Molecular mechanisms of cisplatin and its liposomally encapsulated form, Lipoplatin™. Lipoplatin™ as a chemotherapy and antiangiogenesis drug

Review Article

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Abbreviations: 17-allylamino-17-demethoxygeldanamycin, (17−AAG); 7-ethyl-10-hydroxy-camptothecin, (SN-38); Brain-derived neurotrophic factor, (BDNF); chronic myelogenous leukemia, (CML); copper transporters, (CTR); dipalmitoyl phosphatidyl glycerol, (DPPG); DNA-protein cross-links, (DPC); dose limiting toxicity, (DLT); epidermal growth factor receptor, (EGFR); epidermal growth factor, (EGF); extracellular signal-regulated kinase, (ERK); Focal adhesion kinase, (FAK); glutathione S-transferase P1, (GSTP1); Heat shock protein 90, (Hsp90); heparin-binding epidermal growth factor, (HB-EGF); high mobility group, (HMG); hypoxia-inducible factor-1α, (HIF-1α); MAPK/ERK kinase, (MEK); maximum tolerated dose, (MTD); methoxy-polyethylene glycol-distearylphosphatidylethanolamine, (mPEG2000-DSE); mismatch repair, (MMR); mitogen-activated protein kinase phosphatase-1, (MKP-1); mitogen-activated protein kinase, (MAPK); mitogen-activated protein, (MAP); multi-drug resistance related transporters, (MDR); non-small cell lung cancer, (NSCLC); nucleotide excision repair, (NER); organic cation transporters, (OCTs); overall survival, (OS); partial response, (PR); performance status, (PS); phosphatidylinositol 3-kinase, (PI3K); platelet-derived growth factor receptor, (PDGFR); polyethylene glycol, (PEG); progressive disease, (PD); protein kinase C, (PKC); reactive oxygen species, (ROS); small interfering RNA, (siRNA); sodium dodecyl sulfate, (SDS); soy phosphatidyl choline, (SPC-3); squamous cell carcinoma of the head and neck, (SCCHN); stable disease, (SD); stress-activated protein kinase/c-Jun N-terminal kinase, (SAPK/JNK); transitional cell carcinoma, (TCC); tumor necrosis factor alpha(α)-related apoptosis-inducing ligand, (TRAIL); vascular endothelial growth factor, (VEGF); world health organization, (WHO)

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Summary

Cisplatin continues to be one of the cornerstone drugs in modern chemotherapy thus playing an important role among cytotoxic agents in the treatment of epithelial malignancies. Cisplatin damages, indiscriminately, cancerous and normal tissue. Its severe side effects arise from induction of apoptosis in various cell types in normal tissue in treated patients especially in peripheral nerves, renal tubules, bone marrow and gastrointestinal tract. Apoptosis may arise from the modulation of a number of signaling pathways by cisplatin including the mitochondrial pathway, the DNA damage signaling, stress-related signals, the ERK pathway and others. Patented platform technologies have been used for the liposomal encapsulation of cisplatin (Lipoplatin™) into tumor targeted 110-nm in diameter nanoparticles. Based on the molecular mechanisms of cisplatin including active import / export across the cell membrane, signaling pathway modulation and DNA damage an attempt is made to speculate on the molecular mechanisms of Lipoplatin™. The advantage of Lipoplatin™ over cisplatin is suggested to result from the ability of Lipoplatin™ to target primary tumors and metastases using the permeability of the vasculature of the growing tumor for its preferential extravasation and to cause a greater damage to tumor tissue compared to normal tissue as demonstrated in human studies. The nanoparticles are then avidly taken up by the tumors either via phagocytosis or by direct fusion with the cell membrane. The two mechanisms result to an overall 10 to 400-fold higher intracellular uptake of total platinum in tumor cells compared to cells in normal tissue in human studies. Animal studies shown here suggest that genes wrapped up in Lipoplatin™ shells target not only the tumors after systemic delivery but also their vasculature and result in the expression of a functional gene product after crossing the cell membrane barrier. It is being inferred that Lipoplatin™ is endowed with the properties of cisplatin plus the
ability of its nanoparticles to target and kill endothelial cells of tumor vasculature suggesting that this drug has two properties, that of a chemotherapy drug and that of an antiangiogenesis agent, combined together. Lipoplatin™ is finishing successfully two non-inferiority phase III clinical trials as first line treatment against non-small cell lung cancer (NSCLC) and has received the orphan drug designation by EMEA against pancreatic cancer.

I. Introduction
Cisplatin is the queen of chemotherapy among over 700 FDA-approved drugs with applications in more than 50% of human cancers, including the lucrative non-small cell lung cancer. Cisplatin can be combined with radiation therapy and a variety of other anticancer cytotoxic drugs such as gemcitabine, taxanes or vinca alkaloids. However, wider use of cisplatin is deterred due to severe damage to kidneys, peripheral nerves, bone marrow, gastrointestinal tract, hair follicles and other tissues. These damages may result from apoptotic death of cells in these tissues as a consequence of cisplatin treatment.

The side effects of chemotherapy have prompted academic institutions, biotechnology and pharmaceutical companies to invent new ways of delivering drugs. One fruit of the effort evolved from the genesis of the field of liposomes, pioneered by Gregoriadis and Papahadjopoulos (for example, see Lasic and Papahadjopoulos, 1995; Gregoriadis et al, 2003). An additional attempt that led to the creation of the field of nanotechnology is to wrap up old drugs or new molecules into nanoparticles composed of a variety of polymers including peptides, dendrimers, and hyperbranched polymers (for example, Sideratou et al, 2006). Tumor specific drug targeting involving liposomes, immunoliposomes, microspheres and nanoparticles are now widely used at the experimental level and several have entered the clinic.

In recent years delivery of anticancer drugs using nanoparticles has offered several advantages. Targeting solid tumors and metastases can be achieved at two levels: (i) via passive extravasation of nanoparticle carriers of drugs through the altered endothelium of the vascular wall produced during neoangiogenesis, especially in tumors with high vascularization and (ii) via attachment of ligand molecules on the outer face of the drug-loaded nanoparticles which are able to recognize specific molecules overexpressed on the surface of specific tumors.

Our group has been involved in the liposomal encapsulation of preexisting chemotherapy drugs in order to improve their performance; emphasis has been given to the reduction of side effects but also targeting enhancement to tumors and metastases. A breakthrough was the encapsulation of cisplatin leading to a nanoparticle liposomal formulation, Lipoplatin™ (Boulikas, 2004). These nanoparticles integrate a reverse micelle technology followed by conversion into true liposomes for efficient encapsulation, but also the fusogenic DPPG lipid on the surface of the nanoparticles. DPPG is meant to break the cell membrane barrier by promoting the direct fusion of the nanoparticle with the cell membrane.

The scope of this article is to review the molecular mechanisms of cell and DNA damage by cisplatin and to propose novel mechanisms of cell damage and apoptosis induced by Lipoplatin™.

II. Cisplatin and platinum drugs in chemotherapy
Cisplatin, since its serendipitous discovery in 1965, its identification in 1969 and its clinical application in the early 70’s, continues to be a cornerstone in modern chemotherapy, playing an important role among cytotoxic agents in the treatment of epithelial malignancies (reviewed by Rosenberg, 1977; Hill and Speer, 1982).

Figure 1 shows the structure of cisplatin, the universally approved carboplatin and oxaliplatin and of nedaplatin and lobaplatin, approved in restricted Asian territories. Cisplatin, usually in combination with other drugs, is commonly used as first line chemotherapy against cancers of the lung, head-and-neck, esophagus, stomach, colon, bladder, testis, ovaries, cervix, uterus and as second line treatment against most other advanced cancers, such as cancers of the breast, pancreas, liver, kidney, prostate as well as against glioblastomas, metastatic melanomas, and peritoneal or pleural mesotheliomas.

Well known are the adverse effects of cisplatin including renal toxicity, gastrointestinal toxicity, peripheral neuropathy, myelotoxicity, asthenia, and ototoxicity (Ban et al, 1994; Hanigan and Devarajan, 2003). Cisplatin ototoxicity is caused by apoptotic cell death. Apoptosis is an important mechanism of cochlear hair cell loss following exposure to an ototoxic level of cisplatin (Wang et al, 2004). When P3 rat cochleae were cultured in 1mM cisplatin, enhanced expression of twenty-two cochlear proteins (greater than 1.5-fold) was observed, whereas expression of 17 proteins was significantly decreased (Coling et al, 2007). Reactive oxygen species generated in mitochondria were thought to be the main cause of cellular damage explaining the nephrotoxicity of cisplatin. For example, a single injection of cisplatin (10 mg/kg body weight, i.p.) to rats caused depletion of the antioxidant defense molecules NADPH and glutathione, and increased the activity of the proapoptotic caspase-3 (Santos et al, 2008).

The significant risk of nephrotoxicity caused by cisplatin frequently hinders the use of higher doses to maximize its antineoplastic effects (Humes, 1999; Arany and Safirstein, 2003). Cisplatin, when combined with other cytotoxic agents, has shown an improved response rate and survival in a moderate to high number of patients suffering from epithelial malignancies; nephrotoxicity and neuropathy of cisplatin are often unacceptabe. Cisplatin analogues have been in use (carboplatin, oxaliplatin) but none as yet has achieved a similar broad-spectrum effectiveness.

Lipoplatin™, a liposomal formulation of cisplatin, was developed in order to reduce the systemic toxicity of cisplatin while simultaneously improving the targeting of the drug to the primary tumor and to metastases. Lipoplatin™ nanoparticles display enhanced circulation in
body fluids and evade immune surveillance by their coating with PEG. Furthermore, Lipoplatin™ nanoparticles preferentially extravasate to tumors through their compromised vasculature due to their small particle size (90-130 nm) and long circulation. Lipoplatin™ crosses the cell membrane more readily than cisplatin because fusogenic DPPG lipid is present on its surface. Upon entrance to the cytoplasm, Lipoplatin™ leads to the induction of tumor cell apoptosis; it is proposed here that Lipoplatin™ also induces apoptosis to the endothelium of tumor vasculature, hence, portraying strong antiangiogenesis properties.

III. Lipoplatin™ formulation and clinical development

A. Differentiating features and comparison to SPI-77

Lipoplatin™ is a liposomal formulation of the FDA-approved cisplatin.

The Lipoplatin™ formulation (Figure 2) is based on the formation of reverse micelles between cisplatin and DPPG under special conditions of pH, ethanol, temperature, ionic strength and other parameters. Cisplatin-DPPG reverse micelles are subsequently converted into liposomes by interaction with neutral lipids. This process, involving various steps sensitive to temperature, ethanol concentration, pH, ionic strength, type of salt, type of lipid and other sensitive parameters leads to very high encapsulation efficiencies (95-97%). About 15 repeated extrusions are performed using a Thermobarrel Extruder through membranes of 0.2, 0.1, 0.08 and 0.05 nm pore sizes under pressure in ultra pure nitrogen to an average size of 110 nm. Particles of larger sizes (100-150 nm) might display a better extravasation to tumors over normal tissue compared to smaller particles (60-100 nm).

Lipoplatin™’s liposomes (Figure 2) are composed of dipalmitoyl phosphatidyl glycerol (DPPG), soy phosphatidyl choline (SPC-3), cholesterol and methoxy-polyethylene glycol-distearoyl phosphatidyl-ethanolamine (mPEG2000-DSPE). Lipoplatin™ is composed of: 8.9% cisplatin and 91.1% lipids (w/w). Lipoplatin™ has an opaque appearance reflecting its liposomal nature and is being provided in 50-ml glass vials of 3 mg/ml (concentration refers to cisplatin). Lipoplatin™ is stored at 0-4°C and has an expiration date of three years. The concentration of 3 mg/ml of cisplatin in Lipoplatin™ exceeds the solubility of the free drug, cisplatin, usually provided as a 0.5-1 mg/ml solution for i.v. infusion.

Non-PEGylated liposomes (Figure 2) are taken up by liver macrophages and destroyed with a half-life in body fluids of 20 min (reviewed by Martin and Boulakis, 1998). On the contrary, PEGylated liposomes such as those of Lipoplatin™ display a half-life of 5 days in body fluids (Stathopoulos et al, 2005).

The Lipoplatin™ formulation uses several advancements in its liposome encapsulation: i) the anionic lipid DPPG gives Lipoplatin™ its fusogenic properties presumably acting at the level of entry of the drug through the cell membrane after reaching the target tissue; ii) the total lipid to cisplatin ratio is low (10:1 mg lipid/mg cisplatin) in Lipoplatin™ which means that less lipid is injected into the patient. For comparison, the ratio of lipids to cisplatin in the liposomal formulation SPI-71 is 71:1 (Veal et al, 2001) which is 7-fold higher lipids per mg cisplatin compared to Lipoplatin™; and iii) The PEG polymer coating used on Lipoplatin™ is meant to give the drug particles the ability to pass undetected by the macrophages and immune cells, to remain in the circulation of body fluids and tissues for long periods, to extravasate preferentially and to infiltrate solid tumors and metastatic tissue through the altered and often compromised tumor vasculature.

The Lipoplatin™ formulation differs from the SPI-77 in several basic principles including loading method, type of lipids, and ratio of cisplatin to lipids. Whereas the loading of cisplatin in Lipoplatin™ is based on reverse micelles, the mechanism of cisplatin loading in SPI-71 is passive. The Lipoplatin™ formulation uses anionic and neutral lipids compared to SPI-77 that uses only neutral lipids. Although the mechanism of entry of Lipoplatin™ nanoparticles into cells has not been fully deciphered, tumor cells appear to uptake more avidly Lipoplatin™ particles. In addition, entry of Lipoplatin™ by fusion because of the fusogenic DPPG lipid component has been proposed (see below). Because of the similarity in the shell structure between SPI-77 and a liposomal
formulation of doxorubicin, Doxil (Caelyx in Europe), it can be inferred that SPI-77 nanoparticles are unable to cross the cell membrane barrier and that extracellular lipases degrade the particles over the period of several days, leading to the release of cisplatin outside the tumor cell (see Martin and Boulikas, 1998).

These fundamental differences between the Lipoplatin™ and SPI-77 formulations might explain the low activity of SPI-77 in a phase II clinical study with an overall response rate of 4.5% against advanced NSCLC as first line treatment at doses of 100, 200 and 260 mg/m². The primary end points of this study were response and toxicity, and the secondary end points were survival (White et al, 2006). A previous phase I study used 40-320 mg/m² of SPI-77 i.v. every 4 weeks in children with advanced cancers, which were not amenable to other treatments, and finally no response to treatment was seen. Lipoplatin™ A half-life of up to 134 h, with maximum plasma concentrations approximately 100-fold higher than those reported following comparable doses of cisplatin were observed (Veal et al, 2001).

B. Preclinical studies

In an attempt to decipher the mechanism of Lipoplatin™ action at the molecular level, Fedier and coworkers (2006a) investigated whether the cytotoxic effect of Lipoplatin™ is dependent on the functional integrity of DNA mismatch repair (MMR). MMR is post-replicative DNA repair mechanism implicated in cell cycle control and apoptosis. HCT116 human colorectal adenocarcinoma cells lacking MLH1, one of five proteins crucial to MMR function, were 2-fold resistant to Lipoplatin™ damage compared to MLH1-expressing HCT116 cells. However, proteolytic processing of caspase-3, caspase-7 and poly(ADP-ribose) polymerase-1 following Lipoplatin™ treatment was comparable in MLH1-deficient cells and -proficient cells. It was concluded that MMR function is a relevant determinant accounting for the cytotoxicity of Lipoplatin™ (Fedier et al, 2006a). As an extension of these studies, a possible relationship between MMR-mediated cisplatin DNA damage signaling and the Akt signaling pathway was found (Fedier et al, 2006b). The Akt-specific inhibitor LY294005 decreased the sensitivity of both colorectal adenocarcinoma sublines (deficient or proficient in the function of MLH1) to Cisplatin, Lipoplatin™, Oxaliplatin, and Lipoxal. This decrease was significantly higher in the MLH1-proficient than in the MLH1-deficient subline with Cisplatin and Lipoplatin™, but nearly the same in both sublines with Oxaliplatin and Lipoxal. In the same colon cancer cell lines LY294005 increased the efficacy of Docetaxel and did not affect the efficacy of 6-thioguanine (Fedier et al, 2006b). Preclinical studies have shown the lower nephrotoxicity and other adverse effects of Lipoplatin™, compared to cisplatin, in mice, rats and SCID mice (Boulikas, 2004; Devarajan et al, 2004); whereas animals injected with cisplatin developed renal insufficiency with clear evidence of tubular damage, those injected with the same dose of Lipoplatin™ were almost completely free of kidney injury (Devarajan et al, 2004). Treatment of dogs with Lipoplatin™ led to the conclusion that the drug can be safely administered to clinically normal dogs at dosages of up to 150 mg/m² without the need for concurrent hydration protocols. The maximum tolerated dose (MTD) of unencapsulated cisplatin in dogs has been established as 70 mg/m². Therefore, Lipoplatin™ would allow the safe and repeated administration of doses higher than the MTD of unencapsulated cisplatin and could find application for the treatment of dogs with osteosarcoma (Marr et al, 2004).

In summary, preclinical studies have shown Lipoplatin™’s lower nephrotoxicity in rats, as compared to cisplatin, the plasma pharmacokinetics and therapeutic efficacy in mouse xenografts with breast and prostate human tumors. The data are consistent with an apoptotic death of tumor cells after treatment of xenografts with Lipoplatin™ (Boulikas, 2004).

C. Clinical development of Lipoplatin™

A phase I study has been completed on 27 patients and with dose escalation from 25 mg/m² to 125 mg/m². All 27 patients were at stage IV (19 pancreatic carcinoma, 6 renal cell carcinoma, 1 with gastric cancer and 1 with squamous cell carcinoma of the head and neck). In all cases, Lipoplatin™ was a second- or third-line treatment and was administered when the disease was refractory to standard treatment. Lipoplatin™ was administered as an 8 h infusion diluted in 1 L 5% dextrose, repeated every two weeks. There was no need for pre- or post-hydration of the patient with Lipoplatin™. This is in contrast to cisplatin chemotherapy that requires admittance of the patient the night before infusion for hydration as well as extended

Figure 2. A non-PEGylated liposome (left) and a PEGylated liposome such as that of Lipoplatin™ (right) are depicted.
stay in the hospital after infusion for post hydration to reduce the nephrotoxicity of the drug. The highlights of this study were that Lipoplatin™ has a mild hematological and gastrointestinal toxicity but does not show any nephro- and neurotoxicity, has no ototoxicity, does not cause hair loss and is void of most other side effects (Stathopoulos at al, 2005).

A preliminary Phase II study using 100 mg/m² Lipoplatin™ as an 8 h i.v. infusion on days 1 and 15 and 1 g/m² gemcitabine given on days 1 and 15 in a 28-day schedule for 2 to 10 cycles, has been reported before (Stathopoulos et al, 2002). Patients included 19 with pancreatic cancer, 7 with NSCLC as well as with head and neck cancer and bladder cancer; all patients were resistant to previous first or second line chemotherapy. No renal toxicity was detected in any patient. No neuropathy, otoxicity, hepatotoxicity, cardiotoxicity or allergic reaction was observed. Nausea and vomiting grade I-II was seen in 4 patients (15.3%) and myelotoxicity of grade III was seen in 1 patient and of grade I-II in 15 patients (57.6%). Mild asthenia was common. 6 patients (23%) showed partial response. Stable disease was seen in 65.3% of the patients and clinical benefit in 42.3%. Lipoplatin™ at 125mg/m² and 1 g/m² gemcitabine induced grade III and IV neutropenia and grade III nausea and vomiting.

A phase III dose escalation study of Lipoplatin™ and gemcitabine has been completed in advanced stage pretreated pancreatic cancer patients. The primary objectives were to determine toxicity and the maximum tolerated dose (MTD) and the secondary aims, to determine the response rate and clinical benefit (Stathopoulos et al, 2006). Twenty-four patients were enrolled in the study. The great majority of the patients (79.2%) were at stage IV of their disease. All patients had undergone prior chemotherapy: 11 patients with gemcitabine monotherapy and 13 with gemcitabine combined with irinotecan. Lipoplatin™ was administered as an 8 h i.v. infusion on days 1 and 15 with dose escalation and for most cycles it was at 100 mg/m². Gemcitabine was given on days 1 and 15 at a dose of 1000 mg/m² and cycles were repeated every 4 weeks. Treatment was administered for at least three cycles or until disease progression.

Since both Lipoplatin™ and gemcitabine are myelotoxic, it was not surprising to observe that the main side effect of the combination treatment was myelotoxicity. No neurotoxicity, renal toxicity or febrile neutropenia was observed. Myelotoxicity of grades 3 and 4 was observed at 125 mg/m² of Lipoplatin™ and 1000 mg/m² of gemcitabine and therefore this dose scheme was considered as DLT whereas the dose of 100 mg/m² of Lipoplatin™ and 1000 mg/m² of gemcitabine on days 1,15 in a 28-day cycle for 3 cycles as the MTD.

PR (>50% reduction in all measurable lesions) was achieved in 2 patients (8.3%) with durations of 6 and 5 months. Stable disease (50% reduction to a 25% increase in all measurable lesions) was seen in 14 patients (58.3%) with a median duration of 3 months (range 2-7 months). Clinical benefit mainly due to pain reduction was seen in 8 patients (33.3%). At the end of the study 7 patients (29.2%) were still alive. Median survival from the beginning of second-line treatment was 4 months (range 2 to over 8 months). Taking into account that all of the patients were refractory or in disease progression while on a prior treatment including gemcitabine, the response rate was attributed to the addition of Lipoplatin™ (Stathopoulos et al, 2006a).

There are several ongoing Phase II studies the results of which will be reported including: Lipoplatin™ monotherapy against NSCLC, Lipoplatin™ plus gemcitabine against NSCLC, Lipoplatin™ plus intravenous navelbine against metastatic breast cancer, Lipoplatin™ plus 5-FU plus radiation against gastric cancer, Lipoplatin™ plus doxetaxel against metastatic breast cancer, Lipoplatin™, Lipoxal (liposomal oxaliplatin of Regulon) plus docetaxel against NSCLC and finally, Lipoplatin™ plus radiation against head and neck cancers.

There are three ongoing Phase III studies. The first Phase III (LipoGEM), is a randomized multicenter clinical study that compares 120 mg/m² Lipoplatin™ on days 1,8,15 plus 1g/m² gemcitabine on days 1,8 (Arm A) with 100 mg/m² cisplatin on day 1 plus 1g/m² gemcitabine on days 1,8 (Arm B). The cycle in each arm is 21 days and treatment is given for six cycles or until disease progression as first line treatment in patients with non-small cell lung cancer (NSCLC). Thus, the total cumulative dose in the Lipoplatin™ arm is 1,080 mg cisplatin/m² during 9 weeks compared to 300 mg total cisplatin/m² in the cisplatin arm during 9 weeks. This study was initiated in Greece with the participation of 14 clinical centers in most major hospitals of the country, both public and private. Eligibility criteria include confirmed diagnosis of inoperable or metastatic NSCLC, no previous chemotherapy, WHO PS 0-1, and adequate end-organ function. Planned number of patients is 200 in each Treatment Arm. The primary endpoint is overall survival. Overall response rates, toxicity, progression-free survival and quality of life are also being evaluated.

As of December 2006, 59 patients were treated of whom 33 received Lipoplatin™ and 26 cisplatin. Two patients in the Lipoplatin™ Arm had a hypersensitivity reaction during the first infusion. There were no grade 4 toxicities. The majority of side effects, most notably nephrotoxicity were much lower in the Lipoplatin™ compared to the cisplatin arm. For example, nephrotoxicity of grade 2 was reported in only 6% of Lipoplatin™ patients versus 19% of cisplatin patients, although Lipoplatin™ was administered without hydration as a 6h infusion. Neurotoxicity was also markedly lower in the Lipoplatin™ arm. As of December 2006, 32 patients had been assessed for response to treatment, 16 in each Arm; 4 partial responses (PR) have been reported in each Arm. However, difference has been observed in stable disease (SD, 7/31 or 23% in Lipoplatin™ versus 3/26 or 12% in cisplatin) as well as progressive disease (PD, 5/31 or 16% in Lipoplatin™ versus 9/26 or 35% in cisplatin). Thus, the therapeutic profile in the Lipoplatin™ arm is superior to that of the cisplatin arm. These preliminary data have been reported at the ASCO meeting (1-5 June 2007, Chicago, Illinois, USA) (Boulakis et al, 2007).

Overall, this Phase III study shows that Lipoplatin™ appears to have a better safety profile and equivalent or
slighty improved therapeutic profile than cisplatin, when combined with gemcitabine, in patients with advanced NSCLC as first line treatment. Particularly important might be the significantly lower neuro- and nephrotoxicity of the Lipoplatin™ arm and its administration on an outpatient basis.

The second Phase III (LipoTaxol), was initiated in April 2006 in Greece. This randomized Phase III uses 200 mg/m² Lipoplatin™ plus 135 mg/m² paclitaxel administered on day 1 repeated every 2 weeks (Arm A). Lipoplatin™ was infused for 8 hours in 1 L/5% dextrose. Arm B is 75 mg/m² cisplatin (hydration of 2 L/it) and 135 mg/m² paclitaxel, administered every two weeks. One cycle is 14 days and the plan was to give 9 cycles (treatments) per patient unless disease progression was detected before the 9th cycle. As of December 2006, 61 chemo naive patients were recruited with a median age of 65 (42-80). 54 were evaluable for response and toxicity, 27 in each arm. Response: Arm A: PR: 48.15% SD: 37.03% PD: 3.7% and clinical benefit 11.11%. Arm B: PR 44.44% SD 44.44% PD: 3.7% and clinical benefit 7.41%. Thus, both arms show about the same response rate with a slight superiority in the Lipoplatin™ arm. Toxicity: Arm A: Renal toxicity in 1 patient (3.70%) neurotoxicity grade I-II in 7 patients (25.92%) nausea-vomiting in 5 patients (18.52%) myelotoxicity Grade I-II in 10 patients (37.04%). Arm B: Renal toxicity in 7 patients (25.92%), neurotoxicity Grade I-II in 12 patients (44.44%) nausea-vomiting in 7 patients (25.92%) myelotoxicity Grade I-III in 17 patients (62.96%). Thus, the toxicity differences are very important between the two arms. In particular, the renal toxicity appears to be 7-fold lower (700% less) in the Lipoplatin™ arm. Also significantly lower are the neurotoxicity and myelotoxicity of Grade III (totally absent in the Lipoplatin™ arm). It was concluded that the response rate was similar but toxicity and in particular nephrotoxicity, neurotoxicity, and myelotoxicity was significantly lower in the Lipoplatin™ arm (Stathopoulos et al, 2007). Taken together the phase III studies strongly suggest that Lipoplatin has a higher therapeutic index than cisplatin in first line NSCLC.

One important aspect of Lipoplatin™ chemotherapy is a long time of infusion to further reduce adverse reactions. Recent studies (Stathopoulos et al, unpublished) have shown that a schedule of 200 mg/m² Lipoplatin™ on day 1 given intravenous in 6 to 8-h in combination with 135 mg/m² paclitaxel on day 1 in a 14-day schedule (repeated for 9 cycles) does not show nephrotoxicity; however, rapid infusion of Lipoplatin™ (less than 3h) results in Grade 2-3 nephrotoxicity.

The third Phase III (LipoFU), is a randomized, multicenter phase III trial against squamous cell carcinoma of the head and neck (SCCHN). The study is comparing 100 mg/m²/day Lipoplatin™ (days 1,8,15) plus 1,000 mg/m²/day 5-FU (days 1 to 5) every 21 days (one cycle) for 6 cycles (Arm A). The comparative arm (Arm B) uses 100 mg/m²/day cisplatin (day 1) plus plus 1,000 mg/m²/day 5-FU (days 1 to 5) every 21 days (one cycle) for 6 cycles (Jehn et al, 2007). The overall Lipoplatin™ dose in the LipoFU study is 300 mg/m² every 21 days compared to 360 mg/m² every 21 days in the LipoGEM study (see above). Also the LipoFU trial recruits both chemo naive and previously treated patients compared to LipoGEM that recruits only chemo naive patients. Both 5FU and gemcitabine belong to the class of antimetabolites.

As of December 2006, 62 patients were randomized, of whom 43 were evaluable for outcome and toxicity. Hematotoxicity was more frequent in the Cisplatin arm than in the Lipoplatin™ arm. The rate of anemia was similar between the treatment arms, 13 pts. The Lipoplatin™ arm experienced lower renal toxicity as measured by a reduction of the creatinine clearance (grade I: 99–75 ml/min; grade II: 74–50 ml/min; grade III: <50 ml/min). 5 patients in the cisplatin arm developed grade III renal toxicity whereas no renal toxicity of grade III was developed in the Lipoplatin™ arm. Outcome was as follows: Lipoplatin™ arm: PR: 3 pts; SD: 13 pts; PD: 9 pts; cisplatin arm: PR: 8 pts; SD: 9 pts; PD: 1 pts. Thus, the non-PD pts (PR or SD) was 16/25 (64 %) in the Lipoplatin™ arm vs 17/18 (94%) cases in the cisplatin arm. A report on the overall survival between the two arms is pending. It was concluded that Lipoplatin™ seems to reduce both the renal and hematological toxicity as compared to conventional cisplatin to a clinically relevant extent. This reduction of side effects will influence the chance to preserve the dose density of chemotherapy, and thereby, the efficacy of treatment (Jehn et al, 2007).

The preliminary Phase III studies, as well as additional planned Phase III studies against pancreatic, gastric and breast cancers are expected to establish Lipoplatin™ as an important chemotherapy drug with a broad spectrum of activity against epithelial malignancies, tumor targeting (see below), lower side effects and with an improved quality of life and overall survival.

IV. Import, export and signal transduction by cisplatin and Lipoplatin™

A. Import across the cell membrane

After infusion, cisplatin is rapidly excreted in the urine causing renal tubular damage. When it reaches normal and malignant cells it uses the major copper influx transporter Ctr1 for entry inside the cytoplasm (Figure 3). Ctr1 has been convincingly demonstrated to transport cisplatin and its analogues, carboplatin, and oxaliplatin. Two copper efflux transporters, ATP7A and ATP7B, regulate the efflux of cisplatin (Kuo et al, 2007). While the mechanisms by which hCtr1, ATP7A and ATP7B transport copper ions have been studied extensively, very little is known about the mechanisms by which these transporters shuttle platinum drugs. Cisplatin-resistant ovarian and cervical tumor cell lines exhibited 1.5-1.8-fold lower levels of CTR1, 2.5- and 2.9-fold lower intracellular platinum concentrations and lower DNA platinization compared to the cisplatin-sensitive line with no differences in efflux; expression of Ctr1 could constitute an additional important factor in ex vivo assays to predict cisplatin sensitivity in tumor specimens of patients (Zisowsky et al, 2007). Transfection of cells in culture with constructs expressing the ATP7A gene enhanced resistance not only
to cisplatin but also to vincristine, paclitaxel, 7-ethyl-10-hydroxy-camptothecin (SN-38), etoposide, doxorubicin, mitoxantrone, and CPT-11 (Owatari et al, 2007). Impaired activity in the cisplatin transporter transmembrane proteins including the copper transporters (CTR), organic cation transporters (OCTs) and multi-drug resistance related transporters (MDRs) contribute to cisplatin resistance through the reduction of drug accumulation in the cell (reviewed by Choi and Kim, 2006).

From the clinical experience of Lipoplatin™ (see above), its lipid composition that includes the fusogenic DPPG molecule and the levels of platinum found in saline and sodium dodecyl sulfate (SDS) extracts of tumor and normal specimens representing platinum trapped in tissue versus platinum that has reacted with macromolecules after Lipoplatin™ infusion in patients (Boulikas et al, 2005) we suggest a direct fusion of Lipoplatin™ nanoparticles with the membrane of the tumor cell (Figure 3).

B. Glutathione detoxification.

The S-containing tripeptide glutathione is present in cells at mM concentrations, and the formation of complexes plays an important role in the detoxification and biological activity of platinum compounds (Figure 4). Depletion of glutathione levels has been shown to increase the toxicity of kidney cells to cisplatin and a clinical trial demonstrated that pretreatment with glutathione reduced renal toxicity without affecting antitumor activity. Cancer cells that are resistant to cisplatin often have elevated glutathione levels. Glutathione could quench DNA-Pt monofunctional adducts before they can rearrange to toxic bifunctional adducts on DNA (see below). A direct administration of glutathione ester at a dose of 500 mg/kg, but not glutathione, was shown to protect against cisplatin-induced ototoxicity in a rat model measuring outer hair cell loss and response to click and tone-burst stimuli (Campbell et al, 2003).

Human glutathione S-transferase P1 (GSTP1) contributes to chemoresistance and its suppression, decreasing the cisplatin-induced activation of ERK1/2, might have synergistic therapeutic effects (Huang et al, 2007). The co-overexpression of the two subunits of glutamate cysteine ligase, another key enzyme in glutathione synthesis, was found to correlate to cisplatin sensitivity in xenografts implanted with human NSCLC cells (Fujimori et al, 2004). The thiol dipeptide cysteinyl-glycine, i.e. the GSH catabolite generated by γ-glutamyl transpeptidase, showed a higher reactivity against cisplatin than glutathione and appears to play key a role in modulating cisplatin nephrotoxicity (Paolicchi et al, 2003).

C. Induction of the mitochondrial pathway

Cisplatin and other apoptotic stimuli trigger the release of cytochrome c from the mitochondrial intermembrane space to the cytosol, which induces the

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**Figure 3.** Ctr1, the major copper influx transporter, imports Cisplatin. Two copper efflux transporters, ATP7A and ATP7B, situated at the periphery of the cell membrane regulate the efflux of cisplatin. Instead, Lipoplatin™ bypasses Ctr1 thanks to the fusogenic DPPG lipid which commands direct fusion with the cell membrane and cisplatin deliver across the membrane barrier. In addition, because of its 110-nm particle size (compared to cisplatin with a molecular dimension of less than 1 nm) Lipoplatin™ is taken up by phagocytosis. Tumor cells are known to be more actively engaged in phagocytosis than normal tissue. Thus, Lipoplatin™ acts as a Dorian Horse for tumor cells. Lipoplatin™ is proposed to be able to bypass cisplatin resistance.
Figure 4. Glutathione cisplatin coordination compounds.

formation of the apoptosome and the activation of procaspase-9. The apoptosome is an Apaf-1 cytochrome c complex that activates procaspase-9. The three-dimensional structure of the apoptosome has been determined at 27 Å resolution, to reveal a wheel-like particle with 7-fold symmetry (Acehan et al., 2002; Figure 5). Procaspase-9 molecules can bind to the inner "hub" region of the apoptosome. This complex promotes the efficient activation of procaspase-3. Therefore, the cleavage of procaspase-9 is not required to form an active cell death complex. Cisplatin can activate the proapoptotic protein Bax resulting in cytochrome c release, caspase activation, and apoptosis; Bax activation is implicated in the nephrotoxicity of cisplatin (Wei et al., 2007). Bcl-2 plays an important role in the mitochondrial apoptotic pathway. Although the general role of Bcl-2 is anti-apoptotic, Bcl-2 fragments resulting by caspase cleavage after cisplatin treatment of cells in culture could promote the apoptotic process (Zhu et al., 2007). Lipoplatin™, releasing cisplatin molecules in the cytoplasm of the tumor
cell is also proposed to activate the mitochondrial apoptotic cascade.

**D. Signal transduction pathways and cisplatin**

During signal transduction a cell senses both the external and internal environment and converts a stimulus into an ordered sequence of phosphorylation-dephosphorylation, protease degradation, gene regulation, or ion flux events across the cell membrane. There is a great number of signaling cascades including the epidermal growth factor receptor (EGFR), the p38 mitogen-activated protein kinase (MAPK), the G-protein-coupled receptors/MAPK, the ERK/MAPK, the platelet-derived growth factor receptor (PDGFR), the PKC, the PKA, the growth factor/survival factor/mitogen, the PI3K/AKT/PTEN, the ceramide, the proteasome, the integrin, the Wnt/b-catenin, the insulin, the cholesterol, the RB/E2F, the ubiquitination and the cyclins/p27 regulating the cell cycle, the p53/DNA damage, the oxidative signaling for phosphatidylserine externalization, the survival/BAD, the death receptor/Bcl-2 and many more.

A number of additional properties of cisplatin are now emerging including activation of signal transduction pathways leading to apoptosis. Firing of such pathways may originate at the level of the cell membrane after damage of receptor or lipid molecules by cisplatin, in the cytoplasm by modulation of proteins via interaction of their thiol groups with cisplatin, for example involving kinases, and other enzymes or finally from DNA damage via activation of the DNA repair pathways (reviewed by Boulikas and Vougiouka, 2003, 2004; Wang and Lippard, 2005).

Receptor tyrosine kinases contribute to chemoresistance in tumors. Cisplatin induces a number of signaling pathways (Figure 6). It should be emphasized here that activation of signaling pathways by cisplatin is cell type-specific. The end result is activation of caspases leading to apoptosis, to cell cycle arrest, mitochondrial apoptotic pathway, to DNA damage-induced apoptosis, as well as to upregulation in the expression levels of transcription factors that is tightly linked to apoptosis. Activation of p53 is a key determinant of sensitivity to cisplatin-induced apoptosis.

The p38 mitogen-activated protein kinase (MAPK) signaling pathway plays an important role in stress-induced cell-fate decisions by orchestrating responses that go from cell-cycle arrest to apoptosis. PDGFR signaling might confer selective growth advantage to chemoresistant cells. Ovarian carcinoma and neuroblastoma cell lines derived from PDGFR-expressing tumors were selected in cisplatin to obtain chemoresistant

![Figure 5. Induction of the mitochondrial apoptotic pathway by cisplatin.](image-url)

A cartoon explaining how cisplatin activates the proapoptotic protein Bax resulting in the release of cytochrome c from the mitochondrial intermembrane space to the cytosol to induce the formation of the apoptosome (Apaf-1 cytochrome c complex); this step is being followed by binding and activation of pro-caspase-9 to the inner "hub" region of the apoptosome leading to activation of procaspase-3.
sublines. Co-treatment with inhibitors of PI3K or mitogen-activated protein kinase (MEK) resulted in enhanced growth inhibition in chemoresistant cells; the PDGFR inhibitor ST1571 could have a therapeutic potential in patients with malignant gliomas refractory to chemotherapy (Servidei et al., 2006).

E. Stress signaling by cisplatin

Cisplatin induction of signaling is cell type-, time- and dose-dependent. It induces oxidative stress and is an activator of stress-signaling pathways especially of the mitogen-activated protein (MAP) kinase cascades. The extracellular signal-regulated kinase (ERK) pathway is indeed activated by cisplatin. Acquisition of cisplatin resistance by ovarian carcinoma cells was associated with the loss of ERK activation in response to cisplatin (Villedieu et al., 2007a). ERK activation and DNA-damage induced apoptosis are tightly linked; p53 may act as one of the upstream regulators of ERK activation for the induction of apoptosis in carboplatin-treated cervical cancer cells (Singh et al., 2007).

The c-Abl nonreceptor tyrosine kinase and the c-Jun NH2-terminal kinase (JNK/stress-activated protein kinase) are activated during the injury response to cisplatin (Nehmé et al., 1997). The adaptor protein Shb regulates apoptosis in response to hydrogen peroxide. cisplatin, cytokines or inhibitors of angiogenesis; these signals are propagated with interaction with c-Abl SH3 and SH2 domains (Hägerkvist et al., 2007). Treatment of cells with high cisplatin concentrations (one order of magnitude higher than the IC50) induces cellular superoxide formation and caspase activation independently of nuclear DNA damage. In contrast, cisplatin concentrations at IC50 doses, which do not induce acute apoptosis, are sufficient for induction of DNA damage signaling (Berndtsson et al., 2007). Cisplatin at 500 mM significantly increased the production of reactive oxygen species (ROS) in the early phase via NADPH oxidase, increased the intracellular calcium level by its release from the sites of intracellular calcium storage and caused oxidative stress in renal tubular epithelial cells. This mechanism appears to be largely responsible for the nephrotoxicity of cisplatin (Kawai et al., 2006). Production of reactive oxygen species

Figure 6. Cisplatin induces a number of signaling pathways including the ERK pathway, the PI3K/AKT/PTEN and the Death pathway. These converge on Caspase activation and apoptosis. Activation of the PI3K/AKT pathway by cisplatin leads to the upregulation of proapoptotic genes such as TRAIL and of the tumor suppressor PTEN. Cisplatin also induces adduct and crosslinks in the DNA inducing p53 and the DNA damage repair versus DNA damage-induced apoptotic pathways. Lipoplatin™ is proposed to have a similar signaling activation effects on tumor cells.
(ROS) resulted in EGFR and Akt phosphorylation as well as mobilization of the heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) precursor, proHB-EGF, from the nucleus of bladder cancer cells to a detergent-resistant membrane compartment; the growth factor was cleaved by a metalloproteinase-mediated mechanism and shed into the extracellular space serving as a secreted EGFR ligand. HB-EGF accumulated in the nucleus in aggressive transitional cell carcinoma (TCC) cells and this histologic feature is a marker of poor prognosis in human bladder cancer tissues; cisplatin treatment of TCC cells resulted in protection by HB-EGF toward apoptosis (Kim et al., 2005). Tumor cell lysosomes contain increased levels of cathepsins. Cisplatin damage may result in the release of these enzymes into the cytosol leading to apoptosis or necrosis; cisplatin further induces endoplasmic reticulum stress; thus, organelle damage responses can be used to trigger tumor cell death and could constitute drug targets as mediators of apoptosis signaling (Linder and Shoshan, 2005).

Inflammatory cytokines, UV irradiation or cisplatin induce transient or sustained phosphorylation of EGFR and as a result EGFR internalizes via a Clathrin-mediated process. In cytokine-stimulated cells, EGFR recycles back to the cell surface, whereas in irradiated cells it arrests in Rab5-containing endosomes (Zhang and Yarden, 2006).

F. Cisplatin and the PI3K / AKT1 pathway

The phosphatidylinositol 3-kinase (PI3K) / AKT1 pathway is frequently activated in cancer cells. The PI3K/Akt cascade has an important role in the resistance of ovarian cancer cells to cisplatin and inhibition of PI3K/Akt increases efficacy of cisplatin (Ohta et al., 2006). PTEN is a tumor suppressor gene product believed to promote apoptosis primarily via inactivation of the PI3K/Akt cell survival pathway. However, a p53-mediated apoptotic cascade independent of the PI3K/Akt pathway induced by PTEN has been found and over-expression of PTEN sensitizes ovarian cancer cells to cisplatin-induced apoptosis. PTEN over-expression may represent a novel therapeutic approach for chemoresistant human ovarian cancer (Yan et al., 2006). PI3K-Akt signaling also induced intranuclear translocation of Nr2f2 in auditory cells leading to transcriptional activation of ARE to upregulate heme oxygenase-1 which may be a critically mediator of response to cisplatin (So et al., 2006).

Down-regulation of AKT1 by siRNA could significantly enhance the sensitivity of gastric cancer cells to vincristine, adriamycin, 5-fluorouracil and cisplatin (Han et al., 2006). The Akt-specific inhibitor LY294002 increased the efficacy of Docetaxel, did not affect the efficacy of 6-thioguanine and decreased the efficacy of Cisplatin, Lipopolysaccharide (LPS), Oxaliplatin, and Piroxicam in human colorectal adenocarcinoma sublines suggesting a novel property of Akt in aggravating drug sensitivity (Feder et al., 2006b). Activation of Akt induced by cisplatin (also doxorubicin and H2O2) was repressed by c-Myc expression as a result of action of c-Myc upstream of PI3K activation. c-Myc overexpression impaired the induced association of the p85 subunit of PI3K with phosphotyrosine containing proteins, causing a reduction in the activation of PI3K and recruitment of Akt to the membrane (Bellmann et al., 2006). Brain-derived neurotrophic factor (BDNF) is a biomarker of poor prognosis in tumors from patients with neuroblastoma. BDNF activates TrkB through PI3K/Akt to protect neuroblastoma cells from etoposide/cisplatin-induced cell death. BDNF-induced decrease in the levels of the proapoptotic protein Bim were regulated by MAPK and not PI3K/Akt pathway in neuroblastoma cells (Li et al., 2007).

G. Adhesion molecules, metastatic cascade and cisplatin

The L1 adhesion molecule (CD171), with a role in cell motility and invasion, is overexpressed in ovarian and endometrial carcinomas; L1 expression leads to a sustained ERK, Focal adhesion kinase (FAK) and PAK phosphorylation and is a predictor of poor prognosis; cells expressing L1 are more resistant to apoptosis. Selection of ovarian carcinoma cells in culture in the presence of cisplatin led to upregulated expression of L1 and thus this could conspire a mechanism for the establishment of chemoresistance and of a more malignant tumor phenotype (Stoeck et al., 2007). Focal adhesion kinase (FAK) plays a critical role in ovarian cancer cell survival and in various steps in the metastatic cascade. FAK silencing with siRNA plus docetaxel or platinum might be a novel therapeutic approach against ovarian cancer as indicated from SCID animal studies (Halder et al., 2006).

H. Cisplatin and FasL death ligand signaling

TRAIL (tumor necrosis factor alpha(α)-related apoptosis-inducing ligand) is a potent inducer of apoptosis. TRAIL- and FasL-induced apoptosis might be affected by cisplatin although clearly cisplatin-induced apoptosis involves different pathways. TRAIL induces caspase-3 activation and PARP cleavage. Malignant ascites obtained from women with advanced ovarian cancer protect tumor cells from TRAIL- and FasL-induced apoptosis but not against cisplatin-induced apoptosis; protection involves activation of PI3K and its downstream target Akt by ascerts to increase c-FLIP(S) protein levels without affecting ERK phosphorylation (Lane et al., 2007). Cisplatin appears to exhibit synergistic effects with other potent inducers of apoptosis such as a synthetic isothiocyanate; sequential administration of both agents led to increased intracellular platinum accumulation, glutathione depletion, poly (ADP-ribose) polymerase cleavage, stimulation of caspase-3 activity, upregulation of p53, FasL and Gadd45α, cyclin B1 downregulation and an increase in mitogen-activated protein kinases JNK, ERK and p38 phosphorylation as well as PI3K level alterations (Bodo et al., 2006). Acquired cisplatin resistance in HeLa cells is due to a lower level of induced apoptosis; FasL expression was significantly enhanced in sensitive compared to resistant cells after cisplatin treatment. Thus, activation of the Fas system is critical in induction of apoptosis by cisplatin and in sensitive cells, caspase-8, -9 and -3 were activated by cisplatin. Activation of stress-activated protein kinase/c-
Jun N-terminal kinase (SAPK/JNK) and p38 kinase was cisplatin dose-dependent with significantly lower levels in cells with acquired cisplatin resistance than in the sensitive parental line; furthermore, resistant HeLa cells displayed a reduced level of DNA damage, indicating that long-term stimulation of SAPK/JNK and p38 kinase was triggered by nonrepaired cisplatin-induced DNA lesions (Brozovic et al, 2004).

Cisplatin can damage both extracellular protein domains and cytoplasmic signal transduction molecules. Lipoplatin™ is proposed to be limited to damaging intracellular compartments only. Figure 7 shows a cytotoxic T lymphocyte attached to a tumor cell via binding of the T cell receptor to the major histocompatibility complex molecule on the tumor cell surface. Interaction of Fas Ligand (FasL) on the T lymphocyte with the Fas receptor (CD95) mediates apoptotic signaling to remove transformed and virus-infected cells. The Fas receptor activates the cytoplasmic death domain (TRADD) that interacts with signaling adaptors (FADD) to activate Caspase 8 to cleave downstream caspases. The cascade results in cleavage of lamins, PARP, DNA-PK and a number of molecules leading to apoptosis and elimination of cancer cells. Pretreatment of epithelial ovarian carcinoma OVCAR3 cells with cisplatin significantly improved receptor-dependent apoptotic signaling by up-modulating CD95 receptor expression and increasing the death-inducing signaling complex formation efficiency (Bagnoli et al, 2007).

Cisplatin as well as Lipoplatin™ might modulate these pathways by a direct binding to cysteine residues in these proteins, not studied, thus modulating their activity. Cisplatin is proposed to modulate extracellular surface molecules causing misfiring of signaling cascades as well as intracellular signaling components. Lipoplatin™ is proposed to modulate only intracellular signaling components and protection of interaction with extracellular components from its lipid shell.

There is plenty of evidence suggesting such a role for cisplatin. The induction of apoptosis in L929 cells by cisplatin-treated macrophages is contact dependent and is mediated through Fas-FasL and TNF-TNFRI pathways (Chauhan et al, 2007). TRAIL-resistant melanoma cells

**Figure 7.** Interaction of Fas Ligand (FasL) with the Fas receptor (CD95) mediates apoptotic signaling to remove transformed and virus-infected cells. Cisplatin could affect signaling that involves activation of the death domain (TRADD) to activate caspase 8, lamins, PARP, DNA-PK, Bcl-2, PKC and a number of molecules leading to apoptosis and elimination of cancer cells. Cisplatin as well as Lipoplatin™ might modulate these pathways by a direct binding to cysteine residues in these proteins, not studied, thus modulating their activity.
are cross-resistant to apoptosis induced by the FasL death ligand but are more sensitive to nonapoptotic (necrotic) cell death induced by cisplatin (Zhang et al., 2006). The metastatic potential of osteosarcoma (OS) cells correlates inversely with Fas expression—that is, Fas-negative cells metastasize but Fas-positive cells do not; the explanation proposed is that Fas-positive OS lung metastases are eliminated by engagement with the Fas ligand (FasL) constitutively expressed on the surface of pneumocytes, whereas Fas-negative tumor cells are not. Loss of Fas may be one mechanism by which OS cells evade host resistance in the lung. Furthermore, there was a significant correlation between Fas expression and the administration of preoperative salvage chemotherapy of cisplatin, suggesting that cisplatin may induce regression by upregulating Fas (Gordon et al., 2005). Acquired cisplatin resistance in HeLa cells is due to a lower level of induced apoptosis; FasL expression was significantly enhanced in sensitive cells and remained upregulated up to the onset of apoptosis; in sensitive cells, caspase-8 along with caspase-9 and -3 were activated by cisplatin. On the contrary, cisplatin resistant HeLa cells the levels of Fas, Bax and Bid remained unchanged after cisplatin treatment.

In other cell types cisplatin may not be involved in Fas-FasL induced apoptosis. The expression levels of Fas, FasL, and FADD were not changed in T24 human bladder cancer cells by treatment with cisplatin; instead, cisplatin induced redistribution of Bax and cytochrome c and thus causes apoptosis in a mitochondria-dependent fashion. Furthermore, upregulation of Bcl-2 inhibited cisplatin-induced Bax translocation contributing to the development of cisplatin-resistance in T24 cells (Cho et al., 2006).

I. The protein kinase C (PKC) pathway

The protein kinase C (PKC) pathway may play an important role in cisplatin resistance in various types of tumor cells. This process involves IL-6, which induces dihydriol dehydrogenase expression in NSCLC cells and increases cellular resistance to cisplatin and adriamycin (Wang et al., 2007). Acquisition of resistance of ovarian carcinoma cells to the multinuclear platinum complex BBR3464 was associated with down-regulation of PKCα; however, the regulatory function of PKCα was not apparently implicated in the development of resistance to platinum compounds (Righetti et al., 2006). Cisplatin caused apoptosis in renal proximal tubular epithelial cells which was directly related to the activation of caspase-3 and DNA fragmentation; in addition, cisplatin caused the loss of cell-cell contacts prior to the onset of apoptosis which was associated with the altered localization of the adherens junction-associated protein β-catenin in association with PKC-mediated phosphorylation of the actin-capping protein adducin. PKC inhibitors arrested cisplatin–induced apoptosis (Imamdi et al., 2004).

J. Protein damage by cisplatin

In addition to DNA base modifications and cross-links cisplatin treatment may inhibit a significant number of enzymes, both cytoplasmic and nuclear. Very few such cases have been documented in the literature. Treatment of Chinese hamster AA8 cells with cisplatin resulted in a dose-dependent inhibition of the catalytic activity of DNA topoisomerase II; this inhibition was proposed to result in the development of secondary tumours as a result of cisplatin treatment of primary malignancies (Cantero et al., 2006). Antitumor platinum drugs form DNA-protein cross-links and cisplatin induces such complexes more effectively than the clinically irrelevant transplatin (Chválová et al., 2007).

K. Hsp90 and platinum drug chemotherapy

Heat shock protein 90 (Hsp90) is implicated in stabilizing the conformation and maintaining the function of over 100 identified proteins especially implicated in signaling and chromatin-remodeling pathways including the cell-signaling proteins EGFR, Her2/neu, HIF-1α, ERBB2, C-RAF, CDK4, AKT/PKB, steroid hormone receptors, mutant p53, survivin and telomerase hTERT. The chaperone function of HSP90 requires the formation of a multichaperone complex, which is dependent on the hydrolysis of ATP and ADP/ATP exchange. Cisplatin (also novobiocin) bind to the C-terminal dimerization domain of Hsp90 to inhibit its function (reviewed by Xiao et al., 2006). The ability of Lipoplatin™ in binding to Hsp90 is not known.

Most inhibitors of HSP90 are nucleotide mimetics, which block the intrinsic ATPase activity. Classes of Hsp90 inhibitors developed include the anasamycin geldanamycin and their derivatives 17-AAG and 17DMAG; the macrolide radicicol and their derivatives; purine-scaffold derivatives; pyrazoles; and shepherds that bind to the N-terminal high-affinity ATP-binding domain of Hsp90 (reviewed by Xiao et al., 2006). Sublethal concentrations of radicicol increased the sensitivity to cisplatin and to oxaliplatin in both MLH1-proficient cells and MLH1-deficient cells; however, radicicol might not be the drug to selectively re-sensitize cisplatin-resistant, MLH1-deficient tumor cells (Fedier et al., 2005); MLH1 is one of five proteins crucial to DNA mismatch repair (MMR).

Heat shock transcription factor 1 (HSF1) is the master regulator of heat-induced HSP expression and a promising therapeutic target in cervical carcinoma; small interfering RNA (siRNA) that silence HSF1 caused a dramatic increase in sensitivity to hyperthermo-chemotherapy with cisplatin, leading to massive (>95%) apoptosis of cancer cells (Rossi et al., 2006).

Hsp90 inhibitors accumulate at high levels primarily in tumor cells because tumor cells are "oncogene addicted" and require especially high levels of the high-ATPase form of Hsp90 (reviewed by Xiao et al., 2006). Inhibition of Hsp90 impairs EGFR- and hypoxia-mediated angiogenic signaling in gastric cancer cells (Lang et al., 2007). Modulation of Hsp90 offers the prospect of simultaneously inhibiting multiple signaling pathways and biological processes that have been implicated in the development of the malignant phenotype. HSP90 expression is high in breast cancer cell lines and HSP90-targeting agents are in clinical trials for breast cancer. Evaluation of HSP90 expression in early-stage breast cancer led to the conclusion that high HSP90 expression
was associated with decreased survival (Pick et al, 2007). Hence, Hsp90 is emerging as an exciting new target for the treatment of cancer especially because most of the proteins that interact with Hsp90 are known to function in the cell cycle, signaling and chromatin-remodeling pathways. The first-in-class inhibitor to enter and complete phase I clinical trials was the geldanamycin analogue, 17-allylamino-17-demethoxygeldanamycin (17-AAG) which promotes the proteasomal degradation of its misfolded client proteins. Geldanamycin induces degradation of HSP90 client proteins, which may promote the presentation of degradation peptides with major histocompatibility complex class I on cancer cells. Oncogenic proteins are more dependent on Hsp90 in maintaining their conformation, stability, and maturation than normal proteins. Hsp90 exists in an activated form in malignant cells but in a latent inactive form in normal tissues, suggesting that inhibitors selective for the activated form could provide a high therapeutic index (Maloney et al, 2007; Kasibhatla et al, 2007). A similar model is that drugs that inhibit the function of heat shock protein 90 (Hsp90) are of interest in the treatment of cancers because Hsp90 can deplete the cellular levels of signaling and anti-apoptotic molecules that are important for the growth and survival of many tumors (Bagatell et al, 2005). Hsp90 inhibitors potentiate the activity of drugs in cancer cells lines that are otherwise resistant to the drug. The Hsp90/Hsp70 chaperone complex is important for the survival of cancer cells and tumours. The simultaneous blockade of Hsp70, Hsc70 (a close family member of Hsp70) and Hsp90 was efficient in reducing breast cancer cell viability (Hávik and Bramham, 2007).

An orally administered Hsp90 inhibitor, IPI-504, was evaluated in a murine model of chronic myelogenous leukemia (CML) and proposed as a first line treatment of Philadelphia chromosome-positive CML by inhibiting leukemia stem cells and preventing the emergence of imatinib-resistant clones in patients (Peng et al, 2007).

Co-administration of oxaliplatin and the Hsp90 inhibitor 17-AAG enhanced necrosis and apoptosis in colorectal cancer cell lines (Rakitina et al, 2007). Treatment of cisplatin resistant ovarian adenocarcinoma cells with the Hsp90 inhibitor geldanamycin (GEL) in combination with cisplatin showed Akt depletion and S-phase arrest (Solár et al, 2007).

V. Cisplatin action at the DNA level and consequences

A. DNA damage by platinum drugs

The most important feature of platinum drugs from which their anticancer activity arises is their ability to elicit DNA damage. Figure 8A shows the different type of crosslinks formed between cisplatin (or of the related carboplatin and nedaplatin) with DNA and Figure 8B DNA adduct formation with oxaliplatin. Of the DNA adducts, 60-65% are intra-strand GG diadducts, 25-30% are AG diadducts, 5-10% are GNG diadducts and 1-3% are crosslinks between the two strands (Brabec and Kasparkova, 2005; Chvalová et al, 2007). Platinum crosslinks affect the positioning of the DNA around the histone octamer, forcing it into an asymmetric arrangement with respect to the core histone proteins (Danford et al, 2005). It has been proposed that the asymmetry of the DNA as it wraps around the histone octamer, the accessibility of nucleosomel versus linker DNA as well as of the active versus inactive condensed chromatin domains would greatly affect gene damage and their subsequent repair (Boulikas, 1992).

The antitumor properties of cisplatin are attributed to the kinetics of its chloride ligand displacement reactions leading to DNA crosslinking activities. DNA crosslinks inhibit replication, transcription and other nuclear functions and arrest cancer cell proliferation and tumor growth. DNA lesions generated by cisplatin, oxaliplatin, and JM216, are repaired in vitro with similar kinetics by the mammalian nucleotide excision repair pathway (Reardon et al, 1999). The minor-groove is an important receptor for enzymes and proteins involved in the processing and expression of genomic DNA. Irreversible minor-groove modifying agents acting on adenine-N3, such as a platinum-acridine conjugate have been synthesized; the design of a non-cisplatin type minor-groove pharacophore may opens new avenues in the design of platinum-based therapeutics (Guddneppanavar and Bierbach, 2007). DNA adducts formed by platinum-based anticancer drugs interfere with DNA replication. The eukaryotic DNA polymerases yeast pol zeta, human pol β, and human pol γ bypass oxaliplatin-GG adducts more efficiently than cisplatin-GG adducts (Vaisman et al, 2000). Compared with cisplatin, oxaliplatin formed significantly fewer Pt-DNA adducts and also induced potentially lethal bifunctional lesions, such as interstrand DNA cross-links (ISC) and DNA-protein cross-links (DPC) in CEM cells that were more effective in inhibiting DNA chain elongation (Woynarowski et al, 2000). DNA polymerases, the mismatch-repair system and damage-recognition proteins can all impart specificity to replicative bypass of Pt-DNA adducts (Chaney and Vaisman, 1999).

B. Recognition and repair of cisplatin damage

Binding of chromosomal proteins to cisplatin damaged DNA might either mask damage recognition by repair factors and impair its removal or constitute a damage-recognition signal recruiting components for the assembly of the DNA repair complex.

The XPC-hHR23B complex is a heterodimeric protein required for the initial step of DNA damage recognition in the global nucleotide excision repair (NER) pathway. The heterodimer exhibits a strong preference for UV- and cisplatin-damaged DNA, which is dependent on structural changes in the DNA, and not on adduct chemistry (Trego and Turchi, 2006). A crucial role of the basic region of the p53 C-terminal domain (aa 363-382) in the cisPt-DNA recognition has been demonstrated (Pivonková et al, 2006).

The DNA mismatch repair system appears to function as a detector of cisplatin adducts and mutations or methylation-mediated silencing of hMLH1, hMSH2, or hPMS2

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Figure 8 (A) DNA adduct formation with platinum compounds having two amino groups. (B) DNA adduct formation with oxaliplatin. (C) Structure of a stretch of a turn of the double helix showing different types of adducts and a protein-DNA crosslink. (D) Chemical structure of a GG diadduct formed by molecule of cisplatin in a stretch of six nucleotides. Platinum anticancer agents form bulky DNA adducts which are thought to exert their cytotoxic effect by blocking DNA replication.

Proteins that discriminate between cisplatin-DNA adducts and oxaliplatin-DNA adducts are thought to be responsible for the differences in tumor range, toxicity, and mutagenicity of these two important chemotherapeutic agents. Conformational differences between cisplatin-GG and oxaliplatin-GG adducts may be related to the ability of various DNA repair proteins, DNA binding proteins, and DNA polymerases repair and propagate apoptotic signals (Wu et al., 2007).

Proteins p53 and p73 act as transcription factors in cell cycle control, regulation of cell development and/or in apoptotic pathways. Both proteins bind to response
elements (p53 DNA-binding sites), typically consisting of two copies of a motif RRRCWGGYY. Cisplatin adducts inhibit p53 binding to a synthetic p53 DNA-binding site; bifunctional GG and AG adducts inhibited p53 or p73 sequence-specific DNA binding (Pivonková et al., 2006).

The zinc-finger protein 143 (ZNF143) is a human homolog of Xenopus transcriptional activator staf and its expression, is induced by DNA-damaging agents. ZNF143 preferentially binds to cisplatin-modified DNA. ZNF143 was associated with tumor suppressor p73 but not to p53. p73 could stimulate the binding of ZNF143 to cisplatin-modified DNA, Rad51 and flap endonuclease-1 are target genes of ZNF143 and overexpressed in cisplatin-resistant cells (Wakasugi et al., 2007). DNA polymerase eta can bypass cisplatin-induced intrastrand adducts and is involved in translesion synthesis past these replication-blocking adducts; cell lines with mutations in the DNA polymerase eta gene responsible for the syndrome xeroderma pigmentosum-variant (XP-V) are dramatically more sensitive to cisplatin (Albertella et al., 2005).

Cisplatin adducts are repaired by the nucleotide excision repair (NER) pathway involving, among others, recognition of the damage by High Mobility Group (HMG) nonhistone proteins and mismatch repair proteins as well as ERCC-1, one of the essential proteins in NER. ERCC1 siRNAs to specifically reduce the ERCC1 expression level in human cancer cells enhances their sensitivity to cisplatin (Chang et al., 2005). Defects in DNA mismatch repair produce low-level resistance to cisplatin from the failure to recognize the cisplatin adduct and propagate a signal to the apoptotic machinery. Therapeutic interventions at all these molecular levels, either with gene transfer or with small molecules that interfere with these processes, would greatly affect the ability of cancer cells to cope with cisplatin damage. The discovery of novel platinum molecules could also lead to novel advancements in bypassing cisplatin resistance (McKeage, 2005).

VI. Resistance to cisplatin and platinum compounds

A. Molecular mechanisms of resistance to cisplatin

The major limitation in the clinical applications of cisplatin has been the development of cisplatin resistance by tumors. Resistance to cisplatin developed during first line treatment is a major hurdle in the management of cancer patients leading to clonal expansion of chemotherapy-resistant cells. Both genetic and epigenetic changes explain acquired drug resistance. Alterations in DNA methylation result in the dysfunction of genes involved in cell-cell contact, in apoptosis and in several other pathways giving a survival advantage to chemoresistant cells (Chekhun et al., 2007). Cisplatin modulates a number of signaling pathways including the mitochondrial pathway, the DNA damage signaling, stress-related signals, the ERK pathway and many other. Acquisition of cisplatin resistance has been linked with loss of ERK activation by cisplatin (Villedieu et al., 2007a).

RAD51, XRCC2, and XRCC3 involved in homologous recombination are also implicated in cisplatin resistance; established cell lines with mutations in any of these genes are sensitive to cisplatin and mutations in the human population give a high risk to cancer (Danoy et al., 2007). A great number of additional genes are upregulated in cisplatin-resistant cells including the zinc-finger protein 143, the flap endonuclease-1, and the transcription factors YB-1, ATF4, and Clock. Downregulation of either Clock or ATF4 confers sensitivity of A549 cells to cisplatin and etoposide; on the contrary, ATF4-overexpressing cells show multidrug resistance and marked elevation of intracellular glutathione. Genes for glutathione metabolism are generally downregulated by the knockdown of ATF4 expression (Kohn et al., 2005; Igarashi et al., 2007).

Intrinsic resistance of cells in patients against radiotherapy and DNA-targeted chemotherapy may be linked to the status of the p53 system (Castedo et al., 2006). The hypoxia-inducible factor-1a (HIF-1a) is the key regulator that controls the hypoxic response of mammalian cells. Overexpression of HIF-1a expression by small interfering RNA enhanced the susceptibility of cells to cisplatin (Sasabe et al., 2007). Thirty-eight genes were upregulated and twenty-five were downregulated in a cisplatin-resistant cell line derived from a cisplatin-sensitive tongue squamous cell carcinoma line. Upregulated genes in the cisplatin-resistant cell line included RECG, involved in DNA repair, MAP2K6, involved in the MAP pathway, and CCND1 and CCND3 involved in cell cycle-regulation (Zhang et al., 2006). Activation of inhibitor of apoptosis proteins (IAPs) and their synergy with TNF-a via NF-kB translocation to the nucleus, interaction of hyaluronan with CD44 receptor to promote phospholipase C-mediated calcium ion signaling and topoisomerase II phosphorylation, and release of pro-inflammatory mediators including IL-6 are additional examples of biopathways linked with cisplatin-resistance (reviewed by Boulakis, 2007).

Globally, cisplatin resistance may arise from a decrease in cisplatin uptake by tumor cells across the cell membrane barrier, for example by downregulation of its copper/cisplatin transporter Ctrl or by upregulation of the copper/cisplatin efflux transporters ATP7A and ATP7B. In addition, cisplatin resistance may come by enhanced detoxification of cisplatin from increased levels of glutathione and metallothioneins, by a faster repair of cisplatin-induced DNA lesions, for example, from upregulation of genes involved in lesion recognition and nucleotide excision repair, by inhibition of apoptotic genes or activation of antiapoptotic genes so tumor cells do not perish after drug damage. Additional mechanisms of platinum resistance could involve decreased tumor blood flow, decreased binding of internalized cisplatin to DNA or protein targets. This occurrence could be due to, for example, a higher intracellular pH in chemoresistant cells, decreased mismatch repair, or presence of quiescent non-cycling cells (reviewed by Stewart, 2007).

Lipodipol™ seems to enter tumor cells via fusion with the cell membrane and phagocytosis, thus, proposed to have applications in cisplatin resistant tumors, arising from decreased transport of cisplatin across the cell membrane.

The probability of response to second line chemotherapy following platinum-based treatments is usually related to the platinum-free interval. Patients can be classified as either platinum-sensitive or platinum-resistant depending on whether they have relapsed or progressed within 26 weeks.
of completing first-line platinum-based chemotherapy (Gore et al, 2002). Expression of the mitogen-activated protein kinase phosphatase-1 (MKP-1), involved in inactivation of MAP-kinase pathways, regulation of stress-responses, and suppression of apoptosis, was a prognostic marker for shorter progression-free survival of patients with invasive ovarian carcinomas (Denkert et al, 2002). Apoptotic index can be predictive of treatment outcome in ovarian cancer (Mattern et al, 1998). Salvage monochemotherapy is generally used, but when the platinum-free interval is longer than 24 months, retreatment with platinum compounds and/or taxanes is indicated.

Metallothionein, a thiol-containing protein, is linked with tumor resistance to cisplatin. Overexpression of metallothionein in a cell line by stable gene transfection resulted in 7-fold protection from cisplatin (Holford et al, 2000).

Cisplatin resistance from faster repair of DNA lesions in tumor cells is also an established mechanism. The transcript abundance levels of twelve selected DNA repair or multi-drug resistance genes (LIG1, ERCC2, ERCC3, DDIT3, ABCC1, ABCC4, ABCC5, ABCC10, GTF2H2, XPA, XPC and XRCC1) were related to cisplatin resistance in NSCLC cell lines. The expression profiles of the pairs ABCC5/GTF2H2 and ERCC2/GTF2H2 were proposed as markers suitable to identify cisplatin resistant tumors in fine needle aspirate biopsies (Weaver et al, 2005).

B. Gene expression and cisplatin resistance

Several studies have been done to identify the expression of genes related to cisplatin resistance. The presence of a functional wild-type p53 gene renders cancer cells sensitive to cisplatin. Epithelial ovarian cancer patients undergoing platinum-base chemotherapy showed marked differences in p53 levels; in addition, 83% of nonresponders to chemotherapy had mutations in the p53 gene compared with 16% for responders (Kigawa et al, 2002). The DNA mismatch repair genes, and hMSH2 in combination with one of its heterodimer partners, binds specifically to cisplatin adducts (Niedner et al, 2001).

Transfer of the NPR2L tumor suppressor gene sensitized the response of cells to cisplatin, yielding a 40% greater inhibition of tumor cell viability and resulting in a 2- to 3-fold increase in induction of apoptosis by activation of multiple caspases (Ueda et al, 2006).

Development of techniques to disrupt the expression of single genes in engineered cells has identified a number of previously unsuspected genes that control sensitivity to cisplatin. These include the DNA mismatch repair, the sphingosine-1-phosphate lyase 1, the Golgi vesicular membrane-golvexit, cAMP-specific phosphodiesterases, the regulatory subunit of the cAMP-dependent protein kinase (PKA), the Lyn tyrosine kinase, and a photolyase (reviewed in Niedner et al, 2001). DNA damage leads to simultaneous activation of proapoptotic and survival pathways in a time-dependent, hierarchical manner.

The experimental strategies under investigation aimed at overcoming cisplatin resistance such as introduction of the functional p53 and p21 genes (Di Felice et al, 1998) usually mutated during carcinogenesis, or of genes that intervene with apoptotic pathways such bax, BClXL, bcl-2 are likely to contribute to limiting the disease in combination with regimens using platinum drugs (reviewed by Boulikas and Vougiouka, 2003). For example, p53 is frequently mutated in late-stage cancer and the introduction of a functional wild-type p53 gene in gene therapy applications renders cancer cells sensitive to cisplatin (Buller et al, 2002a,b; reviewed by Boulikas, 1998). Cisplatin mediates killing of cancer cells by activating the intrinsic mitochondrial apoptotic pathway and the status of p53 is a key factor in determining the efficacy of apoptotic signaling (Bagnoli et al, 2007). The HuUO-44 gene plays a role in ovarian cancer cell attachment and proliferation; small interfering RNAs (siRNAs) able to mediate HuUO-44 silencing resulted in the inhibition of cell growth and proliferation and correlated with cisplatin sensitivity (Leong et al, 2007). TIP30 is a tumor suppressor whose expression is altered in human cancers; TIP30 mutants could inhibit cisplatin-induced apoptosis in HepG2 hepatocellular carcinoma cells (Jiang et al, 2007).

VII. Lipoplatin™ as an anti-angiogenesis factor

A major effort against cancer focuses on targeting tumor vasculature. Inhibiting tumor cells of their ability to build vasculature is known to dramatically impair the ability of the tumor for further growth depriving tumor cells of nutrients. Although the efforts are focusing in the potential of angiostatin, endostatin or oncostatin, targeting of the VEGF receptors such as Flk-1 and a number of other approaches, it was reasoned in this study that the ability of "Lipogenes" to preferentially infiltrate tumors after systemic delivery (Figure 9) might arise from their ability to extravasate through imperfections of the leaky and often compromised tumor vasculature. If this mechanism is indeed the case, then our targeting method ought to show a more prominent staining of the tumor vasculature compared to the solid tumor mass. As this is indeed the case is shown in Figure 9.

The photograph shows the SCID mouse implanted with MCF-7 human breast tumor cells that were allowed to develop into large measurable solid tumors at about 30 days post-inoculation. The animal was injected i.p. with a liposomally encapsulated plasmid carrying the β-galactosidase gene under control of the CMV promoter. The encapsulated plasmid had the same shell as Lipoplatin™. Following systemic injection with the reporter β-galactosidase gene, and at 24 h postinjection the carcass was stained with X-Gal to reveal the sites of transgene expression after relocalization of the gene vehicles from the injected peritoneal cavity to the various tissues through the arteries, veins and lymph system. It can be concluded that the liposomally encapsulated gene was dramatically concentrated into tumors, it crossed successfully the cell membrane, survived any lysosomal, endosomal or cytoplasmic nucleases, was imported into nuclei, was successfully expressed into RNA and translated into protein responsible for the blue staining 24-4 h from injection.

It is evident that the sites of gene transfer and expression in vivo are primarily the tumor sites (Figure 9). Even more important, the subcutaneous vasculature developed to supply the tumor with nutrients has a more pronounced staining indicating that cells (presumably endothelial cells) of tumor vasculature are the targets for entry of the liposome and expression of the foreign gene. A control experiment with the same amount of naked plasmid did not reveal gene expression in the tumor and most other tissues presumably as a result of plasmid degradation in the peritoneal cavity.
Since the LipoGene vehicle mediates delivery and expression of the gene, both in tumor cell mass and in tumor vasculature, it is concluded that LipoGenes (and by extension Lipoplatin™) enter through the cell membrane in both cell types. It is known that plasmid DNA is poorly taken across the nuclear membrane barrier. It is concluded that Lipoplatin™ causes apoptotic death to endothelial cells of tumor vasculature in addition to the apoptosis it induces to the tumor cells as deduced from previous studies in xenografts (Boulakas, 2004). All these observations lead to the conclusion that Lipoplatin™ not only kills tumor cells but also cells of the tumor vasculature. It can therefore be classified also as an anti-angiogenesis agent. Lipoxal has the same shell as Lipoplatin™ and LipoGenes; therefore, Lipoxal might also turn out to be an antiangiogenesis drug. A similar targeting of tumors has been shown in human studies after Lipoplatin™ infusion in cancer patients followed by surgery and measurement of platinum levels in tumor specimens and in the adjacent normal tissue; it would be interesting to measure platinum levels in tumor vasculature from human specimens removed surgically after Lipoplatin™ infusion. Blood vessels of tumors carry specific markers that are usually related to angiogenesis that may be beneficial for promoting antiangiogenic therapy. Using phage display peptide libraries peptides can be identified (for example, the peptide CTKNSYLMC) with affinity to gastric cancer vascular endothelial cells. Antiangiogenesis therapy using this and other peptides is a potential candidate for targeted drug delivery in antivascular therapy and diagnosis of gastric cancer (Liang et al, 2006). Antisense VEGF oligodeoxynucleotides formulated in cationic liposomes can downregulate the expression of VEGF and could inhibit the growth of tumors. Canstatin is a newly identified antiangiogenesis protein with a potent inhibitory effect on the proliferation and growth of endothelial cells. A hypoxia-inducible canstatin-expressing vector was designed as a gene therapy tool for antiangiogenesis research (Li et al, 2006).

Antiangiogenic agents alone, cannot eradicate tumors completely and should be combined with other therapy to enhance their effects. For example, Fk-1, a soluble vascular endothelial growth factor (VEGF) receptor, is a potent inhibitor of angiogenesis. Fk-1 gene therapy combined with cisplatin improved antitumor efficacy in animals (Wang et al, 2006). The fact that Lipoplatin™ is endowed with the molecular properties of cisplatin plus the ability of its nanoparticles to target and kill endothelial cells of tumor vasculature, suggests a significant potential of this drug with the two properties, that of a chemotherapy drug and that of an antiangiogenesis agent combined together.

VIII. Tumor targeting in human studies

Lipoplatin™ is preferentially concentrated in the primary tumor and the metastases in human patients undergoing chemotherapy. High tumor levels are seen at about 20 h from infusion of the drug under conditions where blood levels of Lipoplatin™ have dropped. Targeting is done at two levels: (i) after intravenous injection Lipoplatin™ is preferentially (40-times) concentrated into tumors by extravasation through the leaky tumor vasculature; (ii) once inside the tumor Lipoplatin™ is taken up more avidly by the cell membrane of the tumor cell compared to normal cell (5 times more). These two mechanisms together contribute to a 200-fold higher damage to cancer tissue compared to normal.

**Figure 9.** Targeting of the vasculature of the primary tumor and the metastases after systemic delivery of "Lipogenes" using our proprietary liposomal encapsulation technology. The photos (left) show a SCID mouse implanted with MCF-7 human breast tumor cells. Following systemic injection with the reporter b-galactosidase gene, the carcass was stained with X-Gal. Preferential staining of the tumors, especially of the vascular system around the tumors is evident (magnified picture to the right).
tissue and contribute to the low side effects of the drug. This was shown by intravenous infusion of Lipoplatin™ in four independent patient cases (one with hepatocellular adenocarcinoma, two with gastric cancer, and one with colon cancer) who underwent Lipoplatin™ infusion followed by a prescheduled surgery ~20h later. Direct measurement of platinum levels in specimens from the excised tumors and the adjacent normal tissues as well as metastases (colon metastasis from a liver tumor, liver metastasis from a gastric cancer) showed that total platinum levels (and by consequence platinum-bearing drug concentration) were on the average 40 times higher in malignant tissue compared to the adjacent normal tissue specimens; most effective targeting was observed in colon cancer with an accumulation up to 200-fold higher in colon tumors compared to normal colon tissue. Gastric tumor specimens had the highest levels of drug than any other tissue and, thus, Lipoplatin™ may prove effective against stomach cancers in future clinical studies.

One important issue contributing to the therapeutic efficacy of Lipoplatin™ results from its ability to target primary tumors and metastases and to cause a greater damage to tumor tissue compared to normal tissue. During tumor growth neo-angiogenesis is needed to develop tumor vasculature to enable supply with nutrients for growth and expansion in a process known as neoangiogenesis. The tumor uptake of Lipoplatin™ results from the preferential extravasation of the 100-nm liposome nanoparticles through the leaky vasculature of tumors. Indeed, the endothelium of the vascular walls during angiogenesis have imperfections that need a certain period for maturation (Figure 10). During angiogenesis, Lipoplatin™ particles with long circulation properties evade immune surveillance and are able to pass through the leaky vasculature and concentrate in the tumor at about 2- to 40-fold higher concentrations compared to the adjacent normal tissue in human studies. One additional mechanism for the higher accumulation of Lipoplatin™ in tumor tissue, compared to normal tissue, arises from the higher uptake of Lipoplatin™ nanoparticles by tumors presumably arising from a more avid phagocytosis by tumor cells. The second mechanism results to an average of 5- to 10-fold higher uptake of Lipoplatin™ by tumor cells, compared to normal cells in human studies giving an overall 10 to 400-fold higher tumor cell uptake and binding to macromolecules.

Intravenous infusion of Lipoplatin™ resulted in targeting of primary tumors and metastases in four independent patient cases (one with hepatocellular adenocarcinoma, two with gastric cancer, and one with colon cancer) who underwent Lipoplatin™ infusion followed by a prescheduled surgery ~20h later. Direct measurement of platinum levels in specimens from the excised tumors and normal tissues showed that total platinum levels were on the average 10-50 times higher in malignant tissue compared to the adjacent normal tissue specimens (Boulikas et al, 2005). Most effective targeting was observed in colon cancer with an accumulation up to 200-fold higher in colon tumors compared to normal colon tissue. Of the several surgical specimens, gastric tumors displayed the highest levels of total platinum suggesting Lipoplatin™ as a candidate anticancer agent for gastric tumors; gastric tumor specimens had up to 260 micrograms platinum /g tissue that was higher than any tissue level in animals treated at much higher doses. Fat tissue displayed a high accumulation of total platinum in surgical specimens in three different patients correlating to the lipid capsule of cisplatin in its Lipoplatin™ formulation. It was also inferred that normal tissue had more platinum trapped in the tissue but not reacted with macromolecules whereas tumor tissue displayed platinum that reacted with cellular macromolecules; the data were consistent with a model where Lipoplatin™ damages more tumor compared to normal cells. In conclusion, Lipoplatin™ has the ability to preferentially concentrate in malignant tissue both of primary and metastatic origin following intravenous infusion to patients. In this respect, Lipoplatin™ emerges as a very promising drug in the arsenal of chemotherapeutics.

IX. Conclusions and Prospects

Liposomes can be used as carriers of peptide, protein, and antigen-encoding DNA vaccines (Gregoriadis et al, 1999). Liposomes may be effective vehicles to improve the delivery of antisense oligonucleotides to the liver for the therapy of hepatotropic viruses (Soni et al, 1998).

Figure 10. Lipoplatin™ nanoparticles extravasate preferentially through the compromised endothelium of the vasculature of the tumor.
Phospholipid liposomes and charged nanoparticles can be mixed together using sonication can yield particle-stabilized liposomes that repel one another and do not fuse (Zhang and Granick, 2006). A nanoliposomal CPT-11 (irinotecan) formulation has been described with unprecedented drug loading efficiency and in vivo drug retention using a modified gradient loading method; the maximum tolerated dose in normal mice was determined to be 80 mg/kg for free CPT-11 and >320 mg/kg for nanoliposomal CPT-11 (Drummond et al, 2006). Drugs of poor water-solubility and high toxicity, such as Camptothecin, can benefit from nanotechnology formulations.

Others have used sterically stabilized liposomes for various applications; these prevent opsonization and reticular endothelial system uptake. PEGylation is known to greatly enhance the longevity of proteins, liposomes and other molecules in blood circulation (reviewed by Martin and Boulikas, 1998). Naturally occurring polymers of N-acetylleuaraminic acid (polysialic acids) are biodegradable, exhibit long half-lives in the blood circulation and have therefore been proposed as carriers of short-lived drugs and small peptides (Gregoriadis et al, 2000). Poly-(lactide) (PLA), poly-(lactide-co-glycolide) (PLGA) and poly-(lactide-co-caprolactone) (PLCL) microspheres have also been used for the encapsulation of 5-fluorouracil by spray drying and slow-release for inhalation delivery system for adjuvant therapy of lung cancer (Hitzman et al, 2006).

Cisplatin, one of the most widely used and most effective cytotoxic agents in the treatment of epithelial malignancies were encapsulated into 110-nm in diameter liposomes in a stable formulation, Lipoplatin™. One important issue contributing to the therapeutic efficacy of Lipoplatin™ results from its ability to target primary tumors and metastases and to cause a greater damage to tumor tissue compared to normal tissue. Tumor uptake of the Lipoplatin™ nanoparticles results from their preferential extravasation through the leaky vasculature of tumors. Furthermore, a higher uptake of Lipoplatin™ nanoparticles by tumors takes place presumably arising from a more avid phagocytosis by tumor cells compared to adjacent normal tissue in human studies. The two mechanisms result to an overall 10 to 400-fold higher intracellular uptake of total platinum in tumor cells compared to cells in normal tissue. Lipoplatin™ is currently under several Phase III evaluations, A Phase III multicenter clinical trial uses weekly 120 mg/m² Lipoplatin™ in combination with gemcitabine as first line treatment against non-small cell lung cancer (NSCLC) and is being compared to cisplatin plus gemcitabine. Another Phase III study compares weekly Lipoplatin™ plus 5-fluorodeoxyuridine (5-FU) versus cisplatin plus 5-FU against head and neck cancers. A third Phase III uses Lipoplatin™ in combination with paclitaxel as first line treatment against NSCLC and is being compared to cisplatin plus paclitaxel. It is anticipated that chemotherapy regimens integrating Lipoplatin™ will allow higher overall survival of patients suffering with non-small cell lung, pancreatic, gastric and other cancers, with low side effects and improvement in quality of life compared to cisplatin regimens.

Interim analysis of a Phase III study using 120 mg/m² weekly Lipoplatin™ plus gemcitabine as first line in NSCLC using Lipoplatin™ has shown a staggering 84% stable disease and response rate compared to 65% in the cisplatin plus gemcitabine arm with lower side effects and improvement in quality of life. One additional ongoing Phase III study using Lipoplatin™ plus paclitaxel as first line in NSCLC also shows non-inferiority with lower side effects in the Lipoplatin™ compared to cisplatin arm.

Lipoplatin™ and the platform encapsulation technology applied to its manufacturing procedure adds a strong tool in molecular oncology to wrap up preexisting anticancer drugs into nanoparticle formulations that alter the biodistribution, lower the side effects, minimize the toxic exposure to normal tissues while maximizing tumor uptake and penetration of the drug. The shell of the liposome in the Lipoplatin™ formulation has a number of patented features that differentiates it from previous drug formulations. Its loading method is based on reverse micelles whereas the negatively-charged DPPG molecule on the surface gives to the nanoparticles their fusogenic properties, an important feature for cell entry across the nuclear membrane barrier as suggested from gene therapy studies where entrance of the encapsulated gene is needed for expression (Figure 9). In addition, their small size results in passive extravasation to tumors whereas a more avid phagocytosis characteristic of tumor cells further enhances the intracellular and nuclear uptake of the drug. A PEG-coating also gives to the particles long circulation properties in body fluids essential for tumor accumulation. For example, Phase I studies have shown a half-life of 120 h (5 days) for Lipoplatin™ at 100 mg/m² compared to 6h for cisplatin.

A similar technology is applicable to liposomal encapsulation of plasmids carrying therapeutic genes for gene therapy applications in cancer and other disease. Obviously, regimens integrating combination Lipoplatin™ chemotherapy with liposomal gene therapy would have the advantage of targeting both nanoparticles classes to similar tissues in vivo, especially to primary solid tumors and metastases; a more potent anticancer effect is expected than using the drugs separately or in a nonliposomal form, something that needs human testing. So far the human IL-12 has been used in human trials with a liposomally-encapsulated virus expressing this gene; the completed Phase I study has proven safety and has determined the MTD (Stathopoulos et al, in preparation). Once safety has been shown, IL-12 has been replaced by p53 and the new liposomal drug is on its way to Phase I of clinical trials. There are hundreds of genes available for human studies that can also be combined with Lipoplatin™ in future clinical trials.

A putative antiangiogenic activity of Lipoplatin™ has been shown in animal studies where a gene was wrapped up in a capsule identical to that of Lipoplatin™ (Figure 9); the liposomally encapsulated plasmid nanoparticles carrying the gene were injected intravenously and the expression pattern in animal tissue revealed targeting of tumors and tumor vasculature. This implies that Lipoplatin™ particles are primarily targeted to tumors and tumor vasculature. However, further details of cellular uptake of the Lipoplatin™ particles by tumors and normal tissue await further elucidation. One could undertake electron microscopy studies for direct visualization of Lipoplatin™ vesicles entering the cell membrane in animal tissue following Lipoplatin™ infusion. Available animal studies have deciphered the time for maximal platinum accumulation in the major animal tissues after Lipoplatin™ or cisplatin treatment. Studies can also be undertaken to determine the extent and nature of damage caused by Lipoplatin™, versus cisplatin at the DNA level, and the level of other macromolecules.
Studies in human patients who received Lipoplatin™ infusion followed by surgery in about 24 hours and examination of total platinum in tumor specimens and in adjacent tissue also confirmed the tumor accumulation of the drug (Boulikas et al, 2005).

The targeting of Lipoplatin™ to the vasculature of the tumor is an additional desirable anticancer property of the drug. This property of Lipoplatin™, in addition to its tumor concentration by passive extravasation and tumor uptake because of its nanoparticle characteristics could lead to human testing in combination with drugs that have a mechanism of action complementing or synergizing that of Lipoplatin™. For example, ionizing radiation eliciting DNA strand breaks or taxanes stabilizing tubulin polymers could be shown to have a synergistic effect with Lipoplatin™ even better to that with cisplatin. Furthermore, Lipoplatin™ could be combined with a number of other chemotherapy regimens reducing the overall toxicity of the combination therapy.

The advent of taxanes (paclitaxel, docetaxel) stabilizing tubulin, of molecules that can inhibit signaling and a number of new approaches such as those targeting apoptosis or DNA topoisomerases is revolutionizing cancer chemotherapy. A plethora of clinical trials in progress optimizes the different ways drugs can be administered; for example, the addition of cisplatin or carboplatin to paclitaxel results in higher response rates than for each of the drugs as single agents (reviewed by Ranson and Thatcher, 1999).

The present article has reviewed the features and possible clinical applications of a nanoparticle formulation of cisplatin. The same technology was also applied to a liposome formulation of oxaliplatin (Lipoxal™) that has completed successfully a Phase I (Stathopoulos et al, 2006b) currently under Phase II evaluation against gastric cancer and pancreatic cancer. One could envisage application of nanotechnology and the extension of the Lipoplatin™ and Lipoxal formulations to taxanes and other molecules with tumor targeting abilities. Such an achievement and its promotion to the clinic would increase the efficacy of chemotherapy while reducing the side effects. The end goal of an effective anticancer regimen should always be the improvement in the quality of life of the patient and a significant extension in life.

In previously treated patients acquired resistance to chemotherapy is a major hurdle. The major factor of resistance appears to be linked with transport of the chemotherapy drug across the cell membrane barrier. In this capacity, Lipoplatin™, suggested to enter by direct fusion rather than the Ctrl transporter, is proposed to have applications in cisplatin resistant tumors.

Lipoplatin™ is anticipated to successfully complete several Phase III studies and become an important addition to the arsenal of anticancer drugs. It is anticipated that chemotherapy regimens integrating Lipoplatin™ will allow higher overall survival of patients suffering with non-small cell lung, pancreatic, head and neck, gastric and other cancers currently under Phase III evaluation, with lower side effects and improvement in quality of life compared to cisplatin regimens.

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References


Boulikas: Molecular mechanisms of cisplatin and Lipoplatin™


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Wu Y, Bhattacharyya D, King CL, Baskerville-Abraham I, Huh SH, Boysen G, Swenberg JA, Temple B, Campbell SL, Chaney SG (2007) Solution structures of a DNA dodecamer duplex with and without a cisplatin 1,2-d(GG) intrastrand cross-link: comparison with the same DNA duplex containing an oxaliplatin 1,2-d(GG) intrastrand cross-link. Biochemistry. 46, 6477-6487.


