Preclinical activity of the liposomal cisplatin Lipoplatin in ovarian cancer

Naike Casagrande, Marta Celegato, Cinzia Borghese, Maurizio Mongiat, Alfonso Colombatti, Donatella Aldinucci

Experimental Oncology 2, CRO Aviano National Cancer Institute, Aviano, PN, Italy; Department of Medical and Biological Science Technology and MATI (Microgravity Ageing Training Immobility) Excellence Center, University of Udine, Italy.

*Corresponding Author: Donatella Aldinucci, Experimental Oncology 2, CRO Aviano National Cancer Institute, via F. Gallini 2, Aviano I-33081, Italy. Phone: Italy-0434-659234; Fax: Italy-0434-659428; e-mail: daldinucci@cro.it

Running title: Preclinical activity of Lipoplatin in ovarian cancer

Key words: ovarian cancer; liposomal Cisplatin; spheroids; migration; cancer stem cells.

Acknowledgments: This research was supported by Ministero della Salute, Ricerca Finalizzata FSN, I.R.C.C.S., Rome, Italy

The authors declare no potential conflicts of interest.

Word count: 4703  Figures: 5  Table: 1

Statement of translational relevance

At present, standard treatment for ovarian cancer involves tumor debulking with platinum-based chemotherapy. The response to this regimen is at least 70% of patients; however, 60-80% of the first responders relapse within 18 months with a platinum-resistant disease. Lipoplatin is one of the most promising liposomal platinum drug formulations under clinical investigation. Our preclinical data demonstrated that Lipoplatin was active in a panel of ovarian cancer cell lines, including Cisplatin-resistant cells. We have shown that Lipoplatin induced apoptosis and ROS production, reduced spheroid growth and migration, reduced cancer stem cell number, and inhibited more than 90% tumor xenograft growth with low toxicity, while cisplatin resulted either un-effective or effective but too toxic. Lipoplatin showed a synergistic activity with Doxorubicin and Abraxane. This preclinical data provide the rational for the clinical assessment of Lipoplatin in aggressive Cisplatin-resistant ovarian cancer patients.
Abstract

Purpose: Cisplatin and its platinum derivatives are first-line chemotherapeutic agents in the treatment of ovarian cancer; however, treatment is associated with tumor resistance and significant toxicity. Here we investigated the antitumoral activity of Lipoplatin, one of the most promising liposomal platinum drug formulations under clinical investigation.

Experimental design: In vitro effects of Lipoplatin were tested on a panel of ovarian cancer cell lines, sensitive and resistant to Cisplatin, using both 2-dimensional (2D) and 3D cell models. We evaluated in vivo the Lipoplatin anticancer activity using tumor xenografts.

Results: Lipoplatin exhibited a potent antitumoral activity in all ovarian cancer cell lines tested, induced apoptosis, activated the caspases 9, 8 and 3, down-regulating Bcl-2 and up-regulating Bax. Lipoplatin inhibited thioredoxin reductase (TrxR) enzymatic activity and increased Reactive Oxygen Species (ROS) accumulation; reduced EGFR expression and inhibited cell invasion. Lipoplatin demonstrated a synergistic effect when used in combination with Doxorubicin, widely used in relapsed ovarian cancer treatment, and with the albumin-bound Paclitaxel, Abraxane. Lipoplatin decreased both ALDH and CD133 expression, markers of ovarian cancer stem cells. Multicellular aggregates/spheroids are present in ascites of patients and most contribute to the spreading to secondary sites. Lipoplatin decreased spheroids growth, vitality, and cell migration out of pre-formed spheroids. Finally, Lipoplatin inhibited more than 90% tumor xenografts growth with minimal systemic toxicity, and after the treatment suspension no tumor progression was observed.

Conclusion: These preclinical data suggest that Lipoplatin has potential for clinical assessment in aggressive Cisplatin-resistant ovarian cancer patients.
Introduction

Ovarian cancer is the fifth leading cause of cancer-related death in women in developed countries and has one of the highest ratios of incidence to death (1). The standard postoperative chemotherapy for epithelial ovarian cancer is a combination therapy including Cisplatin and taxanes. Most patients are responsive to chemotherapy at first; however, toxicity and acquired resistance to Cisplatin have proven challenging and represent the major obstacle to improve the prognosis of patients with ovarian cancer (1). Cisplatin resistance is due to a broad panel of molecular and functional alterations, including the reduced intracellular accumulation through the copper transporter 1 (Ctr1) and the increased efflux through the cell membrane (2). Thus, the development of new Cisplatin formulations or the encapsulation into liposomes to overcome both resistance and toxicity remains a high priority (3).

While various formulations of Cisplatin encapsulated into liposomes demonstrated a good anticancer activity in vitro, the results obtained in vivo were often disappointing (3). One example is SPI-77, which did not produce significant clinical response rates in several Phase II studies of patients with inoperable head and neck cancer, advanced non-small-cell lung cancer (4), and also in ovarian cancer (5). The lack of therapeutic efficacy was likely due to slow and inefficient release of platinum from SPI-77.

Lipoplatin is one of the most promising liposomal platinum drug formulations under clinical investigation (3, 4, 6). It has shown similar efficacy as Cisplatin in pancreatic, head and neck cancer, NSCLC and HER-2/neu–Negative Metastatic Breast Cancer with a major benefit of a strongly reduced toxicity (6, 7).
Lipoplatin nanoparticles fuse with the cell membrane or are rapidly taken up by cancer cells by their avidity for nutrients, as shown with fluorescent nanoparticles, and Lipoplatin disguises as a nutrient with its lipid shell (6). Thus the toxic payload enters the cytoplasm bypassing active import, explaining Lipoplatin’s efficacy against platinum-resistant tumors (6). Accordingly, we demonstrated that Lipoplatin is active both in vitro and in vivo against Cisplatin-resistant cervical cancer cells (8). Lipoplatin has an enhanced half-life circulation time in body fluids and tissues and can extravasate through the leaky tumor vasculature reaching concentrations 10- to 200-fold higher in the tumor or metastases compared to the adjacent normal tissues. During their extravasation into primary and metastatic tumor tissue shown in human studies (9) Lipoplatin nanoparticles attack the epithelial cancer cells linked to the property of classic Cisplatin chemotherapy, but also the endothelial cells of tumor vasculature because of their lipid nature; thus Lipoplatin is acting as a chemotherapeutic and anti-angiogenesis drug (10). The greater the vascularization of the tumor, the greater the concentration of Lipoplatin in the tumor or metastasis (9).

Given the properties of Lipoplatin to overcome Cisplatin-resistance and to induce low toxicity (6, 8), this drug could represent a good alternative to Cisplatin. The aim of our study was to analyze the efficacy of Lipoplatin in the ovarian cancer setting. Although many drugs show promising results in vitro, the success rate of anti-cancer therapies translating from in vitro culture systems into the clinic is about 5%, suggesting the use of multiple techniques during the preclinical evaluation of new anticancer agents. Thus, using the in vitro traditional two-dimensional (2D) model, the in vitro three-dimensional (3D) cell culture, that seems to better reflect the histological, biological, and molecular features of primary tumors (11), and the in vivo tumor xenograft tumor, we demonstrated that Lipoplatin was active against
Cisplatin-resistant cells in both monolayer cultures (2D model) and spheroids (3D model), it synergized with Doxorubicin and Abraxane, and proved to be very effective in vivo.

Materials and Methods

Drugs

Lipoplatin the liposomal formulation of Cisplatin, and labelled with fluorescein isothiocyanate (FITC) Lipoplatin (Lipoplatin-FITC) (6), were generously provided by Regulon (Regulon Inc., Mountain View, California); Cisplatin was purchased from Mayne Pharma, Carboplatin from Teva (Pharma Italia, S.r.l), Abraxane (Nab-Paclitaxel) from Celgene, Doxorubicin from Pfizer, Docetaxel from Hospira and Paclitaxel from Actavis. Drugs were dissolved in medium at the indicated concentrations immediately before use.

Cell lines and culture conditions

Human ovarian epithelial carcinoma derived cancer cell lines MDAH 2774 (ATCC CRL-10303), OVCAR3 (ATCC HTB-161), SKOV3 (ATCC HTB-77), TOV21G (ATCC CRL-11730) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), A2780 and its Cisplatin-resistant clone A2780cis from Sigma, Inc., St. Louis, MO, USA. OVCAR5 (NIH) cells were provided by Dr. Baldassarre (CRO, Aviano, Italy). Cell lines were further authenticated for their origin by BMR Genomics (Padua, Italy) on January 2012 according to Cell ID™ System (Promega) protocol and using Genemapper ID Ver 3.2.1 to identify DNA STR profiles. Histology origins: ovarian carcinoma from an untreated patient (A2780 and its Cisplatin-resistant clone A2780cis), clear cell carcinoma (TOV21G), endometriod carcinoma (MDAH), and malignant cells derived from the ascites (OVCAR3, OVCAR5, and SKOV3). Cells were cultured in RPMI medium (Sigma-Aldrich-Italy) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Sigma), 0.2 mg/mL penicillin/streptomycin (Sigma) and 0.1% (w/v) L-glutamine (Sigma) at 37°C in a 5% CO2 fully humidified atmosphere.
Cytotoxicity assay

Cells (4.0×10^3) were cultured in 96-well flat-bottom and treated with increasing concentrations of Lipoplatin (2.5-100 µM), Cisplatin (2.5-100 µM) or Carboplatin (2.5-200 µM) at 37°C for 72 h. Triplicate cultures were established for each treatment. Cytotoxicity was measured by using MTT assay. The half maximal inhibitory concentration (IC₅₀) value was calculated using the CalcuSyn software (Biosoft, Ferguson, MO, USA) (12).

Experimental design for drug combinations and Chou–Talalay analysis for synergy

First, we determined the IC₅₀ for Doxorubicin, Abraxane, Docetaxel and Paclitaxel for OVCAR5 and SKOV3. Then, 4.0×10^3 cells were incubated with each drug alone or in combination for 72 h and cytotoxicity was evaluated by MTT assay. The combined drug effects was calculated using the diagonal constant ratio combination (12). Synergy was determined calculating the combination index (CI) using Calcusyn software. A CI value of 1 indicates an additive effect between two drugs. CI values less than 1 indicate synergy, the lower the value the stronger the synergy. On the contrary, CI values more than 1 indicate antagonism.

Flow cytometry

Cells (5.0×10^4) were incubated for 72 h on 6-well plates in complete medium in the presence of (30 µM) Lipoplatin. Annexin-V binding (Becton-Dickinson [BD] Pharmeden, San Jose, CA), DNA fragmentation (Apo-Direct kit, BD), changes in mitochondrial membrane potential (MitoTracker® Red CMXRos, Invitrogen, Milan, Italy), cytochrome-c (Cyt-c) release (BD) (Materials and Methods, Supplementary), caspases 3, 8, and 9 activation (Chemicon International, Milan, Italy), mitochondrial reactive oxygen species (ROS) (MitoSox reagent working solution, Molecular Probes, Invitrogen), B-cell lymphoma/leukemia-2 (Bcl-2) (DAKO Cytomation, Milan, Italy), B-cell lymphoma-extra large (Bcl-xL) (Cell Signalling, Danvers, MA, Research).
USA) and Bcl-2 associated x protein (Bax) (BD), epithelial growth factor receptor (EGFR) (anti-EGFR monoclonal antibody (mAb) 528 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and CD133 (AC133, Miltenyi Biotec, Auburn, CA, USA.) were evaluated as previously described (8, 13). Cell cycle was evaluated by PI staining. Aldehyde dehydrogenase (ALDH) activity was evaluated using Aldeflour reagent based method (Stem Cell Technologies, Vancouver, Canada). Briefly, cells (2x10^5/mL) were incubated for 40 min at 37 °C with Aldeflour reagent with and without the ALDH-inhibitor (DEAB). Viable antibody-labeled cells were identified according to their forward and right-angle scattering, electronically gated and analyzed on a FACSCalibur flow cytometer (BD), using cell quest software (BD).

**Thioredoxin Reductase (TrxR) enzymatic activity assay**

Cells (7.5x10^4) were treated with Lipoplatin (20, 30 µM) for 72 h. TrxR activity was assessed using the Thioredoxin Reductase Assay Kit (Sigma-Aldrich) (13).

**Invasion assay**

Invasion was assessed by the FATIMA assay (8). After drug treatment (72 h with 10 µM Lipoplatin) cells (1.0x10^5 cells/insert) tagged with the lipophylic dye Fast Dil (Molecular Probes) were seeded in 150 µL of serum free medium in the upper side of collagen type I-coated Boyden chamber inserts. Complete medium with 20% FCS was used as chemoattractant. Invasion was monitored using a computer-interfaced GeniusPlus microplate reader (Tecan, Rapperswit, Switzerland) and the percentage of transmigrated cells was determined by FATIMA software.

**Three Dimension (3D)-multicellular spheroids formation assays, growth and migration**

To obtain spheroids, 48-well plates were coated twice with 20 mg/mL of poly(2-hydroxyethyl methacrylate) (poly-HEMA; Sigma) (14) in 95% ethanol and washed once with PBS before cell seeding. Spheroids were generated plating 5x10^4 SKOV3 cells in complete medium. To
evaluate the effect of Lipoplatin on spheroid formation, cells were cultured in poly-HEMA coated 48 wells in the presence of the drug (10, 25 or 50 µM). After 72 h, spheroids were photographed, harvested and dissociated into single-cell suspensions by trypsinization, then the extent of apoptosis (AnnexinV/PI staining) was determined (15). Alternatively, Lipoplatin or Cisplatin activity was evaluated on preformed single spheroids as described (14). Briefly, 1.0x10^4 SKOV3 cells were dispensed into poly-HEMA coated round-bottom 96-well. After 4 days spheroids were treated with increasing concentrations of Lipoplatin (25, 50 µM). Spheroid size was measured up to 15 days after Lipoplatin treatment initiation. A 50% medium replacement was performed on days 3, 7, 10 and 15. Responses were evaluated by the measurement of spheroid size at regular intervals (16) using an inverted microscope (Eclipse TS/100, Nikon) with photomicrographic systems DS Camera Control Unit DS-L2. Spheroid volumes were calculated using the formula: (width^2 x length x 3.14)/6 (17). To assess cell viability, spheroids were incubated for 30 min with 2 µg/ml PI and then observed under laser fluorescence microscope (DMI 600013, Leica) (original magnification 4×). Single spheroids were incubated with 1:50 Lipoplatin-FITC (6). Spheroid images were acquired using the confocal microscope (Leica DM IRE2) to trace the penetration of Lipoplatin-FITC.

Migration/dissemination (14): this assay was performed in 96-well plates pre-coated with 10 μg/mL collagen I (Sigma Aldrich) and blocked with BSA (1 mg/mL) for 2 h. Pre-formed spheroids were layered (3-5 spheroids/well) in the 96-well plates in the absence or in the presence of Lipoplatin (25, 50 µM). Image analysis software was used to calculate the spheroid size. The extent of migration was determined using Adobe Photoshop by outlining the entire area of the dispersed cells (14). The fold-change in area was calculated dividing the pixel area of the spheroid at 24 and 48 h by the pixel area at time 0.

**Tumor xenograft experiments**
All the in vivo studies were approved by the Institutional Ethics Committee.

Six-week-old female athymic nu/nu (nude) mice were purchased from Charles River (Lecco, Italy) and 2.7x10⁶ OVCAR5 cells suspended in 0.1 mL of matrigel (1:3 in PBS) were inoculated in the right flank of each mouse. When tumors reached ~44 mm³ in volume, mice were divided randomly into two groups of 8 mice each and were treated three times/week with intraperitoneal (IP) injection of 20 mg/kg Lipoplatin or drug-free vehicle. Tumor size was measured over time using a caliper, and volumes were calculated according to the standard formula: (width² x length x 3.14)/6. At day 39 the treatment was suspended for 14 days. When control tumors had reached a volume of ~1000 mm³, mice were sacrificed. The mouse organs were excised and fixed in formalin for tissue toxicity analyses. Sections were cut and counterstained with haematoxilin and eosin according to standard procedures.

Software and statistical analysis of data

Values are presented as the mean with the standard error of not less than three measurements (unless otherwise stated) (mean ± SEM). To estimate the equal sample size for the mouse study groups, the experiment was designed to be able to detect a 0.60 difference with 0.90 power and a α error of 0.05. Statistical analysis was performed using GraphPad Prism 6 Software (GraphPad, San Diego, CA, USA). The statistical significance of differences was determined by Student’s t-test for comparison between two groups. Analysis of variances (ANOVA) was used to evaluate the correlation of data among three or more groups; consecutive multiple comparison analysis was performed using Dunnett’s or Tukey’s tests. Differences were considered statistically significant at P<0.05.
Results

**Lipoplatin inhibited proliferation and induced apoptosis in Cisplatin-sensitive and -resistant ovarian cancer cell lines**

First, we evaluated the *in vitro* cytotoxic effects of Lipoplatin, Cisplatin and Carboplatin (Fig. 1A) on a panel of ovarian cancer cell lines with different sensitivity to Cisplatin. Treatment with Lipoplatin induced a dose-dependent inhibition of cell proliferation with IC$_{50}$ ranging from 14.6 in MDAH to 32.1 µM in OVCAR3 cells (Fig. 1A upper panel). IC$_{50}$ for Lipoplatin was higher (about 4-fold) than that of Cisplatin (Fig. 1A, central panel) in all cell lines tested excluding OVCAR5 that had a similar sensitivity to both drugs (Fig. 1A). On the contrary, A2780 (Cisplatin, IC$_{50}$= 1.46 µM) and its Cisplatin-resistant clone A2780cis (Cisplatin, IC$_{50}$= 10.3 µM) showed a comparable sensitivity to Lipoplatin (IC$_{50}$=17.8 µM and 17.7 µM, respectively) (Fig. 1A upper panel). Thus, Lipoplatin exhibited a similar cytotoxic effect in cell lines with different histological origins and with a wide range of sensitivity to Cisplatin, including cell lines considered Cisplatin-resistant (18).

The sensitivity of ovarian cancer cell lines to Carboplatin resulted very similar to that of Cisplatin, excluding TOV21G cells (Fig. 1A, lower panel). A2780 cells were more sensitive to Carboplatin than A2780cis, that showed the highest IC50 together with OVCAR5 and TOV21G cells.

Next, to measure Lipoplatin efficacy to induce cell cycle modifications and apoptosis we used OVCAR5 (the least sensitive cell line to Cisplatin) and SKOV3 cells (intrinsic Cisplatin-resistant (18) and forming spheroids). Treatment for 24 h with Lipoplatin (30 µM) induced an increase in the S and G$_2$M phases in OVCAR5 cells and a block in G$_2$M phase in SKOV3 cells (data not shown). Lipoplatin induced apoptosis in a dose-dependent manner, as evaluated
by the Annexin-V/PI staining (Fig. 1B), the activation of caspases 9, 8, and 3 (Fig. 1C) and DNA fragmentation (Fig. 1D, left panels). In analogy with Cisplatin (19), Lipoplatin decreased the mitochondrial membrane potential (Fig. 1D, central panels) and induced Cyt-c release (Fig. 1D, right panels). Lipoplatin increased the pro-apoptotic molecule Bax, decreased the anti-apoptotic Bcl-2, and only slightly decreased Bcl-xL expression (Fig. 1E). Lipoplatin increased ROS production (Figs. 2A and B) and reduced the enzymatic activity of TrxR (Fig. 2C), a selenoenzyme essential to maintain the cellular redox status and to protect against oxidative damage due to ROS accumulation (20), in a dose-dependent manner in both cell lines.

In addition, we evaluated Lipoplatin activity in A2780 and its Cisplatin-resistant clone A2780cis. Even if A2780 and A2780cis had the same IC_{50} for Lipoplatin, the drug induced significant apoptosis (Supplementary Figs. S1A, B and C) and ROS formation (Supplementary Figs. S1D and E) only in A2780 cells. On the contrary, while Lipoplatin was able to stop cell growth, it only slightly increased Annexin-V staining and ROS formation in A2780cis (Supplementary Fig. S1S). A2780cis and OVCAR5 cells had similar IC_{50}, however Cisplatin induced apoptosis and ROS formation only in A2780 and A2780cis, but not in OVCAR5 cells (Supplementary Fig. S2).

Taken together our results suggest that different mechanisms of resistance are involved in A2780cis, obtained by in vitro selection with Cisplatin, and OVCAR5 cells, derived from ascitic fluid of a patient with progressive ovarian adenocarcinoma without prior cytotoxic treatment.

**Lipoplatin synergized with Doxorubicin and Abraxane**

The standard treatment of patients with advanced ovarian cancer is cytoreductive surgery followed by combination chemotherapy with taxanes or Doxorubicin and platinating agents (21, 22). We evaluated if also the combination of Lipoplatin with Doxorubicin (Table 1) or
with either one of the three different taxanes, Docetaxel, Paclitaxel and the albumin-stabilized Paclitaxel Abraxane (23, 24) (Table 1), resulted more effective than each agent used separately. OVCAR5 cells were less sensitive (IC50=0.87 μM) to Doxorubicin than SKOV3 cells (IC50=0.13 μM) (Table 1); however, Lipoplatin and Doxorubicin synergized in both cell lines (CI < 1.0) (Table 1). While the combination of Lipoplatin with Paclitaxel or Docetaxel resulted in additive effects in OVCAR5 and in low synergy in SKOV3 cells, Abraxane showed clear synergistic activity in both cell lines (CI <1) (Table 1).

In OVCAR5 we observed a high synergistic activity of Cisplatin with Doxorubicin and Abraxane, but not with Paclitaxel and Docetaxel. In SKOV3 Cisplatin strongly synergized with all the four drugs and especially with Abraxane (Supplementary Table S1). Finally, the combination of Carboplatin with Doxorubicin exerted very low synergistic (OVCAR5) or additive (SKOV3) effects. In SKOV3 cells all the three taxanes exerted a significant synergistic activity, whereas in OVCAR5 only Paclitaxel displayed synergy (Supplementary Table S2).

Lipoplatin inhibited invasion and down-modulated EGFR expression

To exclude that a lower migration rate could be attributable to a decreased cell proliferation, cells were cultured for 72 h in the presence of less drug (10 μM) and at low serum concentration. Then, we evaluated cell invasion through a type I collagen-coated Boyden chamber. Already at 5 h, Lipoplatin decreased invasion of about 45% and 51% in OVCAR5 and SKOV3 cells, respectively (Fig. 3A), and this level of inhibition was maintained at 24 h. EGFR is usually over-expressed in ovarian carcinoma, and its activation is related not only to survival but also to invasion and metastasis (25). Both OVCAR5 and SKOV3 had similar invasive properties (Fig. 3A) and expressed high levels of EGFR (Figs. 3B and C). Lipoplatin down-regulated in a dose-dependent manner EGFR expression (Figs. 3B and C).

Lipoplatin reduced ALDH+ and CD133+ cells and inhibited both growth and migration of
cells from pre-formed spheroids

Together with the ability to form spheroids (26), the enzymatic activity of ALDH and CD133 expression (27) are considered markers of ovarian cancer stem cells (CSCs) and of drug resistance. OVCAR5 expressed higher amounts (about 2 fold) of ALDH+ cells than SKOV3 (Fig. 4A). CD133 expression was similar (about 5%) in both OVCAR5 and SKOV3 cells. Treatment with Lipoplatin decreased in a dose-dependent manner ALDH enzymatic activity (Figs. 4A and B) and CD133 expression (Figs. 4C and D) in both cell lines.

Spheroids represent a 3D in vitro system that more closely resembles the in vivo tumor microenvironment and a more efficacious first-line approach to study drug activity and the invasive phenotype (11, 28). In agreement with Lee et al. (11) OVCAR3 and MDAH could spontaneously form large loose aggregates or spheroids, OVCAR5, TOV21G, A2780 and A2780cis formed small aggregates (data not shown), and the SKOV3 cell line formed large dense aggregates (LDAs)/spheroids. Based on their ability to form LDAs spheroids, we used SKOV3 cells to quantify Lipoplatin activity also in 3D conditions.

Spheroids obtained by SKOV3 cells increased their volume in a time-dependent manner, reaching a 6-fold increase after 15 days of cultivation (Figs. 5A and B). Lipoplatin, as Cisplatin (Supplementary Fig. S3A), inhibited the spheroids growth (Figs. 5A and B) in a dose-dependent manner. Lipoplatin increased the PI positive dead cells (Fig. 5C). Consistently, we found that Lipoplatin-FITC deeply penetrated into spheroids (Fig. 5D). Lipoplatin decreased the capability of SKOV3 cells to migrate/disseminate out of the spheroids with a 50% reduction of the area covered by migrating cells (Figs. 5E and F).

Lipoplatin inhibited the growth of ovarian cancer xenografts

We analyzed the anticancer activity of Lipoplatin also in vivo. For this purpose, OVCAR5 cells (2.7x10⁵) were injected into the right flank of 6-week-old female athymic nude mice and,
once tumors reached a volume of ~44 mm$^3$, the mice were treated intraperitoneally three times a week with vehicle alone or with vehicle containing Lipoplatin (20 mg/Kg). Significant tumor growth inhibition by Lipoplatin was apparent already after 17 days of treatment (Fig. 5G). At day 41 the control tumors grew to a mean 417.2 ± 5 mm$^3$ in size, whereas Lipoplatin showed a 82% inhibitory effect, since the treated tumors reached a mere size of 73.12± 8 mm$^3$ (**p < 0.01; Fig. 5G). The treatment was suspended (Fig. 5F dashed lines) and mice were followed for 14 more days. Tumors of untreated mice continued to grow (Fig. 5G) and after 14 days doubled their size and reached a volume of 969.74 ± 8 mm$^3$, whereas tumors of Lipoplatin treated mice were inhibited by more than 90%. While treatment with the same concentration of Cisplatin was lethally toxic for mice, there was no histological detectable cytotoxicity involving the animals’ heart, spleen, liver and kidney in mice treated with Lipoplatin (data not shown). A sublethal dose of Cisplatin (6 mg/kg)(29) led to a significant decrease of tumor growth (Supplementary Fig. S3B) but was still severely toxic for the animals (Supplementary Fig. S3C). Mice experienced a significant weight loss (about 50% of control), two mice out of 5 died (at day 22) during the treatments and 2 had to be euthanized (at day 28) to avoid further suffering, prior to the end of the experiment (at day 42). Only an even lower dose of Cisplatin (3 mg/kg) was not toxic for the animals but did not affect tumor growth (Supplementary Figs. S3B and C).
Discussion

Cisplatin is very effective for the treatment of ovarian cancers; however, its severe toxicity and the emergence of primary or acquired resistance limit its efficacy. In this study, we investigated the biological activity and molecular mechanisms of action of a new formulation of Cisplatin, Lipoplatin, the most clinically active formulation of liposomal encapsulated Cisplatin to date (3, 6).

Lipoplatin affected cell proliferation exhibiting a similar cytotoxic effect in ovarian cancer cell lines of different histological origins and with a wide range of Cisplatin sensitivity, including Cisplatin-resistant cell lines. Lipoplatin exerted its cytotoxic effect by inducing apoptosis, as previously demonstrated in cervical cancer (8), determined mitochondrial membrane depolarization, Cyt-c release and the activation of both caspase 9 and 3. Consistent with recent studies demonstrating that another formulation of liposomal Cisplatin induced the extrinsic apoptotic pathway in the Cisplatin-resistant A2780cis cells (30, 31), we found that also Lipoplatin activated caspase 8, indicating that the activity of this drug was exerted through the mitochondrial intrinsic and the extrinsic apoptotic pathways. Lipoplatin significantly affected the expression of two regulators of the mitochondrial apoptotic pathway: it decreased the pro-survival protein Bcl-2 and increased the pro-apoptotic Bax protein (32, 33).

The IC50 for Lipoplatin was higher than that for Cisplatin but similar in OVCAR5 and in A2780cis resistant cells. However, it should be considered that other liposomal cisplatin showed similar IC50 in A2780 and A2780cis cells (30, 31). Moreover, we found that Lipoplatin but not Cisplatin, used at the same concentration, induced apoptosis and ROS generation in OVCAR5 but not in A2780cis, thus suggesting that distinct liposomal
formulations could result in different cytotoxic activity and that different mechanisms of resistance to cisplatin are involved between OVCAR5 and A2780cis.

The antioxidant Trx system maintains the intracellular redox state and defends cells against oxidative damage due to ROS overproduction, leading to the formation of pro-apoptotic molecules (34). TrxR is upregulated in many malignant tumors (34, 35) and plays a central role against drug-induced oxidative stress, suggesting that this enzyme could become a suitable target for anti-cancer therapy. Lipoplatin, in analogy with Cisplatin (36), inhibited TrxR enzymatic activity and induced the generation of large amounts of mitochondrial superoxide.

Lipoplatin, and especially Cisplatin, demonstrated a synergistic effect with Doxorubicin, a chemotherapeutic drug widely used in relapsed ovarian cancer treatment (21) and with the albumin-bound Paclitaxel, Abraxane. Abraxane was the only taxane of the three tested capable of synergizing with Lipoplatin, as Docetaxel and Paclitaxel essentially exerted additive effects. Abraxane was more active than Docetaxel and Paclitaxel also in combination with Cisplatin, but not with Carboplatin. It is of note that Abraxane is used in Phase II clinical trials in patients with recurrent platinum-resistant primary epithelial ovarian or primary peritoneal carcinoma since it displays reduced toxicity respect to Paclitaxel (37).

EGFR over-expression and activation is related to ovarian cancer progression and increased migration (38). However, small molecule inhibitors of EGFR tyrosine kinase activity, such as Erlotinib, exhibited very limited activity as single agents in patients with recurrent or persistent ovarian cancer (39). Recently, we demonstrated that Lipoplatin decreased EGFR expression and cell migration in cervical cancer cells (8). Similarly we found here that Lipoplatin down-regulated EGFR expression and decreased cell migration, thus suggesting that it could not only exert direct cytotoxic effects on ovarian cancer cells, but
also decrease tumor invasion and/or proliferation induced by EGFR activation. Since ovarian carcinoma has a very poor rate of survival and is characterized by the presence of diffuse peritoneal metastases (40), this significant activity of Lipoplatin could also be useful in highly aggressive, poor-prognosis subgroup of high-grade malignant ovarian cancer characterized by the coexpression of ALDH/EGFR (41).

Together with the enzymatic activity of ALDH, the expression of CD133 is considered a marker of ovarian CSCs and is associated with drug resistance (27, 42, 43). Accordingly, Lipoplatin reduced the percentage of ALDH+ and CD133+ cells, suggesting that Lipoplatin could eliminate ovarian CSCs that are more chemo- and radio-resistant than the bulk of tumor cells and likely responsible for tumor relapse, the major clinical problem in cancer treatment.

Ovarian cancer cells are present in ascitic fluids either as single cells or as less or more compact macro aggregates, the latter contributing most to the spreading to secondary sites (28). In fact, these aggregates can travel through the ascitic fluid and attach to organs within the peritoneal cavity, a process that requires invasion of the mesothelial cell layer covering these organs (44). The 3D in vitro growth conditions (spheroids) recall several characteristics of ovarian cancer ascites cellular macro aggregates, including resistance to Cisplatin, and represent a more reliable model than 2D cell cultures (45). Moreover, the present finding that Lipoplatin inhibited the growth and the dissemination of cells from pre-formed spheroids is important. The positive relationship found between compact spheroid formation and invasive behavior (28) implies a preferential survival of an invasive subpopulation of ovarian cancer cells, as cells in spheroids are more resistant to several chemotherapeutics (11). Preventing/reducing ovarian cancer spheroids or reducing CSCs may represent a novel strategy to decrease metastases and to improve the efficacy of
existing therapeutics. The data in this study support an additional property of Lipoplatin, that of an anti-metastasis drug.

Finally, while Cisplatin used at the same concentration of Lipoplatin (20 mg/Kg) caused a severe toxicity in nude mice (46), Lipoplatin inhibited tumor xenografts of OVCAR5 with minimal systemic toxicity and even if the treatment was discontinued no tumor progression was observed, suggesting that the schedule used was very effective. Moreover, lower doses of Cisplatin (3 and 6 mg/kg) were either un-effective or effective but too toxic.

Monotherapy studies in lung cancer showing almost negligible (grade I) toxicity in human studies and a very high efficacy (38% partial response, 43% stable disease) as second-line treatment (47) establish Lipoplatin as a very exciting drug of a high potential in the chemotherapy arsenal.

In conclusion, replacing Cisplatin with Lipoplatin in aggressive Cisplatin-resistant ovarian cancer patients, would add the advantage of lower toxicities as already shown in randomized Phase II and Phase III studies in NSCLC (48-50). Adding the advantage of reducing the metastatic potential and the putative ovarian cancer stem cells, and its synergistic activity with Abraxane and Doxorubicin, Lipoplatin in combination with Abraxane or Doxorubicin should be compared to Cisplatin + Abraxane/Doxorubicin in a randomized clinical study against ovarian cancer.
Reference List


Legends to figures

Figure 1. Lipoplatin induces mitochondria-mediated apoptosis and modulates Bax, Bcl-2 and Bcl-xL expression. (A) Cells were cultured for 72 h in the presence of increasing concentrations of Lipoplatin, Cisplatin or Carboplatin. IC$_{50}$ was obtained using the CalcuSyn software. Values in the bar graph represent the mean IC$_{50}$ ± SEM of different experiments. (B) FACS analysis of cells after 72 h incubation with different concentrations of Lipoplatin and double stained with Annexin-V-FITC and PI. Values in the bar graph represent the mean ± SEM of three different experiments. * P<0.05; ****P<0.0001 drug vs. medium. (C) Analysis of caspase-9, -8 and -3 activation after incubating cells with Lipoplatin (30 µM) for 24 and 48 h. Cells were harvested, washed and resuspended in complete medium supplemented with FLICA for 1 hour at 37°C, then washed again and analyzed by flow cytometry. Dotted lines indicate background fluorescence of cells. X- and Y-axes indicate the logarithms of the relative fluorescence intensity and relative cell number, respectively. FACS histograms are representative of one of three different experiments. (D) DNA fragmentation (Apo-Direct), mitochondrial membrane permeabilization (CMXRos), and Cyt-c release were assessed by flow cytometry after treatment for 72 h with Lipoplatin (30 µM). E, analysis of Bax, Bcl-2 and Bcl-xL expression. Cells were incubated with Lipoplatin (30 µM) for 72 h and then Bax, Bcl-2, and Bcl-xL expression was assessed by flow cytometry.

Figure 2. Lipoplatin induces ROS accumulation and inhibits TrxR activity. (A) ROS production: cells were treated with Lipoplatin for 72 h and the bar graphs represent the percentage of ROS as the mean ± SEM of three different experiments. (B) Representative FACS dot plots of one of three independent experiments showing ROS generation. (C) TrxR enzymatic activity:
values in the bar graph represent the mean ± SEM of three different experiments

***P<0.001, ****P<0.0001 drug vs. medium, ^^ P<0.0001 OVCAR5 vs. SKOV3.

**Figure 3.** Lipoplatin inhibits invasion and decreases EGFR expression. (A) Invasion of ovarian cancer cells through a collagen type I-coated Boyden chamber after treatment for 72 h with 10 µM Lipoplatin. Values in the bar graph represent the mean ± SEM of three different experiments. **P<0.01, drug vs. medium. (B) EGFR surface expression: cells were treated for 72 h with Lipoplatin and analyzed by flow cytometry using the anti-EGFR mAb 528. Values in the bar graph represent the mean ± SEM of three different experiments. ****P<0.0001 drug vs. medium; (C) representative FACS histograms of one of three independent experiments showing the decrease of EGFR expression after drug treatment.

**Figure 4.** Lipoplatin reduces CD133+ and ALDH+ cells. Cells were treated with different concentrations of Lipoplatin (10, 20, 30 µM). (A) Quantification of ALDH as percentage of positive cells. Values in the bar graph represent the mean ± SEM of three different experiments. The ALDH inhibitor diethylamino-benzaldehyde (DEAB) was used as negative control. (B) Representative FACS dot plots of one of three independent experiments showing ALDH quantification (% of positive cells) after Lipoplatin treatment. (C) Quantification of CD133 as percentage of positive cells. Values in the bar graph represent the mean ± SEM of three different experiments. (D) Representative FACS histograms of one of three independent experiments showing CD133 expression after Lipoplatin treatment. *P<0.05 and ****P<0.0001 drug vs. medium.
**Figure 5.** Lipoplatin inhibits spheroids growth, migration and tumor xenograft. (A) SKOV3 single pre-formed spheroids were cultured for 15 days in the absence or in the presence of Lipoplatin (25, 50 µM). Responses were evaluated by spheroid volume measurements at regular intervals. Values in the bar graph represent the mean ± SEM of three different experiments. ****P<0.0001 drug vs. medium. (B) Representative phase contrast microphotographs showing volume decrease by Lipoplatin treatment (original magnification 4x). (C) After Lipoplatin treatment, spheroids were incubated with PI and then observed under fluorescence microscope. (D) Representative confocal images of FITC-labeled Lipoplatin (Lipo-FITC) penetration into single SKOV3 spheroids. (E and F) Inhibition of migration/dissemination on matrix protein of SKOV3 spheroids by Lipoplatin. SKOV3 single spheroids were placed on collagene-I coated plates in the presence of Lipoplatin (25, 50 µM). (E) Histograms showing the migration rate of spheroids, evaluated as the area covered by migrating cells from spheroids and represented as fold increase respect to the area (pixel) covered at time=0 ****P < 0.0001, drug vs. medium. Values in the bar graph represent the mean ± SEM of three different experiments. (F) Images were captured after 24 h and 48 h using an inverted microscope (phase contrast microphotographs, original magnification 4x). (G) *In vivo* anticancer activity of Lipoplatin (OVCAR5 xenograft). Tumor volumes were measured in female athymic nude mice after IP injection of either drug-free medium or containing 20 mg/kg Lipoplatin three times a week using a caliper. At day 39, treatment was suspended for 14 days (dashed lines). Each value represents the mean ± SEM of 8 animals per group. ****P < 0.0001 Lipoplatin vs. control.
Table 1. Combination index (CI) values for OVCAR5 and SKOV3 cell lines treated with Lipoplatin and Doxorubicin, Abraxane, Docetaxel or Paclitaxel.

<table>
<thead>
<tr>
<th></th>
<th>Lipoplatin (μM)</th>
<th>DOXO (μM)</th>
<th>CI</th>
<th>ABX (ng/ml)</th>
<th>CI</th>
<th>DTX (nM)</th>
<th>CI</th>
<th>PTX (ng/ml)</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVCAR5</td>
<td>4.23</td>
<td>0.21</td>
<td>0.617</td>
<td>8.42</td>
<td>0.687</td>
<td>4.51</td>
<td>0.947</td>
<td>6.32</td>
<td>1.282</td>
</tr>
<tr>
<td></td>
<td>8.46</td>
<td>0.43</td>
<td>0.839</td>
<td>16.84</td>
<td>0.415</td>
<td>9.02</td>
<td>1.105</td>
<td>12.64</td>
<td>0.623</td>
</tr>
<tr>
<td></td>
<td>*16.93</td>
<td>*0.87</td>
<td>0.488</td>
<td>*33.68</td>
<td>0.325</td>
<td>*18.04</td>
<td>0.683</td>
<td>*25.27</td>
<td>0.752</td>
</tr>
<tr>
<td></td>
<td>33.86</td>
<td>1.73</td>
<td>0.718</td>
<td>67.36</td>
<td>0.539</td>
<td>36.08</td>
<td>0.729</td>
<td>50.54</td>
<td>0.808</td>
</tr>
<tr>
<td></td>
<td>67.72</td>
<td>3.46</td>
<td>1.222</td>
<td>134.72</td>
<td>1.220</td>
<td>72.16</td>
<td>1.260</td>
<td>101.08</td>
<td>1.119</td>
</tr>
<tr>
<td>SKOV3</td>
<td>4.26</td>
<td>0.03</td>
<td>1.390</td>
<td>11.76</td>
<td>0.381</td>
<td>10.23</td>
<td>0.524</td>
<td>34.11</td>
<td>0.682</td>
</tr>
<tr>
<td></td>
<td>8.53</td>
<td>0.06</td>
<td>0.877</td>
<td>23.52</td>
<td>0.536</td>
<td>20.45</td>
<td>0.728</td>
<td>68.23</td>
<td>0.586</td>
</tr>
<tr>
<td></td>
<td>*17.06</td>
<td>*0.13</td>
<td>0.385</td>
<td>*47.05</td>
<td>0.507</td>
<td>40.90</td>
<td>0.696</td>
<td>*136.45</td>
<td>0.706</td>
</tr>
<tr>
<td></td>
<td>34.12</td>
<td>0.26</td>
<td>0.586</td>
<td>94.10</td>
<td>0.723</td>
<td>81.80</td>
<td>0.636</td>
<td>272.90</td>
<td>0.617</td>
</tr>
<tr>
<td></td>
<td>68.24</td>
<td>0.52</td>
<td>1.420</td>
<td>188.20</td>
<td>1.380</td>
<td>163.60</td>
<td>1.190</td>
<td>545.80</td>
<td>0.950</td>
</tr>
</tbody>
</table>

Cells were incubated with each drug alone or in combination for 72 h and then cell viability was determined by the MTT assay and the CI calculated using CalcuSyn software.

* indicates the IC\textsubscript{50} for each drug. DOXO, Doxorubicin; ABX, Abraxane; DTX, Docetaxel; PTX, Paclitaxel.
Figure 1

A

Lipoplatin

Cisplatin

Carboplatin

IC_{50} (µM)

AZ270
AZ270cis
MDA-MB-231
OVCAR3
OVCAR5
SKOV3
TOV21G

B

Annexin-V + PI

Annexin-V

OVCAR5

SKOV3

FITC-Annexin-V (%)

Lipoplatin (µM)

C

OVCA5
SKOV3

OVCAR5
SKOV3

OVCAR5
SKOV3

Caspase 9

Caspase 8

Caspase 3

medium medium medium medium

C

medium medium medium medium

D

DNA FRAGMENTATION

CMXros

Cyt-c

medium medium medium medium

medium medium medium medium

fluorescence intensity

E

Bax

Bcl-2

Bcl-xL

medium medium medium medium

medium medium medium medium

medium medium medium medium

medium medium medium medium

fluorescence intensity
Figure 2

A

![Bar chart showing ROS (%) vs Lipoplatin (µM) for OVCAR5 and SKOV3 cells.](image)

B

![Flow cytometry plots showing ROS levels for OVCAR5 and SKOV3 cells at different lipoplatin concentrations.](image)

C

![Bar chart showing TrxR activity U/mg vs Lipoplatin (µM) for OVCAR5 and SKOV3 cells.](image)
Figure 3

A

![Graph showing invasion rate (%)](image)

B

![Graph showing EGFR (MFI)](image)

C

![Graph showing cell count](image)
Figure 4

A

% ALDH+ (MFI %)

B

Lipoplatin (µM)

C

% CD133+ (MFI %)

D

Lipoplatin (µM)

SSC

DEAB median 10 20 30

ALDH (fluorescence intensity)

OVCAR5

SKOV3

0.51% 5.51% 4.91% 4.15% 1.85%

0.59% 5.31% 8.62% 3.11% 1.30%

medium 10 20 30

CD133 (fluorescence intensity)

OVCAR5

SKOV3

Author Manuscript Published OnlineFirst on September 17, 2014; DOI: 10.1158/1078-0432.CCR-14-0713

Downloaded from clincancerres.aacrjournals.org on November 1, 2017. © 2014 American Association for Cancer Research.
Figure 5
Preclinical activity of the liposomal cisplatin Lipoplatin in ovarian cancer

Naike Casagrande, Marta Celegato, Cinzia Borghese, et al.

*Clin Cancer Res* Published OnlineFirst September 17, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-0713

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/09/18/1078-0432.CCR-14-0713.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.