Preclinical Activity of the Liposomal Cisplatin Lipoplatin in Ovarian Cancer

Naike Casagrande1, Marta Celegato1, Cinzia Borghese1, Maurizio Mongiat1, Alfonso Colombatti1,2, and Donatella Aldinucci1

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 two-dimensional (2D) and 3D cell models. We evaluated in vivo the lipoplatin antitumor activity using tumor xenografts.

Results: Lipoplatin exhibited a potent antitumoral activity in all ovarian cancer cell lines tested, induced apoptosis, and activated caspase-9, -8, and -3, downregulating Bcl-2 and upregulating Bax. Lipoplatin inhibited thioredoxin reductase enzymatic activity and increased reactive oxygen species accumulation and reduced EGF receptor (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 preformed spheroids. Finally, lipoplatin inhibited more than 90% tumor xenograft 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 patients with ovarian cancer.

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).

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 (NSCLC; ref. 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).
Translational Relevance
At present, the 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% to 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, and reduced cancer stem cell (CSC) number. Lipoplatin inhibited xenograft tumor growth to more than 90% and with low toxicity, whereas the effective dose of cisplatin was too toxic for the animals. Lipoplatin showed a synergistic activity with doxorubicin and Abraxane. This preclinical data provide the rationale for the clinical assessment of lipoplatin in aggressive cisplatin-resistant patients with ovarian cancer.

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 the efficacy of lipoplatin 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 than in the adjacent normal tissues. During their extravasation into primary and metastatic tumor tissue shown in human studies (9), lipoplatin nanoparticles attack not only 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 antiangiogenic 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 anticancer 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 2-dimensional (2D) model, the in vitro 3D cell culture, which seems to better reflect the histologic, biologic, 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 labeled with fluorescein isothiocyanate (FITC), and lipoplatin (lipoplatin-FITC; ref. 6) were generously provided by Regulon Inc. Cisplatin was purchased from Mayne Pharma, carboplatin from Teva (Pharma Italia, Srl), Abraxane (Nab-Paclitaxel) from Celgene, doxorubicin from P6zer, 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 ATCC; A2780 and its cisplatin-resistant clone A2780cis from Sigma, Inc. OVCAR5 (NIH) cells were provided by Dr. Baldassarre (CRO, Aviano, Italy). Cell lines were further authenticated for their origin by BMR Genomics on January 2012 according to Cell ID System (Promega) protocol and using Genemapper ID Ver 3.2.1 to identify DNA short tandem repeat profiles. Histology origins included ovarian carcinoma from an untreated patient (A2780 and its cisplatin-resistant clone A2780cis), clear cell carcinoma (TOV21G), endometrioid carcinoma (MDAH), and malignant cells derived from the ascites (OVCAR3, OVCAR5, and SKOV3). Cells were cultured in RPMI (Sigma–Aldrich) supplemented with 10% heat-inactivated 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 × 10^4) were cultured in a 96-well, flat-bottom plate and was treated with increasing concentrations of lipoplatin (2.5–100 μmol/L), cisplatin (2.5–100 μmol/L), or carboplatin (2.5–200 μmol/L) at 37°C for 72 hours. Triplicate cultures were established for each treatment. Cytotoxicity was measured by using the MTT assay. The half maximal inhibitory concentration (IC50) value was calculated using the CalcuSyn software (Biosoft; ref. 12).

Experimental design for drug combinations and Chou–Talalay analysis for synergy
First, we determined the IC50 values for doxorubicin, Abraxane, docetaxel, and paclitaxel for OVCAR5 and SKOV3. Then, 4.0 × 10^4 cells were incubated with each drug alone or in combination for 72 hours, and cytotoxicity was evaluated by MTT assay. The combined drug effects
were 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 2 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 × 10⁴) were incubated for 72 hours on 6-well plates in complete medium in the presence of (30 μmol/L) lipoplatin. Annexin V binding [Becton Dickinson (BD) Pharmingen], DNA fragmentation (Apo-Direct kit, BD), changes in mitochondrial membrane potential (MitoTracker Red CMXRsos, Invitrogen), cytochrome c (cyt c) release (BD; see Supplementary Materials and Methods), caspase-3, -8, and -9 activation (Chemicon International), mitochondrial reactive oxygen species (ROS; MitoSox reagent working solution, Molecular Probes, Invitrogen), Bcl-2 (DAKO Cytomation), Bcl-xL (Cell Signalling), Bax (BD), anti-EGFR monoclonal antibody (mAb) 528 (Santa Cruz Biotechnology Inc.), and CD133 (AC133, Miltenyi Biotec) were evaluated as previously described (8, 13). Cell cycle was evaluated by propidium iodide (PI) staining. Aldehyde dehydrogenase (ALDH) activity was evaluated using Aldeflour reagent–based method (Stem staining. Aldehyde dehydrogenase (ALDH) activity was evaluated using Aldeflour reagent–based method (Stem (8, 13)). Cell cycle was evaluated by propidium iodide (PI) staining. Aldehyde dehydrogenase (ALDH) activity was evaluated using Aldeflour reagent–based method (Stem Cell Technologies). Briefly, cells (2 × 10⁶/mL) were incubated for 40 minutes 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 CellQuest Software (BD).

Thioredoxin reductase enzymatic activity assay

Cells (7.5 × 10⁴) were treated with lipoplatin (20–30 μmol/L) for 72 hours. Thioredoxin reductase (TrxR) activity was assessed using the Thioredoxin Reductase Assay Kit (Sigma-Aldrich; ref. 13).

Invasion assay

Invasion was assessed by the FATIMA assay (8). After drug treatment (72 hours with 10 μmol/L lipoplatin), cells (1.0 × 10⁵ cells/insert) tagged with the lipophilic 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), and the percentage of transmigrated cells was determined by FATIMA software.

3D multicellular spheroid formation assays, growth, and migration

To obtain spheroids, 48-well plates were coated twice with 20 mg/mL of poly-HEMA [poly(2-hydroxyethyl methacrylate); Sigma; ref. 14] in 95% ethanol and washed once with PBS before cell seeding. Spheroids were generated by plating 5 × 10⁴ 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 μmol/L). After 72 hours, spheroids were photographed, harvested, and dissociated into single-cell suspensions by trypsinization, then the extent of apoptosis (Annexin V/PI staining) was determined (15). Alternatively, lipoplatin or cisplatin activity was evaluated on preformed single spheroids as described (14). Briefly, 1.0 × 10⁶ SKOV3 cells were dispensed into poly-HEMA–coated round-bottom, 96-well plates. After 4 days, spheroids were treated with increasing concentrations of lipoplatin (25, 50 μmol/L). 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: \(V = \frac{4}{3}\pi r^3\) (17). To assess cell viability, spheroids were incubated for 30 minutes with 2 μg/mL PI and then observed under laser fluorescence microscope (DMi 6000 BD, 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) assay was performed in 96-well plates pre-coated with 10 μg/mL collagenase I (Sigma-Aldrich) and blocked with BSA (1 mg/mL) for 2 hours. Preformed spheroids were layered (3–5 spheroids per well) in the 96-well plates in the absence or presence of lipoplatin (25, 50 μmol/L). 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 hours 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, and 2.7 × 10⁶ OVCARS5 cells suspended in 0.1 mL of Matrigel (1:3) in PBS) were inoculated in the right flank of each mouse. When tumors reached about 44 mm³ in volume, mice were divided randomly into 2 groups of 6 mice each and were treated 3 times per week with intraperitoneal injection of 20 mg/kg lipoplatin or drug-free vehicle. Tumor size was measured over time using a calliper, and volumes were calculated according to the standard formula: \(V = \frac{4}{3}\pi r^3\). At day 39, the treatment was suspended for 14 days. When control tumors had reached a volume of about 1,000 mm³, mice were sacrificed. The mouse organs were excised and fixed in formalin for tissue toxicity analyses. Sections were cut and counterstained with hematoxylin 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 3 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 an α error of 0.05. Statistical analysis was performed using GraphPad Prism 6 Software (GraphPad). The statistical significance of differences was determined by the Student t test for comparison between 2 groups. ANOVA was used to evaluate the correlation of data among 3 or more groups; consecutive multiple comparison analysis was performed using Dunnett or Tukey 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 sensitivities to cisplatin. Treatment with lipoplatin induced a dose-dependent inhibition of cell proliferation with IC50 value ranging from 14.6 to 32.1 μmol/L in OVCAR3 cells (Fig. 1A, top). The IC50 for lipoplatin was higher (about 4-fold) than that of cisplatin (Fig. 1A, middle) in all cell lines tested excluding OVCAR5 that had a similar sensitivity to both drugs (Fig. 1A). On the contrary, A2780 (cisplatin, IC50 = 1.46 μmol/L) and its cisplatin-resistant clone A2780cis (cisplatin, IC50 = 10.3 μmol/L) showed a comparable sensitivity to lipoplatin (IC50 = 17.8 and 17.7 μmol/L, respectively; Fig. 1A, top). Thus, lipoplatin exhibited a similar cytotoxic effect in cell lines with different histologic 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 was very similar to that of cisplatin, excluding TOV21G cells (Fig. 1A, bottom). 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 [intrinsically cisplatin-resistant (ref. 18) and forming spheroids]. Treatment for 24 hours with lipoplatin (30 μmol/L) induced an increase in the S- and G2-M phases in OVCAR5 cells and a block in G2-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 caspase-9, -8, and -3 (Fig. 1C), and DNA fragmentation (Fig. 1D, left). In analogy with cisplatin (19), lipoplatin decreased the mitochondrial membrane potential (Fig. 1D, middle) and induced cytochrome c release (Fig. 1D, right). Lipoplatin increased the proapoptotic molecule Bax, decreased the antiapoptotic Bcl-2, and only slightly decreased Bcl-xl expression (Fig. 1E). Lipoplatin increased ROS production (Fig. 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 IC50 for lipoplatin, the drug induced significant apoptosis (Supplementary Fig. S1A–S1C) and ROS formation (Supplementary Fig. S1D and S1E) 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. S1). A2780cis and OVCAR5 cells had similar IC50 values; 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 also evaluated whether the combination of lipoplatin with doxorubicin (Table 1) or with either of the 3 different taxanes, docetaxel, paclitaxel, and the albumin-stabilized paclitaxel Abraxane (refs. 23, 24; Table 1), was more effective than each agent used separately. OVCAR5 cells were less sensitive (IC50 = 0.87 μmol/L) to doxorubicin than SKOV3 cells (IC50 = 0.13 μmol/L; 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 4 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 3 taxanes exerted a significant synergistic activity, whereas in OVCAR5, only paclitaxel displayed synergy (Supplementary Table S2).

Lipoplatin inhibited invasion and downmodulated EGFR expression

To exclude that a lower migration rate could be attributable to a decreased cell proliferation, cells were cultured for...
72 hours in the presence of less drug (10 µmol/L) and at low serum concentration. Then, we evaluated cell invasion through a type I collagen–coated Boyden chamber. Already at 5 hours, 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 hours. EGFR is usually overexpressed 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 (Fig. 3B and C). Lipoplatin downregulated in a dose-dependent manner EGFR expression (Fig. 3B and C).

Lipoplatin reduced ALDH\(^+\) and CD133\(^+\) cells and inhibited both growth and migration of cells from preformed spheroids

Together with the ability to form spheroids (26), the enzymatic activity of ALDH and CD133 expression (27) are considered markers of ovarian CSCs and of drug resistance. OVCAR5 expressed higher amounts (~2-fold) of ALDH\(^+\) cells than SKOV3 (Fig. 4A). CD133 expression was
similar (~5%) in both OVCAR5 and SKOV3 cells. Treatment with lipoplatin decreased in a dose-dependent manner the ALDH enzymatic activity (Fig. 4A and B) and CD133 expression (Fig. 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 the study by Lee and colleagues (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 (LDA)/spheroids. On the basis of 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 (Fig. 5A and B). Lipoplatin, like cisplatin (Supplementary Fig. S3A), inhibited the spheroid growth (Fig. 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

Table 1. CI values for OVCAR5 and SKOV3 cell lines treated with lipoplatin and doxorubicin, Abraxane, docetaxel, or paclitaxel

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Lipoplatin, µmol/L</th>
<th>Doxorubicin, µmol/L</th>
<th>CI</th>
<th>Abraxane, ng/mL</th>
<th>CI</th>
<th>Docetaxel, nmol/L</th>
<th>CI</th>
<th>Paclitaxel, 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>8.46</td>
<td>0.42</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>
<td></td>
</tr>
<tr>
<td>16.93a</td>
<td>0.87a</td>
<td>0.488</td>
<td>33.68a</td>
<td>0.325</td>
<td>18.04a</td>
<td>0.683</td>
<td>25.27a</td>
<td>0.752</td>
<td></td>
</tr>
<tr>
<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>
<td></td>
</tr>
<tr>
<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>
<td></td>
</tr>
<tr>
<td>SKOV3</td>
<td>4.26</td>
<td>0.03</td>
<td>1.390</td>
<td>11.76</td>
<td>0.831</td>
<td>10.23</td>
<td>0.524</td>
<td>34.11</td>
<td>0.682</td>
</tr>
<tr>
<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>
<td></td>
</tr>
<tr>
<td>17.06a</td>
<td>0.13a</td>
<td>0.385</td>
<td>47.25a</td>
<td>0.507</td>
<td>40.90</td>
<td>0.696</td>
<td>136.45a</td>
<td>0.706</td>
<td></td>
</tr>
<tr>
<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>
<td></td>
</tr>
<tr>
<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>
<td></td>
</tr>
</tbody>
</table>

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

*aThe IC50 for each drug.
Lipoplatin (10 µmol/L) was investigated as a potential therapeutic agent in ovarian cancer. The treatment was suspended (Fig. 5F, inset). The mice were treated intraperitoneally 3 times a week with lipoplatin. Values in the bar graph represent the mean ± SEM of 3 different experiments. **P < 0.001, drug versus medium.

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 biologic activity and molecular mechanisms of action of a new formulation of cisplatin, lipoplatin, the most clinically active formulation of liposomal-encapsulated cisplatin to date. Lipoplatin inhibited invasion and decreases EGFR expression. 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 hours with 10 µmol/L lipoplatin. Values in the bar graph represent the mean ± SEM of 3 different experiments. **P < 0.001 vs. control, drug versus medium. B, EGFR surface expression: cells were treated for 72 hours with lipoplatin and analyzed by flow cytometry using the anti-EGFR mAb 528. Values in the bar graph represent the mean ± SEM of 3 different experiments. ***P < 0.0001 drug versus medium. MFI, mean fluorescence intensity. C, representative FACS histograms of 1 of 3 independent experiments showing the decrease of EGFR expression after drug treatment.

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 hours with 10 µmol/L lipoplatin. Values in the bar graph represent the mean ± SEM of 3 different experiments. **P < 0.001 vs. control, drug versus medium. B, EGFR surface expression: cells were treated for 72 hours with lipoplatin and analyzed by flow cytometry using the anti-EGFR mAb 528. Values in the bar graph represent the mean ± SEM of 3 different experiments. ***P < 0.0001 drug versus medium. MFI, mean fluorescence intensity. C, representative FACS histograms of 1 of 3 independent experiments showing the decrease of EGFR expression after drug treatment.

The antioxidant TrxR system maintains the intracellular redox state and defends cells against oxidative damage due to reactive oxygen species (ROS) that are generated during chemotherapy. Lipoplatin, a new formulation of cisplatin, has been shown to be effective in ovarian cancer cell lines. In this study, we investigated the biologic activity and molecular mechanisms of action of lipoplatin in ovarian cancer cell lines.

Tumors of untreated mice continued to grow (Fig. 5G) and after 14 days doubled their size and reached a volume of 969.74 ± 8 mm³, 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 histologically 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; ref. 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 (~50% of control), 2 of 5 mice died (at day 22) during the treatments and 2 had to be euthanized (at day 28) to avoid further suffering, before the end of the experiment (at day 42). An even lower dose of cisplatin (3 mg/kg) was not toxic for the animals and did not affect tumor growth (Supplementary Fig. S3B and S3C).

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 biologic activity and molecular mechanisms of action of a new formulation of cisplatin, lipoplatin, the most clinically active formulation of liposomal-encapsulated cisplatin to date. Lipoplatin inhibited invasion and decreases EGFR expression. A, invasion of ovarian cancer cells through a collagen type I-coated Boyden chamber after treatment for 72 hours with 10 µmol/L lipoplatin. Values in the bar graph represent the mean ± SEM of 3 different experiments. **P < 0.001 vs. control, drug versus medium. B, EGFR surface expression: cells were treated for 72 hours with lipoplatin and analyzed by flow cytometry using the anti-EGFR mAb 528. Values in the bar graph represent the mean ± SEM of 3 different experiments. ***P < 0.0001 drug versus medium. MFI, mean fluorescence intensity. C, representative FACS histograms of 1 of 3 independent experiments showing the decrease of EGFR expression after drug treatment.

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 hours with 10 µmol/L lipoplatin. Values in the bar graph represent the mean ± SEM of 3 different experiments. **P < 0.001 vs. control, drug versus medium. B, EGFR surface expression: cells were treated for 72 hours with lipoplatin and analyzed by flow cytometry using the anti-EGFR mAb 528. Values in the bar graph represent the mean ± SEM of 3 different experiments. ***P < 0.0001 drug versus medium. MFI, mean fluorescence intensity. C, representative FACS histograms of 1 of 3 independent experiments showing the decrease of EGFR expression after drug treatment.

The antioxidant TrxR system maintains the intracellular redox state and defends cells against oxidative damage due to reactive oxygen species (ROS) that are generated during chemotherapy. Lipoplatin, a new formulation of cisplatin, has been shown to be effective in ovarian cancer cell lines. In this study, we investigated the biologic activity and molecular mechanisms of action of lipoplatin in ovarian cancer cell lines. In particular, we investigated the effects of lipoplatin on cell proliferation, invasion, and EGFR expression.

We also analyzed the anticancer activity of lipoplatin in vivo. For this purpose, OVCAR5 cells (2.7 × 10⁶) were injected into the right flank of 6-week-old female athymic nude mice and, once tumors reached a volume of about 44 mm³, the mice were treated intraperitoneally 3 times a week with lipoplatin (20 mg/kg). Significant tumor growth inhibition by lipoplatin was apparent after 17 days of treatment (Fig. 5G). At day 41, the control tumors grew to a mean 417.2 ± 5 mm³ in size, whereas lipoplatin showed a 82% inhibitory effect, as the treated tumors reached a mere size of 73.12 ± 8 mm³ (**, P < 0.01; Fig. 5G). The treatment was suspended (Fig. 5F, dashed lines) and mice were followed for 14 more days.

Lipoplatin inhibited the growth of ovarian cancer xenografts

We also analyzed the anticancer activity of lipoplatin in vivo. For this purpose, OVCAR5 cells (2.7 × 10⁶) were injected into the right flank of 6-week-old female athymic nude mice and, once tumors reached a volume of about 44 mm³, the mice were treated intraperitoneally 3 times a week with lipoplatin (20 mg/kg). Significant tumor growth inhibition by lipoplatin was apparent after 17 days of treatment (Fig. 5G). At day 41, the control tumors grew to a mean 417.2 ± 5 mm³ in size, whereas lipoplatin showed a 82% inhibitory effect, as the treated tumors reached a mere size of 73.12 ± 8 mm³ (**, P < 0.01; Fig. 5G). The treatment was suspended (Fig. 5F, dashed lines) and mice were followed for 14 more days.

Lipoplatin inhibited the growth of ovarian cancer xenografts

We also analyzed the anticancer activity of lipoplatin in vivo. For this purpose, OVCAR5 cells (2.7 × 10⁶) were injected into the right flank of 6-week-old female athymic nude mice and, once tumors reached a volume of about 44 mm³, the mice were treated intraperitoneally 3 times a week with lipoplatin (20 mg/kg). Significant tumor growth inhibition by lipoplatin was apparent after 17 days of treatment (Fig. 5G). At day 41, the control tumors grew to a mean 417.2 ± 5 mm³ in size, whereas lipoplatin showed a 82% inhibitory effect, as the treated tumors reached a mere size of 73.12 ± 8 mm³ (**, P < 0.01; Fig. 5G). The treatment was suspended (Fig. 5F, dashed lines) and mice were followed for 14 more days.

Lipoplatin inhibited the growth of ovarian cancer xenografts

We also analyzed the anticancer activity of lipoplatin in vivo. For this purpose, OVCAR5 cells (2.7 × 10⁶) were injected into the right flank of 6-week-old female athymic nude mice and, once tumors reached a volume of about 44 mm³, the mice were treated intraperitoneally 3 times a week with lipoplatin (20 mg/kg). Significant tumor growth inhibition by lipoplatin was apparent after 17 days of treatment (Fig. 5G). At day 41, the control tumors grew to a mean 417.2 ± 5 mm³ in size, whereas lipoplatin showed a 82% inhibitory effect, as the treated tumors reached a mere size of 73.12 ± 8 mm³ (**, P < 0.01; Fig. 5G). The treatment was suspended (Fig. 5F, dashed lines) and mice were followed for 14 more days.
to ROS overproduction, leading to the formation of proapoptotic 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 anticancer 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 3 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, as it displays reduced toxicity with respect to paclitaxel (37).

EGFR overexpression 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 downregulated 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. Because 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 co-expression 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 radioresistant than the...
Figure 5. Lipoplatin inhibits spheroid growth, migration, and tumor xenograft. A, SKOV3 single preformed spheroids were cultured for 15 days in the absence or presence of lipoplatin (25, 50 µmol/L). Responses were evaluated by spheroid volume measurements at regular intervals. Values in the bar graph represent the mean ± SEM of 3 different experiments. , , , , , , , P < 0.0001 drug versus medium. B, representative phase contrast microphotographs showing volume decrease by lipoplatin treatment (original magnification, 4×). 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 collagenase I–coated plates in the presence of lipoplatin (25, 50 µmol/L). 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 versus medium. Values in the bar graph represent the mean ± SEM of 3 different experiments. F, images were captured after 24 and 48 hours using an inverted microscope (phase contrast microphotographs; original magnification, 4×). G, in vivo anticancer activity of lipoplatin (OVCAR5 xenograft). Tumor volumes were measured in female athymic nude mice after intraperitoneal injection of medium, either drug-free- or containing 20 mg/kg lipoplatin, 3 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 versus control.
bulky 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 macroaggregates, 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 as it ascites cellular macroaggregates, 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 preformed 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 antimetastasis 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 ineffective 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 patients with ovarian cancer would add the advantage of lower toxicities as already shown in randomized phase II and III studies in NSCLC (48–50). Adding the advantage of reducing the metastatic potential and the putative ovarian CSCs, and its synergistic activity with Abraxane and doxorubicin, lipoplatin in combination with Abraxane or doxorubicin should be compared with cisplatin + Abraxane/doxorubicin in a randomized clinical study against ovarian cancer.

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

Authors’ Contributions
Conception and design: N. Casagrande, D. Aldinucci
Development of methodology: N. Casagrande, M. Colegato, C. Borghese, M. Mongiat
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): N. Casagrande
Writing, review, and/or revision of the manuscript: N. Casagrande, A. Colombatti, D. Aldinucci
Study supervision: D. Aldinucci

Grant Support
This research was supported by Ministero della Salute, Ricerca Finalizzata FSN, I.R.C.C.S., Rome, Italy.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 25, 2014; revised July 25, 2014; accepted August 10, 2014; published OnlineFirst September 17, 2014.

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

Clinical Cancer Research


Preclinical Activity of the Liposomal Cisplatin Lipoplatin in Ovarian Cancer

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


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

Cited articles
This article cites 50 articles, 10 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/21/5496.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/20/21/5496.full.html#related-urls

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.