Preclinical evaluation of a new liposomal formulation of cisplatin, lipoplatin, to treat cisplatin-resistant cervical cancer

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Objective. Cisplatin-based chemotherapy has been shown to improve survival in cervical cancer; however, treatment is associated with tumor resistance and significant toxicity. Lipoplatin is a new liposomal formulation of cisplatin, developed to reduce cisplatin toxicity, to improve drug accumulation at tumor sites and to overcome drug resistance.

The aim of this study is to analyze the antitumoral activity of lipoplatin against cisplatin-resistant cervical cancer cells and to investigate its mechanism of action.

Methods. The activity and mechanism of action of lipoplatin were studied in the ME-180 cervical cancer cell line and its cisplatin-resistant clone R-ME-180 and HeLa cells using cell proliferation assays, flow cytometry, ELISA assay, cell migration, spheroids and tumor xenograft.

Results. We demonstrated that lipoplatin exhibited a potent antitumoral activity on HeLa, ME-180 cells and its cisplatin-resistant clone R-ME-180. Lipoplatin inhibited cell proliferation in a dose-dependent manner and was more active than the reference drug cisplatin in R-ME-180 cells and induced apoptosis, as evaluated by Annexin-V staining and DNA fragmentation, caspases 9 and 3 activation, Bcl-2, and Bcl-xL down-regulation, but Bax up-regulation inhibited thioredoxin reductase (TrxR) enzymatic activity and increased reactive oxygen species (ROS) accumulation; reduced EGFR expression and inhibited both migration and invasion. R-ME-180, but not ME-180 cells, generated three-dimensional (3D)-multicellular spheroids expressing the cancer stem cell marker ALDH. The ability of R-ME-180 cells to form spheroids in vitro and tumors in nude mice was also remarkably decreased by lipoplatin.

Conclusions. Overall, our results suggest that lipoplatin has potential for the treatment of cisplatin-resistant cervical cancer.

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Introduction

Cervical cancer is the third most common cancer worldwide with an annual incidence of 530,000 cases [1]. Based on Phase III results, the combination of cisplatin and paclitaxel remains the standard of care for treatment of recurrent cervical cancer, although clinical benefit is limited. In fact, although cisplatin is the most effective agent for metastatic cervical cancer, prolonged treatment induces multiple mechanisms of tumor resistance and is associated with significant toxicity [1]. Therefore, the development of new cisplatin formulations to overcome both resistance and toxicity remains a high priority [2] and is crucial for a better treatment and a more prolonged survival.

Presently, one of the main goals is to identify compounds with superior efficacy, reduced toxicity and a lack of cross-resistance as...
compared with the parent compound cisplatin. In recent years different formulations of cisplatin encapsulated into liposomes were developed [3] demonstrating in vitro activity, but not very promising clinical results [4]. One remarkable exception is lipoplatin [5] that was developed to reduce the systemic toxicity of cisplatin, to escape immune surveillance, to improve drug targeting to the primary tumor and metastases, and to overcome cisplatin resistance at the cell membrane level. One of the mechanisms contributing to cisplatin resistance is the reduced intracellular accumulation or the increased efflux through the cell membrane [5]. Since liposomes directly fuse with the tumor cell membrane or are phagocytized inside the cells, lipoplatin could overcome drug resistance.

The lipids of lipoplatin are composed of soy phosphatidyl choline (SPC-3), cholesterol, dipalmitoyl phosphatidyl glycerol (DPPG), and methoxy-polyethylene glycol distearoyl phosphatidylethanolamine (mPEG 2000-DSPE). These nanoparticles, displaying an enhanced half-life circulation time in body fluids and tissues, have the ability to target tumors and metastasis through the compromised endothelium of the tumor vasculature sprouted during angiogenesis, and there to reach concentration at levels up to 200-fold higher compared to the adjacent normal tissue [5]. Lipoplatin clinical trials have shown similar efficacy to those of cisplatin in pancreatic, head and neck cancer and in NSCLC, with the benefit of a reduced toxicity [6]. Similarly, in a Phase II trial the lipoplatin/vinorelbine combination demonstrated substantial reduced toxicities and enhanced or similar efficacy on HER-2/neu-Negative Metastatic Breast Cancer [7]. However, no data are available on cervical cancer. Indeed, despite survival improvement with the introduction of chemoradiation, the prognosis for patients with cervical cancer remains poor. Therefore, lipoplatin could represent a new therapeutic option in this pathology. The aim of our study was to analyze the antitumor activity of lipoplatin against cisplatin-resistant cervical cancer cells and to investigate its mechanism of action.

Materials and methods

Drugs

Lipoplatin™, the liposomal formulation of cisplatin, was generously provided by Regulon (Regulon Inc., Mountain View, California); cisplatin was purchased from Mayne Pharma, Napoli, Italy.

Cell lines and culture conditions

HeLa (ATCC CCL-2) cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The highly invasive cervical cancer-derived ME-180 (HPV+) cell line was purchased by Dr. G. Toffoli (CRO, Aviano). Cisplatin-resistant R-ME-180 cells were developed in our laboratory by continuous exposure of ME-180 cells to cisplatin (1 μM) at 37 °C for 3 weeks. Triplicate cultures were established for each treatment. Cytotoxicity was measured by using MTT assay. IC50, IC75 and IC90 values were calculated using the CalcuSyn software (Biosoft, Ferguson, MO, USA) [8].

Flow cytometry

1.0 × 10^5 tumor cells were incubated for 72 h on 6-well plates in complete medium in the presence of lipoplatin or cisplatin. Then Annexin-V binding (Becton-Dickinson [BD] Pharmingen, San Jose, CA), DNA fragmentation (BD), changes in mitochondrial membrane potential (Invitrogen, Milan, Italy) cytochrome-c release (BD), caspases 3 and 9 activation (Chemicon International, Milan, Italy), mitochondrial reactive oxygen species (ROS) (Invitrogen, B-cell lymphoma/leukemia-2 (Bcl-2) (DAKO Cytomation, Milan, Italy), B-cell lymphoma-extra large (Bcl-xl) (Cell Signalling, Danvers, MA, USA) and Bcl-2 associated x protein (Bax) (BD) analysis and the surface expression of EGFR were evaluated as previously described (anti-EGFR monoclonal antibody (mAb) 528 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)) [9]. Analyses were carried out using a FACSCalibur flow cytometer (BD).

Thioredoxin reductase (TrxR) enzyme activity assay

1.0 × 10^5 cells were treated with 20 and 30 μM lipoplatin or cisplatin for 72 h. TrxR activity was assessed using the Thioredoxin Reductase Assay Kit (Sigma-Aldrich), according to the manufacturer’s instructions [9].

Cell migration and invasion assay

Cell migration was assessed using the scratch wound healing assay, as described elsewhere [10]. Briefly, cells were grown to confluence in tissue culture dishes, then 10 μM of lipoplatin, cisplatin or drug-free medium were added. After 72 h, cells were scraped up and cultured in DMEM with 0.5% FCS. Invasion was assessed by FATIMA assay after drug treatment (12 h with 30 μM lipoplatin) as previously described [11].

ALDH assay

Identification of cancer stem cells (CSCs) [11] was performed using the Aldeflour reagent based (Stem Cell Technologies, Vancouver, Canada) flow cytometry method according to the manufacturer’s instructions. Briefly, cells (2 × 10^7/ml) were incubated for 40 min at 37 °C with Aldeflour reagent with and without the ALDH-inhibitor diethylamino-benzaldehyde (DEAB). Analyses were carried out using a FACSCalibur flow cytometer (BD).

3D-Multicellular spheroid formation assays

To obtain spheroids, 24-well plates were coated twice with 20 mg/ml of poly(2-hydroxyethyl methacrylate) (poly-HEMA; Sigma, Inc., St. Louis, MO, USA) [12] in 95% ethanol and washed once with PBS before cell seeding [13]. To evaluate lipoplatin activity on spheroid formation, R-ME-180 cells (5 × 10^5) were cultured for 72 h on poly-HEMA coated wells with increasing concentrations of lipoplatin (10, 25 or 50 μM). To evaluate apoptosis or ALDH activity, spheroids (72 h) were trypsinized in a single cell suspension. To obtain a second generation, formed spheroids (72 h) were dissociated and then replated on poly-HEMA coated wells.

Human cervical tumor xenograft experiments

Six-week-old female athymic nu/nu (nude) mice were purchased from Charles River (Lecco, Italy). 2.5 × 10^5 R-ME-180 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 32 mm^3 in volume, mice were divided randomly into two groups of 5 mice each and treated every other day with intraperitoneal injection of 10 mg/kg lipoplatin or drug-free vehicle. Tumor size was measured over time.
using a caliper. Tumor volumes were calculated according to the standard formula: $\pi L W^2 / 6$, where $L$ indicates length and $W$ indicates width. Mice were sacrificed after 28 days of treatment when control tumors had reached a volume of about 600 mm$^3$. The mouse organs were excised and fixed in formalin for tissue toxicity analyses.

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 $\alpha$ error of 0.05.

**Results**

*Lipoplatin inhibited cervical cancer cell proliferation and induced apoptosis*

We compared the *in vitro* cytotoxic effects of lipoplatin (Fig. 1A) on HeLa, ME-180 cells and its cisplatin-resistant clone R-ME-180. Treatment with lipoplatin or cisplatin induced a dose-dependent inhibition of cell proliferation. HeLa (cisplatin, $IC_{50} = 8.6 \mu M$), ME-180 (cisplatin, $IC_{50} = 6.5 \mu M$) and R-ME-180 cells (cisplatin, $IC_{50} = 55.5 \mu M$) showed a comparable sensitivity to lipoplatin ($IC_{50} = 19.6 \mu M$, 24.8 $\mu M$, and 18.1 $\mu M$, respectively) (Fig. 1A). Lipoplatin induced apoptosis (Fig. 1B) in both cisplatin-sensitive (ME-180) and -resistant (R-ME-180) cells. On the contrary, cisplatin, used at the same concentrations, did not induce apoptosis in R-ME-180 cells. Consistently, cisplatin induced a remarkable DNA fragmentation only in ME-180 cells, whereas lipoplatin was active in both cell lines (Fig. 2A, left panel).

*Lipoplatin affected mitochondrial functions, ROS formation and TrxR activity*

Cisplatin damages tumors via induction of apoptosis that is mediated by various signals including the activation of mitochondrial pathways, and the formation of reactive oxygen species (ROS) [14]. Similarly, lipoplatin (30 $\mu M$) led to a decline in the mitochondrial membrane potential, increased the expression of Bax (pro-apoptotic), decreased...
the anti-apoptotic molecules Bcl-xL and Bcl-2 and cytochrome-c release from the mitochondria in both cell lines (Figs. 2A, B). Cisplatin, used at the same concentration, affected mitochondrial functions only in ME-180 cells (Fig. 2A). Consequently, lipoplatin (30 μM) led to activation of caspases 3 and 9 in both cell lines (Fig. 2C).

Lipoplatin induced ROS production in both cell lines while cisplatin was active only in ME-180 cells (Figs. 3A, B). TrxR is a selenoenzyme essential to maintain the balance of the cellular redox status and to protect the cells against oxidative damage due to ROS accumulation [15]. R-ME-180 cells expressed higher activity of TrxR than ME-180 cells (about 3 fold) (Fig. 3C). Both lipoplatin and cisplatin reduced TrxR activity in a dose-dependent manner (20, 30 μM) in both cell lines, but only lipoplatin (30 μM) was capable to reduce TrxR activity in R-ME-180 cells to levels lower than those of sensitive ME-180 cells.

Lipoplatin inhibited cancer cell migration, invasion and down-modulated EGFR expression

The effect of lipoplatin on cancer cell migration and invasion was measured using the scratch wound healing assay and the FATIMA assay, respectively. To exclude that a lower migration rate could be attributable to a decreased cell proliferation, cells were cultured in the presence of less drug (10 μM) and at low serum concentration. The migration rate (the percentage of the surface area covered by tumor...
cells 24 and 48 h after the scratch) was reduced by lipoplatin in both cell lines whereas by cisplatin only in ME-180 cells (Figs. 4A, B). Then, we evaluated invasion of tumor cells treated for 12 h with 30 μM lipoplatin through a type I collagen-coated Boyden chamber. R-ME-180 cells exhibited enhanced invasive properties compared to ME-180 cells (Fig. 4C). At 24 h lipoplatin decreased cell invasion of about 35% and 50% in ME-180 and R-ME-180 cells, respectively (Fig. 4C).

EGFR is over-expressed in approximately 85% of invasive cervical tumors, it is associated with higher stages and poor prognosis [16], and its inhibition significantly decreases tumor cell metastases [16]. We detected higher EGFR levels in R-ME-180 cells (mean fluorescent intensity (MFI) = 387.26 ± 42) than in ME-180 cells (MFI = 201.68 ± 30). Lipoplatin (30 μM) decreased EGFR surface expression in both cell lines, while cisplatin (30 μM) remarkably down-modulated EGFR in ME-180 cells and only minimally affected R-ME-180 cells (Fig. 4D). A representative experiment is shown in Fig. 4E.

Spheroid-forming efficiency and ALDH are increased in R-ME-180 cells: lipoplatin inhibited spheroid formation and reduced ALDH+ cells

Spheroids represent a three-dimensional in vitro system that more closely resembles the in vivo tumor microenvironment. A greater ability to form spheroids [17] and the expression of ALDH enzymes [18] usually indicate an increase in the cancer stem cell (CSC) population. R-ME-180, but not ME-180 cells, spontaneously formed spheroids when cultured on poly-HEMA coated wells. Consistently, we found that the percentage of cells expressing ALDH was 0.2% ± 0.01 in ME-180 and 0.51% ± 0.04 in R-ME-180 cells grown in monolayer. Then, we evaluated ALDH activity in R-ME-180 spheroids and found that it significantly increased in the first (I gen) (4.45-fold) and especially in the second generation (II gen) (6.82-fold) spheroids (Fig. 5A). A representative FACS dot plot showing ALDH expression is shown in Fig. 5B. Finally, we investigated whether lipoplatin could prevent spheroid formation. Lipoplatin (10–25–50 μM) but not cisplatin inhibited spheroid formation in poly-HEMA coated wells. R-ME-180 cells formed several large and dense spheroids, whereas lipoplatin, but not cisplatin, treated cells formed small spheroids, with dead cells interspersed among cell aggregates (Fig. 5C). At the highest lipoplatin concentration (50 μM) we detected only dead cells (Fig. 5C). Consistently, lipoplatin induced apoptosis in a dose dependent manner (Fig. 5D) and decreased the percentage of ALDH+ cells (Figs. 5E, F).

Lipoplatin inhibited the growth of cervical cancer xenografts

Cisplatin-resistant R-ME-180 cells (2.5 × 10^6) were injected into the right flank of 6-week-old female athymic nude mice. After 4 days (tumor size of about 32 mm^3) mice were treated intraperitoneally with lipoplatin. Treatment for 28 days resulted in a significant (P < 0.01)
tumor growth inhibition. The tumors of the untreated control group grew to a mean tumor size of about 615.4 ± 50 mm³, while in lipoplatin treated mice tumors reached a mean size of about 192 ± 15 mm³ (Fig. 6).

There was no histological detectable cytotoxicity involving the animals’ heart, spleen, liver and kidney, whereas treatment with the same concentration of cisplatin was lethally toxic for mice (data not shown).

Discussion

Cisplatin has been very effective for the treatment of gynecological cancers, such as ovarian and cervical cancers, but the development of resistance in initially responsive tumors and its severe toxicity represent the most important problems in cisplatin-based chemotherapy. Here lipoplatin potently inhibited the proliferation of the cisplatin-resistant R-ME-180 cells with an IC₅₀ comparable to that of the parent cisplatin-sensitive cells (ME-180) and of HeLa cells, and strongly decreased xenograft tumor growth of the cisplatin-resistant R-ME-180 cells without apparent toxicity.

Lipoplatin induced its potent cytotoxic effect by activating apoptosis, inducing mitochondrial membrane depolarization, cytochrome-c release, and caspases 3 and 9 activation, indicating that its activity was exerted through the mitochondrial intrinsic apoptotic pathway. Accordingly, the pro-survival protein Bcl-2 and Bcl-xL were reduced and the pro-apoptotic Bax protein increased.

TrxR is a ubiquitous enzyme over-expressed in many cancer cells, identified as a potential target of anticancer drugs. TrxR exhibits protective effects against various cellular stresses, including growth inhibition and cell death induced by ROS and chemotherapeutic agents. Resistance to cisplatin increases Trx and TrxR levels in HeLa cervical cancer cells as shown here for the R-ME-180 cells. The cisplatin-
resistant variants of HeLa cells, established by continuous exposure to cisplatin exhibited an increased expression and activity of TrxR as well as Trx compared with the parental cells [23]. Consistently, we found that R-ME-180 cells express more TrxR than ME-180 cells. Lipoplatin remarkably reduced TrxR enzymatic activity with up-regulation of ROS, possibly overcoming the resistance mediated by TrxR over-expression. On the contrary, cisplatin decreased TrxR levels to a lesser extent than lipoplatin, and to levels comparable to those of untreated cisplatin-sensitive cells, suggesting that cisplatin resistance could be due to the reduced capability to increase ROS levels because of the higher TrxR enzymatic activity in R-ME-180 cells.

The increased expression of EGFR is associated with bad prognosis in cervical cancer [24, 25]. HB-EGF, a ligand of EGFR produced by stromal fibroblasts in uterine cervical cancer, contributes to ME-180 cell proliferation [26]. Moreover, treatment with cisplatin led to EGFR degradation in sensitive head and neck cancer cell lines, and this degradation strongly correlates with cytotoxicity [27]. We demonstrated that the R-ME-180 cells
expressed higher amounts of EGFR than ME-180 cells and lipoplatin, but not cisplatin, down-modulated EGFR expression in cisplatin-resistant cells. Our results suggest that lipoplatin, by reducing EGFR, could exert not only direct cytotoxic effects on cervical cancer cells, but also affect the proliferation induced by HB-EGF secreting stromal cells of the tumor microenvironment. Lipoplatin and also cisplatin decreased EGFR expression in cisplatin sensitive cells. This implies that an appropriate treatment schedule should be considered when cisplatin and gefitinib or other similar drugs are used together since cisplatin treatment might hinder targeted therapy against molecules susceptible to downregulation.

Cervical cancer usually spreads to the adjacent organs through the angiolymphatic system [28]. Although uncommon at initial diagnosis, metastasis will develop in a high percentage, usually within the first two years of completing treatment and in the majority of cases metastatic cervical cancer is not curable [28]. Therefore, suppressing not only cell proliferation, but also tumor metastasis is desirable to obtain clinically useful results in advanced cervical cancer. High levels of EGFR also promote cancer cell invasion and metastases. The angiolymphatic system elevates oxidative stress in HeLa cells. Toxicol Appl Pharmacol 1999;27:504–514.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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References

[25] Nakayama K, Tanaka Y, Nagai S, Takeuchi T, Hiraoka Y, et al. Curcumin targeting the thioredoxin system inhibits spheroid formation and reduces the percent- age of CSCs (ALDH+ cells) suggesting that it could be used to treat in vivo tumors. Accordingly, lipoplatin inhibited tumor xenografts of R-ME-180 cells with minimal systemic toxicity, while cisplatin at the same concentration caused a severe toxicity in nude mice [29]. These results are promising in the light of using this drug, single or in combination therapy, as a novel therapeutic strategy for cisplatin-resistant recurrent cervical cancer.

Because cisplatin is the standard care for the treatment of cervical cancer in combination with radiation therapy (RT), replacement of cisplatin by lipoplatin against cervical cancer would add the advantage of lower toxicities to patients as shown in randomized Phase II and Phase III studies against NSCLC [30–32]; A better radiosensitizing activity of lipoplatin compared to cisplatin has also been shown in preclinical studies [33]. Adding the advantage of reducing the metastasis potential from lipoplatin treatment suggested from our current work we recommend that lipoplatin in combination with RT should be compared to cisplatin + RT in a randomized clinical study against cervical cancer.

Fig. 6. In vivo anticancer activity of lipoplatin (xenograft). Tumor volume was measured in female athymic nude mice after intraperitoneal (i.p.) injection of either drug-free medium or containing 10 mg/kg lipoplatin, three times a week using a caliper. Points represent the mean ± SEM of five animals per group. "P < 0.01 lipoplatin vs. control."


