficacy of cisplatin as a single agent in head and neck cancer was initially found to be only modestly better than other single chemotherapy drugs, it was soon appreciated that chemotherapy combinations based on cisplatin were highly active in head and neck cancers. Overall responses of 70–90% with complete responses of 20–30% have been reported when cisplatin is combined with 5-fluorouracil (Kish et al., 1984) or other agents (Hong et al., 1979). These high response rates led to tremendous enthusiasm that cisplatin-based combination chemotherapy would be highly effective as an adjuvant treatment in head and neck cancer, but unfortunately several well-organized clinical trials proved that this was not the case (Head and Neck Contacts Program, 1987; Jacobs, 1989; Jacobs and Makuch, 1990).

Despite the lack of efficacy for cisplatin-based combination chemotherapy in an adjuvant setting, a considerable body of data has emerged in the past decade validating the use of this treatment in the *neoadjuvant* setting, primarily for organ preservation (Department of Veterans Affairs Laryngeal Cancer Study Group, 1991; Jacobs et al., 1987; Hong et al., 1993). In this approach, chemotherapy is given as the initial treatment in selected patients as an alternative to surgery. Patients who have a good response to chemotherapy can then be radiated to complete their treatment.

Patients with advanced laryngeal cancer treated with up front cisplatin and 5-FU chemotherapy followed by definitive radiation, have survival equivalent to standard surgery and radiation therapy, but 2/3 of these patients preserve laryngeal function (Hong et al., 1979). While the use of *neoadjuvant* chemotherapy for organ preservation has been most clearly documented for larynx, similar data from smaller studies are beginning to emerge for other anatomic sites including tongue and pharynx (Athanasiadis and Vokes, 1995). More recently, concomitant cisplatin/radiation therapy has been demonstrated to be highly effective in a variety of head and neck cancers. This strategy has been tested with positive results in a number of clinical trials (Tan et al., 1997; Vokes, 1997). Recently, two high profile American and European intergroup studies confirmed that concomitant chemoradiotherapy with cisplatin enhance organ preservation and survival compared with radiotherapy alone in the postoperative adjuvant setting (Cooper et al., 2004; Bernier et al., 2004). Concomitant chemoradiotherapy utilizing cisplatin has thus emerged as the standard for locally advanced unresectable tumors and for postoperative adjuvant therapy.

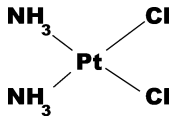

Fig. 1 Cisplatin (cis-diamminedichloroplatinum II). Introduced more than 30 years ago, cisplatin remains one of the most widely used chemotherapy drugs in the world. It is active in a wide range of malignancies including lung, ovarian, head and neck as well as testicular and germ cell cancers

Molecular mechanisms of cisplatin action in head and neck cancer

Despite the importance of cisplatin in the treatment of head and neck cancer and a broad range of other malignancies, there are many uncertainties about its molecular pharmacology and ultimate mechanism of action. Cisplatin (*cis*-diamminedichloroplatinum (II)) is a relatively simple compound consisting of a platinum atom complexed by two ammine groups and two chloride ions (Fig. 1; Chabner and Myers, 1985).

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between cisplatin and nuclear DNA, only approximately 1% of intracellular platinum is bound to nuclear DNA, with the great majority of the intracellular drug available to interact with nucleophilic sites on other molecules, including but not limited to phospholipids, cytosolic, cytoskeletal and membrane proteins, RNA, and mitochondrial DNA (Fuertes et al., 2003; Gonzalez et al., 2001).

Further evidence that nuclear DNA adduct formation may not be the sole determinant of cisplatin-induced cytotoxicity comes from recent clinical studies demonstrating that combined therapy with other agents such as taxanes significantly enhances the clinical efficacy of cisplatin, while actually inhibiting formation of cisplatin adducts with nuclear DNA (Crul et al., 2002).

Resistance to cisplatin can result from a number of mechanisms, including decreased uptake, inactivation by nucleophilic compounds such as glutathione, or accelerated DNA repair (Kartalou and Essigmann, 2001).Inhibiting glutathione synthesis with buthionine sulfoximine (BSO) has been known for some time to enhance cisplatin cytotoxicity in tumor cells as well as increasing normal cell toxicity (Bier, 1991; Andrews et al., 1988).

We previously demonstrated that BSO treatment of head and neck tumor cell lines was accompanied by complete loss of detectable glutathione and marked increase in cisplatin cytotoxicity. However, this markedly enhanced apoptotic cell killing was not accompanied by significant changes in cisplatin DNA adduct formation (Yang et al., 2000). This was a surprising finding, as glutathione was felt to inhibit cisplatin cytotoxicity by preventing formation of cisplatin/DNA adducts. We now believe that in the case of cisplatin, glutathione serves to protect the cell from reactive oxygen species rather than inhibiting formation of platinum/DNA adducts.

Cisplatin and mitochondria

Overexpression of Bcl-2 is associated with cisplatin resistance in several model systems (Isonishi et al., 2001). Recently, we demonstrated that while Bcl-2 transfection was associated with significant acquired cisplatin resistance, it did not produce measurable alterations in nuclear cisplatin/DNA adducts (Rudin et al., 2003). Because of the lack of clarity on the role of nuclear cisplatin/DNA adducts in mediating cytotoxicity, and because of the Bcl-2 data suggesting the importance of mitochondrial pathways in cisplatin action, we elected to look more carefully at cisplatin interactions with mitochondria and mitochondrial DNA.

Limited studies have examined cisplatin activity in cells selectively depleted of mitochondrial DNA, with conflicting results. Loss of mitochondrial DNA has been associated with increased sensitivity to cisplatin-induced apoptosis (Liang and Ullyatt, 1998),but more recent literature has shown that cells depleted of mitochondrial DNA show significant resistance to cell death mediated by a range of chemotherapeutic agents (Park et al., 2004). Indeed, mitochondrial DNA is significantly more sensitive than nuclear DNA to the damage induced by a range of agents. This is in part because mitochondrial DNA lacks nucleotide excision repair, the principal mechanism which mitigates nuclear DNA damage by cisplatin (Preston et al., 2001).

Mitochondrial damage by cisplatin has increasingly been studied as a mediator of toxicity in normal tissues in animals receiving cisplatin. Gastrointestinal toxicity, (Yanez et al., 2003) ototoxicity (Devarajan et al., 2002) and nephrotoxicity (Park et al., 2002; Schwerdt et al., 2003) have all been attributed to mitochondrial damaging effects of cisplatin.

While there has been comparatively little study of direct cisplatin action on mitochondria, some studies have indicated that mitochondrial DNA-cisplatin adducts may be significantly more common than cisplatin adducts with nuclear DNA in the same cell line treated with the same concentration of cisplatin (Murata et al., 1990; Olivero et al., 1995). This has been attributed to a lack of mitochondrial DNA repair following cisplatin exposure (Singh and Maniccia-Bozzo, 1990).

Mitochondria, as the sites of aerobic respiration, are the principal generators of reactive oxygen species in the cell. Mitochondria are dependent upon glutathione to detoxify reactive oxygen species, preventing oxidative damage (Davis et al., 2001; Anderson, 1998). Despite this dependence, mitochondria are unable to synthesize glutathione. Glutathione stores within mitochondria are derived from active transport across the mitochondrial membrane against an electrochemical gradient (Griffith and Meister, 1985). Mitochondrial glutathione concentrations are regulated and have been implicated in apoptotic regulation (Martensson and Meister, 1989; Martensson et al., 1991). At baseline, the concentration of glutathione in mitochondria has been found to be similar to that of the cytoplasm. However, in cells exposed to oxidative stress, the concentration of mitochondrial glutathione is maintained at the expense of a decreasing cytoplasmic pool (Fernandez-Checa et al., 1997; Colell et al., 1997). Depletion of the mitochondrial (but not cytoplasmic) glutathione pool is associated with markedly increased sensitivity to antimycin A, which blocks oxidative phosphorylation in complex III of the electron transport chain, leading to generation of reactive oxygen species (Garcia-Ruiz et al., 1995). These observations suggest that mitochondrial glutathione stores are highly regulated by the cell and may affect the cellular sensitivity to apoptotic stimuli.

Mitochondrial cytochrome *c* and apoptosis

Mitochondrial cytochrome *c* is a positively charged, soluble protein present in the mitochondrial intermembrane space.

In normal physiology, cytochrome *c* functions in the respiratory chain by interacting with redox partners of complex III and complex IV (Hatefi, 1985; Mathews, 1985). In response to a variety of apoptosis-inducing agents, including cisplatin, cytochrome *c* is released from mitochondria to the cytosol (Yang et al., 1997; Kluck et al., 1997). A number of investigators have examined mechanisms by which cytochrome *c* can be released from mitochondria in response to apoptotic stimuli arising elsewhere in the cell. Several studies have indicated that in at least some circumstances, the translocation of the pro-apoptotic protein Bax from the cytoplasm to the mitochondria is a critical step in overcoming mitochondrial stabilization by Bcl-2 (Hsu et al., 1997; Wolter et al., 1997). Bax and Bak interact with mitochondrial permeability transition (PT) pores. That interaction leads to a conformational change in a voltage-dependent anion channel, releasing the positively charged cytochrome *c* from the mitochondrion into the cytoplasm (Narita et al., 1998; Bossy-Wetzel et al., 1998; Shimizu et al., 1999).

Once in the cytoplasm, cytochrome *c* becomes part of a complex composed of cytochrome *c* itself (Apaf-2, 15 kDa), Apoptosis protease activating factor (Apaf-1, 130 kDa) and caspase-9 (Apaf-3, 46 kDa). Within this complex, caspase-9 is activated, leading to the downstream activation of other caspases, including caspase-3 and caspase-7 (Li et al., 1997; Zou et al., 1997). Activated caspase-3 ultimately leads to cell death (Nicholson et al., 1995; Tewari et al., 1995; Takahashi et al., 1996; Song et al., 1996).

Although this understanding of the role of cytochrome *c* release from mitochondria as an intermediate step in apoptosis is the result of many elegant studies, this body of work has presumed that drug induced apoptosis results from (in the case of cisplatin) a nuclear event which ultimately leads to the translocation of Bax to the mitochondria. The full nature of intermediate signaling resulting from the formation of adducts between cisplatin and nuclear DNA is not known.

> The possibility that a positively charged drug like cisplatin could interact with and damage the mitochondria directly, resulting in cytochrome *c* release and triggering apoptosis, has not been studied.

Recent studies have supported the notion that mitochondria may be a direct and important target of cisplatin and its analogues. Analysis of cisplatin sensitivity in an animal model and in a series of 8 cell lines shows that cisplatin sensitivity is positively correlated with mitochondrial density. That is, for both normal and tumor cell lines, increased mitochondrial content was associated with increased cisplatin sensitivity. This group also reported that ρ^0 cells are cisplatin resistant as we show in our preliminary data (Qian et al., 2005). Other investigators examining a leukemia model, showed that c-FLIP overexpression did not alter cisplatin sensitivity, but did result in cells which were less sensitive to TRAIL-induced apoptosis. This implies that cisplatin induced apoptosis is independent of the death receptor pathway (Wang et al., 2006). Another group of investigators recently reported that although cisplatin, carboplatin and oxaliplatin show similar tumoricidal effect *in vitro*, this cannot be explained by similar formation of DNA adducts. At equivalent inhibitory doses, cisplatin adducts to nuclear (mitochondrial not measured) DNA were 10 times more numerous than oxaliplatin adducts (Goodisman et al., 2006). The authors imply that oxaliplatin DNA lesions are more potent than cisplatin DNA lesions, but an alternative explanation would be that the nuclear DNA adducts are not the primary source of cellular toxicity.

The voltage dependent anion channel (VDAC)

The voltage-dependent anion channel (VDAC, also known as porin) is the most abundant mitochondrial membrane protein (Krimmer et al., 2001). Three isoforms have been described, (Decker and Craigen, 2000) and RT PCR analysis in our lab demonstrates all three isoforms are expressed in human head and neck tumor cells (data not shown). The release of cytochrome *c* from the mitochondria is mediated by the mitochondrial permeability transition pore, which is a protein complex composed of the VDAC, members of the pro- and anti-apoptotic Bax/Bcl-2 family, cyclophilin D and possibly adenine nucleotide translocators (Asakura and Ohkawa, 2004; Kokoszka et al., 2004). The pore size of the VDAC is increased by Bax and tBid, which may lead to rupture of the outer mitochondrial membrane and release of cytochrome *c* (Banerjee and Ghosh, 2004). However, recent studies have questioned whether Bax interacts with VDAC directly (Rostovtseva et al., 2004). In contrast, Bcl-2 and cyclophilin D inhibit release of cytochrome *c* through the permeability transition pore, and so act to prevent apoptosis (Schubert and Grimm, 2004; Tsujimoto, 2002).

Since cisplatin forms adducts with proteins as well as DNA, we hypothesize that interactions with specific mitochondrial membrane proteins such as VDAC may significantly alter the mitochondrial permeability transition pore, leading to release of cytochrome *c*. Two reactive, sulfur-containing amino acids have been identified as sites of platinum adduct formation on specific proteins: cysteine (hemoglobin, Mandal et al., 2003) and methionine (albumin, Ivanov et al., 1998; cytochrome *c*, Lijuan et al., 1997; Boswell et al., 1982; and ubiquitin, Gibson and Costello, 1999). VDAC contains two cysteines and two methionines, thus four potential targets for platinum binding. Our preliminary data are consistent with this hypothesis. Three VDAC isoforms have been described (VDAC1-3) and all appear to be expressed in head and neck cell lines.

Recent studies

Our laboratory recently undertook a series of experiments to further define the role of mitochondrial interactions in cisplatin induced apoptosis. (Yang et al., 2006). We examined comparative levels of cisplatin binding to nuclear DNA versus mitochondrial DNA in a series of head and neck cancer cell lines.

Regardless of duration of cisplatin treatment (one or two hours), pretreatment with BSO to deplete glutathione, or removal of drug for 24 hours to allow DNA repair, the level of cisplatin adducts in mitochondrial DNA was consistently at least two orders of magnitude greater than the level in nuclear DNA.

If the presence of nuclear DNA is essential for cisplatin induced cytotoxicity, cells without nuclear DNA should become resistant to cisplatin. We examined head and neck tumor cell cytoplasts in which the cell nucleus has been removed to established centrifugation techniques. Despite the absence of a nucleus, the cell cytoplasts retained dose dependent cisplatin sensitivity, as determined by caspase-3 activation. While the cytoplasts show a somewhat elevated basal level of caspase-3 activation, they maintained a statistically significant response to escalating doses of cisplatin when compared to the parental cell line.

We then studied the effect of cisplatin on isolated mitochondria. If mitochondria are a direct target for cisplatin action, we would expect to see cytochrome *c* release from mitochondria treated directly with cisplatin. Mitochondria isolated from either rat liver or human head and neck cancer cell lines treated with irinotecan showed rapid and dose-dependent release of cytochrome *c* following as little as five minutes of exposure to cisplatin at concentrations seen in serum following standard clinical dosing. Control drugs including the inactive isomer transplatin and the topoisomerase inhibitor irinotecan did not lead to release of cytochrome *c*. Cells transfected with the anti-apoptopic protein Bcl-2 were significantly resistant to cytochrome *c* release after cisplatin treatment compared with wild type controls.

Conversely, cells depleted of mitochondrial DNA through chronic culture in ethidium bromide become significantly resistant to cisplatin, but not to drugs of other classes.

Atomic absorption spectroscopic analysis of cellular fractions isolated from cisplatin treated HNSCC showed that the cisplatin concentration in the mitochondrial protein fraction was 10 fold higher than in the whole cell protein fraction. Interestingly, the amount of cisplatin bound to VDAC (purified chromatographically from mitochondrial protein) was 24 times higher than in the mitochondrial protein fraction and more than 200 fold higher than in the whole cell protein fraction (Table 1).

Table 1 Cisplatin binding to whole cell protein, mitochondrial protein and voltage dependent anion channel protein isolated from cisplatin treated HNSCC

Fraction	fmol CDDP/μg protein
Whole cell lysate (included DNA)	256
Whole cell protein[a]	7
Mitochondrial protein[a]	65
VDAC[a]	1633

[a] DNAse/RNAse treated.

Conclusions

While there has been comparatively little study of direct cisplatin action on mitochondria, some studies have indicated that mitochondrial DNA-cisplatin adducts may be significantly more common than cisplatin adducts with nuclear DNA in the same cell line treated with the same concentration of cisplatin (Murata et al., 1990; Olivero et al., 1995). This has been attributed to a lack of mitochondrial DNA repair following cisplatin exposure (Singh and Manicia-Bozzo, 1990).

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Our findings support and expand recent literature reports which have hinted that cisplatininduced cytotoxicity may be independent of nuclear DNA binding. Mandic and colleagues recently demonstrated that enucleated cells retain dose-dependent cisplatin induction of caspase-3 activation

in colon and melanoma cell lines. Interestingly, in that study, while the enucleated cells retained sensitivity to cisplatin, they became resistant to the DNA-damaging topoisomerase-II inhibitor etoposide (Fuertes et al., 2003). Similar results have been reported in colon cancer cell cytoplasts treated with oxaliplatin (Gourdier et al., 2004). Other agents may also exert proapoptotic effects through direct interactions with mitochondria (Kluza et al., 2006).

While we demonstrate that head and neck tumor cells lacking mitochondrial DNA become cisplatin resistant, they are not completely cisplatin insensitive. A previous study with ρ^0 osteosarcoma cells demonstrated that the loss of mitochondrial DNA does not result in a complete absence of cytochrome *c* release from tumor mitochondria when damaged by the toxin staurosporine (Jiang et al., 1999). This result combined with our own suggests that cisplatininduced mitochondrial toxicity is not entirely DNA dependent. Although we demonstrate that mitochondrial platinum adducts are measured at a concentration at least two orders of magnitude greater than is measured in the nucleus, the observations that cytochrome *c* release from isolated mitochondria can be seen within five minutes of cisplatin exposure and that, in whole cells, ultrastructural damage of mitochondria is clearly visible within a few hours of cisplatin treatment both argue that a direct impact on mitochondrial gene transcription is not necessary for cisplatin's mitochondrial toxicity. Importantly, we demonstrate that cytochrome *c* release is seen at cisplatin concentrations attainable following bolus injection of the drug clinically.

Corresponding to our observation that Bcl-2 transfection is associated with resistance to cisplatin induced release of cytochrome *c* from the mitochondria, a recent study demonstrated that Bcl-2 overexpression was associated with diminished disruption of mitochondrial ultrastructure following cisplatin treatment as determined by electron microscopy (de Graaf et al., 2004).

Although mitochondria lack the capability for nucleotide excision repair, the marked elevations in mitochondrial versus nuclear DNA platinum adducts are not explained by differences in repair alone. Adduct levels in both mitochondrial and nuclear DNA decreased at similar rates after cisplatin exposure (data not shown). The electrochemical gradient resulting in a net negative charge within mitochondria may play a role in the significant accumulation of the positively charged cisplatin that we measured. Indeed, alterations in mitochondrial membrane potential have been associated with significant shifts in cisplatin sensitivity (Andrews and Albright, 1992; Zinkewich-Peotti and Andrews, 1992).

While we have demonstrated a direct effect of cisplatin on mitochondria which is not dependent on nuclear or cytoplasmic signaling, our results do not exclude the importance of such effects in generating apoptotic signals following cisplatin exposure. Cisplatin exposure is associated with localization of p53 to the mitochondria which enhances binding of mitochondrial transcription factor A to cisplatin-damaged mitochondrial DNA (Yoshida et al., 2003). The p38 map kinase (MAPK) pathway may also have a role in cisplatin-induced apoptosis. Activation of p38 has been demonstrated following cisplatin exposure in a number of studies and this can be inhibited by RACK1 and AKT2 (Losa et al., 2003; Jeong et al., 2002; Yuan et al., 2003).

Cells deficient in nuclear DNA repair are sensitive to cisplatin, but this may be secondary to DNA damage caused by reactive oxygen species generated following release of mitochondrial cytochrome *c*, accelerating apoptosis.

In addition to interacting with nucleophilic sites in DNA, cisplatin binds to nucleophilic amino acid residues in proteins, including cysteine, methionine and histidine. Crosslinking or modification of critical intracellular proteins by cisplatin is a potentially important, and little studied, cytotoxic mechanism. The effect of cisplatin on mitochondria may be mediated through protein modification or crosslinking. Components of the permeability transition pore, such as the voltage-dependent anion channel and the adenine nucleotide translocator (ANT), are interesting candidates. Both contain vulnerable residues, and modification of a cysteine residue of ANT by thiol crosslinking agents has been shown to cause mitochondrial membrane permeabilization and apoptosis (Costantini et al., 2000). Our finding that the concentration of cisplatin bound to VDAC is more than 200 fold higher than the amount bound by total cellular proteins raises the interesting possibility that cisplatin binding to this key mitochondrial membrane protein could significantly alter its structure or function, facilitating the release of cytochrome *c*.

In conclusion, recent literature as well as data from our laboratory indicate that in HNSCC cell lines, the cytotoxic effect of cisplatin is, in significant part, mediated through direct action on mitochondria. While cisplatin interactions with nuclear DNA may have important cellular effects contributing to apoptosis, cisplatin binding to nuclear DNA is not necessary for induction of apoptosis. Indeed, the data presented here, in combination with recent findings discussed above, suggest that mitochondria may be the principal and sufficient target of cisplatin in this group of diseases. Further studies will be necessary to determine the precise mitochondrial target(s) of cisplatin and whether these observations are applicable to other tumor types. In the future, mitochondria-based drug screening assays may be important in the evaluation of new chemotherapeutic agents.

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