Activity of lipoplatin in tumor and in normal cells *in vitro*

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Lipoplatin is a novel liposomal cisplatin formulation with reduced adverse side effects compared with its parental compound, cisplatin. The aims of this preclinical study were to compare lipoplatin and cisplatin cytotoxicity *in vitro* in established cell lines derived from non-small cell lung cancer, renal cell carcinoma, and in normal hematopoietic cell precursors, and to identify biological markers associated with sensitivity and resistance. Our results showed a superior cytotoxicity in all tumor cell models and a much lower toxicity in normal cells for lipoplatin compared with cisplatin, suggesting a higher therapeutic index for the liposomal compound. Moreover, RT-PCR analysis of molecular markers known to be related to cisplatin resistance showed a direct correlation between cisplatin and lipoplatin resistance and ERCC1 and LRP expression. In conclusion, lipoplatin showed a higher antitumor activity in both tumor histotypes investigated and was found to be safer than the parent compound, cisplatin. Moreover, ERCC1 and LRP expression levels would seem to be valid predictors of sensitivity or resistance to these drugs. *Anti-Cancer Drugs* 19:983–990 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2008, **19**:983–990

Keywords: ERCC1, lipoplatin, LRP, MDR-1, non-small cell lung cancer, renal cell carcinoma

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Received 21 March 2008 **Revised form accepted** 19 July 2008

Introduction

Although cisplatin is one of the most effective and widely used chemotherapeutic agents for the treatment of epithelial malignancies [1,2], it is frequently responsible for immunosuppression and for severe adverse renal, gastrointestinal, and neurological toxicities [3–6]. Since the introduction of cisplatin into clinical practice, thousands of platin-derived analogs have been synthesized and evaluated for their antitumor activity. Over 30 drugs have been used in human clinical trials [7,8], but only carboplatin has received worldwide approval and is used in clinical practice [9]. Although carboplatin is less toxic than cisplatin and can be administered at higher doses, it is only active in cisplatin-sensitive tumor histotypes [10]. Great efforts have been made to try to understand the mechanisms of resistance to platinum compounds, whether intrinsic, as observed for colorectal, prostate, breast and lung cancer, or acquired, as observed during treatment for ovarian cancer. It has emerged that resistance to cisplatin is multifactorial and mainly consists of mechanisms limiting the formation of DNA adducts, such as nucleotide excision repair (NER) and mismatch repair systems [11]. DNA adduct formation by platinum compounds may also be limited by a reduction in drug accumulation due to enhanced efflux and/or inactivation [12]. The latter mechanism occurs when cisplatin, intravenously administered, interacts with various compounds including sulfur-containing molecules before reaching the DNA of tumor cells [12].

Cisplatin resistance may also be associated with the inhibition of DNA damage signal propagation to the apoptotic machinery due to loss of p53 function, overexpression of antiapoptotic bcl-2, and interference in caspase activation [13]. More recently, other analogs, oxaliplatin and nedaplatin, received formal approval in some countries but have not yet clearly demonstrated any advantages over cisplatin [10]. The search therefore continues for improved platin-based antitumor agents that are orally active, less toxic than and not cross-resistant to cisplatin or carboplatin.

Other interesting platinum compounds are currently undergoing clinical evaluation, such as the oral platinum drug JM216, the active transplatinum complex JM335, the sterically hindered platinum (II) complex ZD0473, the active trinuclear platinum complex BBR3464, and the liposomal forms SPI-77 and lipoplatin [7]. Lipoplatin is a new formulation of cisplatin, encapsulated into liposomes composed of dipalmitoyl phosphatidyl glycerol (DPPG), soy phosphatidyl choline (SPC-3), cholesterol, and methoxy-polyethylene glycol–distearoyl phosphatidylethanolamine (mPEG 2000-DSPE). Preclinical studies on nude mice, rats, and severe combined immunodeficiency mice have highlighted the milder adverse effects, especially to the kidney, of lipoplatin compared with cisplatin [14,15]. Moreover, clinical phase I and II studies have reported that this novel cisplatin formulation, used singly or in combination with gemcitabine, does not show

0959-4973 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e3283114fb2

nephrotoxicity, neurotoxicity, hepatotoxicity, or ototoxicity [16–18]. Preliminary results from ongoing phase III studies also strongly suggest that lipoplatin has a higher therapeutic index than cisplatin in the first-line treatment of non-small cell lung cancer (NSCLC) [19]. Initial results from a phase III study on patients with head and neck cancer have confirmed the lower toxicity profile of the lipoplatin-based regimen compared with that of the cisplatin-containing schedule. A report on overall survival in the two regimen arms is pending [19,20]. Interestingly, this novel formulation, in addition to showing promising therapeutic efficacy and fewer adverse side effects than cisplatin, has demonstrated a capacity to preferentially act on tumor cells [21]. In this study, we compared the activity of lipoplatin and cisplatin *in vitro* NSCLC and renal cell carcinoma (RCC) cell lines and in normal hematopoietic cell precursors, and explored the clinical relevance of a number of biomarkers supposedly related to platinum compound resistance.

Materials and methods

Biological systems

Tumor cells

The study was performed on established cell lines representative of different human NSCLC histotypes. CAEP and RAL, derived from an epidermoidal carcinoma and adenocarcinoma, respectively, were established and characterized in our laboratory [22]. ChaGo-K1, a bronchiogenic cell line, was obtained from the American Type Culture Collection, and ChaGo-CPL, a cisplatin-resistant cell line, was derived from ChaGo-K1 cells after repeated pulsation with a 20-μmol/l concentration of cisplatin. Two RCC cell lines, CAKI-1 and CAKI-2, obtained from the American Type Culture Collection, were also used. Cells were maintained as a monolayer at 37°C and subcultured weekly. Culture medium was composed of Dulbecco's modified Eagle's medium/Ham's F12 (1 : 1) supplemented with fetal calf serum (10%), glutamine (2 mmol/l), nonessential amino acids (1%) (Mascia Brunelli S.p.A., Milan, Italy), and insulin (10 mg/ml) (Sigma Aldrich, Milan, Italy). Cells were used in the exponential growth phase for all of the experiments.

Normal cells

Peripheral blood stem cells were obtained by leukapheresis from four healthy donors after informed written consent. Mononuclear cells were enriched by Histopaque-1077 gradient centrifugation (Sigma, St Louis, Missouri, USA). Light-density cells were washed twice in phosphate-buffered saline with 1% bovine serum albumin (Sigma) [23].

Drugs

Cisplatin (Bristol-Myers Squibb, Milan, Italy) was dissolved in sterile water and conserved at room temperature in the dark. Lipoplatin (Regulon Inc., Mountain View, California, USA) was dissolved in sterile water and stored at 0–4°C.

In-vitro chemosensitivity assay

Tumor cells

The sulforhodamine B assay was used according to the method of Skehan *et al.* [24]. Drugs were used at scalar concentrations of 0.1, 1, 10, and 20 μmol/l for exposure times corresponding to half-life values in humans, that is, 6 h for cisplatin and 72 h for lipoplatin, followed by a 72-h culture in drug-free medium (washout). The cytotoxic effect was evaluated at the end of the washout time.

Normal cells

The colony-forming cell assay was used as previously described [25]. In brief, 1×10^6 peripheral blood mononuclear cells were incubated with different concentrations (0.2, 2, and 20 μmol/l) of cisplatin or lipoplatin for 6 and 72 h, respectively, in Iscove's modified Dulbecco medium (Gibco Invitrogen Carlsbad, California, USA) supplemented with fetal calf serum (10%), glutamine (2 mmol/l), penicillin and streptomycin (1%), and maintained at 37°C in a humidified atmosphere of 5% CO_2. Control cells were incubated under the same conditions but in drug-free medium. After drug exposure, cells were washed in Iscove's modified Dulbecco medium and plated (50 000 cells) in duplicate in a complete culture medium (MethoCult H4434, StemCell Technologies, Vancouver, Canada). After 14 days of incubation, granulocyte macrophage colony-forming unit (GM-CFU) aggregates of more than 50 cells were counted.

Data analysis

Dose response curves were created by Excel software and the 50% inhibiting concentration (IC_{50}) values were determined graphically from the plots. Paired proportions were compared using McNemar's test and statistical analyses were carried out with SAS Statistical Software (SAS Institute Inc., Cary, North Carolina, USA).

Comet assay

The assay was performed according to the manufacturer's protocol (Trevigen, Gaithersburg, Maryland, USA). In brief, 5×10^5 normal stem cells were incubated with 20 μmol/l of cisplatin or lipoplatin for 6 and 72 h, respectively. At the end of the treatments, cells were suspended in LMAgarose at 37°C at a ratio of 1 : 10 (v/v), and 75 μl were immediately transferred onto the comet slide. The slides were immersed in lysis solution for 1 h at 4°C and washed in the dark for 1 h at room temperature in alkaline solution. Samples were washed twice in Tris–borate–EDTA electrophoresis solution and then electrophoresed for 10 min at 20 V. Slides were then dipped in 70% ethanol and stained with the Silver Staining kit (Trevigen). The extent of DNA damage was evaluated quantitatively by using an Axiovert microscope (Zeiss, Arese, Italy) (× 40) and scoring at least 1000 nucleoids. Damage was expressed, as previously described [26], as the percentage of comet-shaped

nucleoids with respect to the total number of nucleoids scored. All samples were evaluated blindly by two independent observers.

mRNA RT-PCR analysis

Total cellular RNA was isolated from 2×10^6 untreated tumor cell samples using an RNeasy Minikit (Qiagen, Hilden, Germany). One microgram of RNA was reverse-transcribed into cDNA using iScript (Bio-Rad Laboratories, Hercules, California, USA), in accordance with the manufacturer's recommendations and analyzed by real time polymerase chain reaction (RT-PCR) (MyiQ System, Bio-Rad Laboratories) to detect the expression of multidrug resistance 1 (MDR-1), excision repair cross-complementing 1 (ERCC1), lung resistance protein (LRP), and β_2-microglobulin. The standard reaction volume was 25 µl containing 2 µl of cDNA template, 1 × SYBR Green Mix (Bio-Rad) and 5 µmol/l of forward and reverse primers.

The mixture was subjected to the following cycling conditions: 95°C for 1 min and 30 s, followed by 40 cycles of amplification for 15 s at 95°C and 30 s at 59°C (for MDR-1) or 60°C (for β_2-microglobulin, ERCC1, and LRP). Primer sequences were designed using Beacon Designer software (version 4, BioRad) and are described in Table 1. The amount of each mRNA of each marker was normalized to the endogenous reference β_2-microglobulin using Gene Expression Macro Software (Version 1.1) (BioRad).

The baseline levels of the different markers were expressed as relative values considering as reference the most sensitive cell line for each cancer histotype, in particular, ChaGo-K1 for NSCLC and CAKI-1 for RCC.

TUNEL assay

The percentage of apoptotic cells in all tumor cell lines was evaluated by flow cytometric analysis, according to the previously described TUNEL assay procedure [26], after a 6-h exposure to cisplatin (0.1, 1, 10, and 20 µmol/l) or a 72-h exposure to lipoplatin (0.1, 1, 10, and 20 µmol/l) followed by a 72-h washout. In brief, after treatment, cells were trypsinized, fixed, exposed to the TUNEL reaction mixture (Roche Diagnostic GmbH, Mannheim, Germany), counterstained with propidium iodide and analyzed by FACS Vantage flow cytometer (Beckton Dickinson, Franklin Lakes, New Jersey, USA).

Results

Cytotoxicity

Tumor cells

In NSCLC, a dose-dependent cytotoxic effect was always observed, albeit with a modulation in the different cell lines. In particular, cytotoxicity was similar but lower in CAEP and RAL, intermediate in ChaGo-CPL, and higher in ChaGo-K1 cell lines after a 6-h exposure to cisplatin or a 72-h exposure to lipoplatin followed by a 72-h washout (Fig. 1). The IC_{50} was only reached in ChaGo-K1, at a 10-µmol/l concentration for cisplatin, corresponding to the peak plasma level in humans, and at a 8-µmol/l concentration for lipoplatin, about 2.5-fold lower than the peak plasma level (Table 2).

In the RCC cell lines, both drugs produced highly dose-dependent cytotoxic effects and the IC_{50} values were always reached at concentrations lower than the peak plasma levels (Fig. 2). IC_{50} concentrations for cisplatin were similar (8 and 10 µmol/l) in the two cell lines, whereas the IC_{50} value for lipoplatin was 8 µmol/l in CAKI-2 but 4-fold lower (2 µmol/l) in CAKI-1 cell line (Table 2).

Normal Cells

After exposure to cisplatin, an important cytotoxic effect in terms of inhibition of stem cell clonogenicity was observed starting at the lowest drug concentration (0.2 µmol/l) (Fig. 3a), with IC_{50} reached at 0.05 µmol/l. The liposomal compound induced a much lower toxicity and the IC_{50} was never reached within the range of concentrations tested.

DNA damage

The lower toxicity of lipoplatin with respect to cisplatin was further confirmed by the results obtained from the Comet assay. In particular, DNA damage was higher after exposure to cisplatin than to lipoplatin, as highlighted by the increase in the percentage of comet-shaped nucleoids (6 vs. 51%; $P < 0.05$ by *t*-test) (Fig. 3b).

Apoptosis

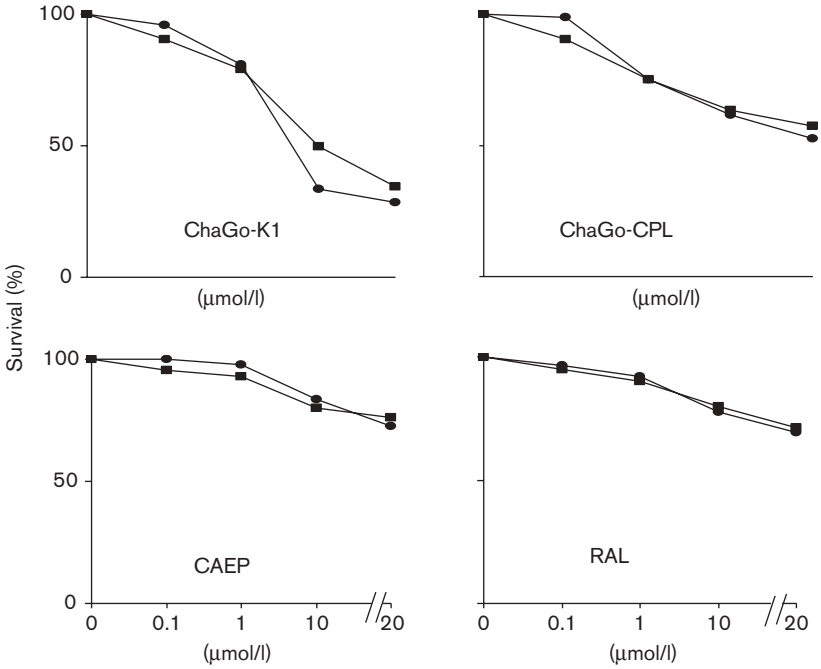
Apoptotic cells, which only rarely exceeded *2%* in control cultures, significantly increased in all cell lines after exposure to cisplatin or lipoplatin. At a concentration of 10 µmol/l, the parental drug induced a 1.7-fold to 6-fold increase in apoptosis in the different NSCLC cell cultures, whereas lipoplatin (20 µmol/l) produced

Table 1 Primers used in RT-PCR

Gene name	5′–3′ Forward primer	5′–3′ Reverse primer	Annealing temperature (°C)
MDR-1	ATATGGTGGTGGGAACTTTGG	GGCATACCTGGTCATGTCTTC	59
ERCC1	TCAGTCAACAAAACGGACAGTCAG	TCCTTGGGTTCTTTCCCAGAGC	62
LRP	ACAACTACTGCGTGATTCTC	AAGACTTCTCTCCCTTGACC	62
Beta2-microglobulin	CGCTACTCTCTCTTTCTGGC	AGACACATAGCAATTCAGGAAT	61

ERCC1, excision repair cross-complementing 1; LRP, lung resistance protein; MDR-1, multidrug resistance 1.

Fig. 1



Cytotoxic activity of cisplatin and lipoplatin in NSCLC cell lines. All the cell lines were exposed to cisplatin for 6 h (■) or to lipoplatin for 72 h (●) at concentrations of 0.1, 1, 10, and 20 μmol/l followed by a 72-h washout. Each point indicates the mean of at least three experiments. Standard deviation never exceeded 5%. NSCLC, non-small cell lung cancer.

Table 2 Correlation between marker expression levels, IC_{50}, and apoptosis

	Markers[c]			IC_{50}values (μmol/l)		Apoptotic Cells (%)		
Cell lines	LRP	ERCC1	MDR-1	Cisplatin	Lipoplatin	Control	Cisplatin	Lipoplatin
NSCLC								
ChaGo-K1[c]	1	1	1	10	8	1.8	11.5[a]	23.4[a,b]
ChaGo-CPL	5.8	3.0	0.8	NR	NR	2.1	4.7[a]	10.0[a,b]
CAEP	6.4	2.1	1.1	NR	NR	1.9	4.5[a]	5.3[a]
RAL	7.0	2.2	0.9	NR	NR	2.2	3.7[a]	5.6[a]
RCC								
CAKI-1[c]	1	1	1	8	2	2	6.6[a]	14.4[a,b]
CAKI-2	2.8	1.6	2.0	10	8	1.4	3.3[a]	4.3[a]

NSCLC, non-small cell lung cancer; NR, not reached.
[a]Control vs. cisplatin or lipoplatin $P<0.0001$.
[b]Cisplatin vs. lipoplatin $P<0.0001$.
[c]Relative expression.

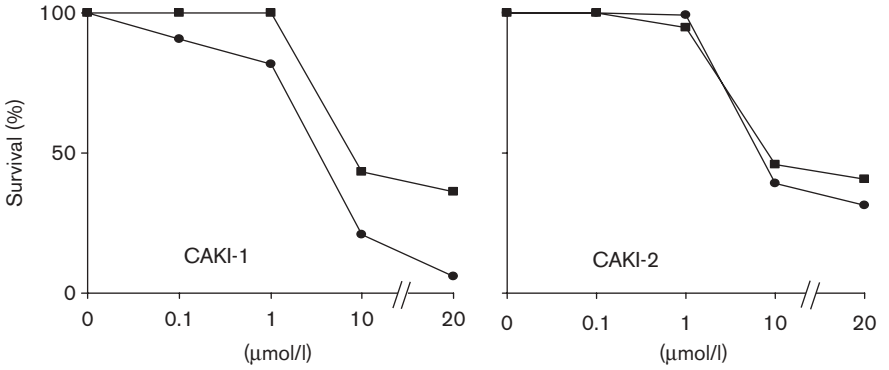
a 2.5-fold to 13-fold increase in programmed cell death. Similarly, in RCC cell cultures, the percentage of apoptotic cells was between 2.3-fold and 3.3-fold higher than that of control cells after cisplatin treatment and about 3-fold to 7-fold higher after exposure to lipoplatin (Table 2, Fig. 4). No appreciable apoptosis was observed at lower concentrations (data not shown).

Resistance-related biomarkers

Biomarker expression was analyzed in relation to drug sensitivity and/or resistance, as expressed by IC_{50}. The basal level of the membrane-bound ATP-dependent efflux transporter, MDR-1, which confers resistance to a number of cytotoxic drugs, was fairly similar but not significantly different among cell lines, regardless of their resistance to either drug. Conversely, the basal expression of both ERCC1, an enzyme involved in the NER process, and LRP, an MDR-related protein involved in mediating multidrug resistance in lung cancer, was significantly higher in the most resistant cell line (ChaGo-CPL) compared with the most sensitive line (ChaGo-K1) (Table 2).

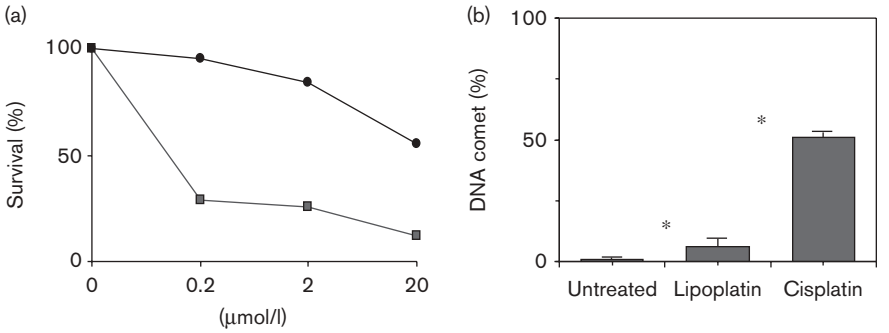
In RCC, the expression of all the markers was significantly higher in the more resistant cell line, CAKI-2.

Fig. 2



Cytotoxic activity of cisplatin and lipoplatin in RCC cell lines. The two renal carcinoma cell lines (CAKI-1 and CAKI-2) were exposed to cisplatin for 6 h (■) or to lipoplatin for 72 h (●) at concentrations of 0.1, 1, 10, and 20 μmol/l followed by a 72-h washout. Each point indicates the mean of the least three experiments. Standard deviation never exceeded 5%.

Fig. 3



Cytotoxic activity of cisplatin and lipoplatin in peripheral blood stem cells. (a) Cytotoxic activity of cisplatin (■) and lipoplatin (●) in normal cells was evaluated as the ability of either drug to inhibit clonogenic growth of normal stem cells derived from human peripheral blood stem cells. Each point indicates the mean of the least three experiments. Standard deviation never exceeded 5%. (b) Percentage of cells with damaged DNA (comets) evaluated after exposure to cisplatin and lipoplatin. *Significance at $P<0.05$ by *t*-test.

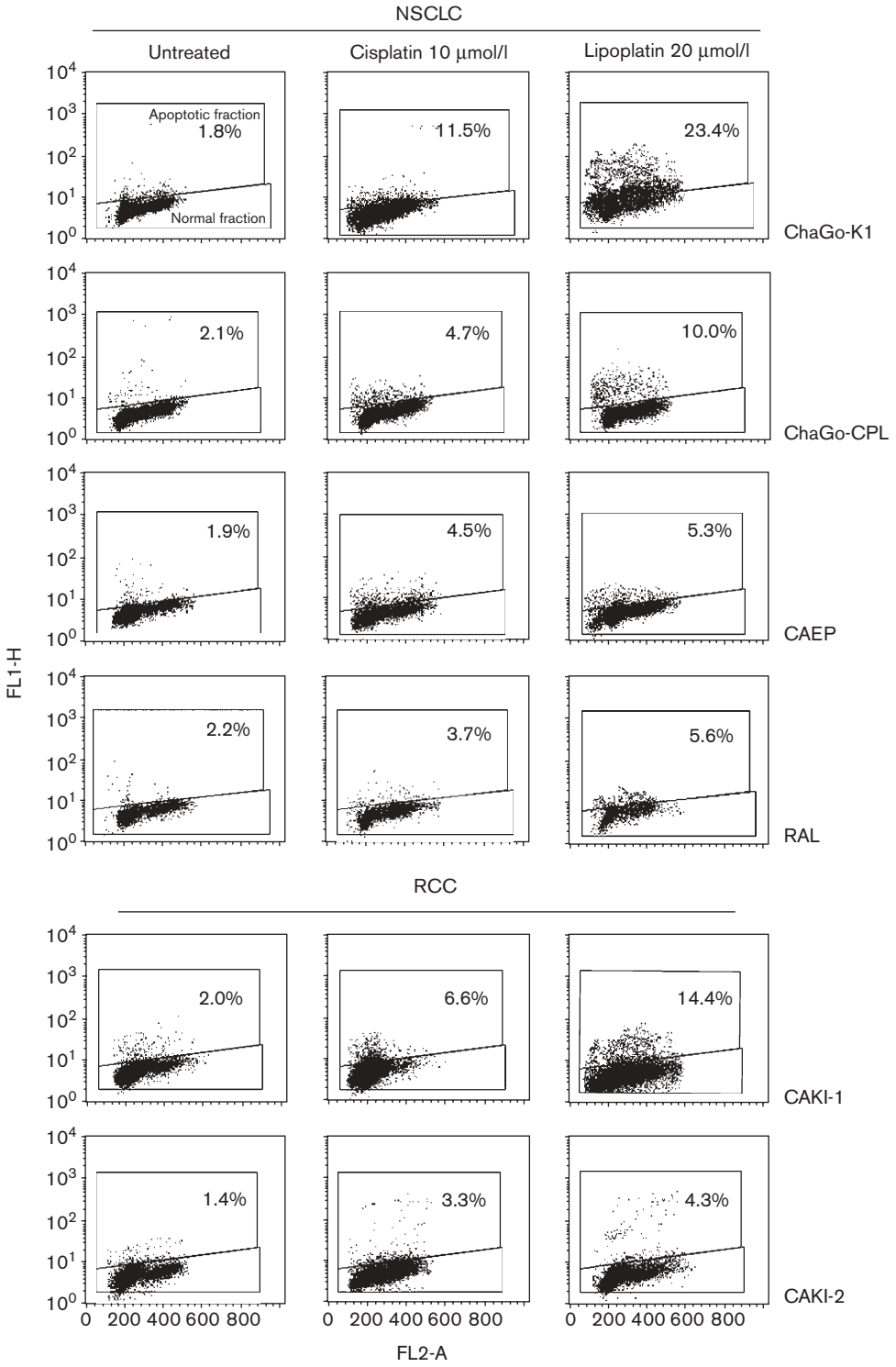
Discussion

Although platinum-derived compounds have been shown to be active in various cancer histotypes, high clinical toxicity severely limits their widespread clinical use. In this study, the antitumor activity of a new liposomal cisplatin-derived drug, lipoplatin, was compared with that of cisplatin in four NSCLC and two RCC cell lines. Lipoplatin showed a cytotoxic activity similar to the parental compound in three NSCLC cell lines (ChaGo-CPL, CAEP, and RAL) and a higher activity in the remaining cell line (ChaGo-K1), reaching IC_{50} values at a concentration at least 1.25-fold lower than that observed for cisplatin. Conversely, lipoplatin proved more active than cisplatin in both RCC cell lines, with IC_{50} values up to 4-fold lower than those registered for the parental compound.

The cytotoxic activity of the two drugs was associated, in both tumor models, with a significant increase in programmed cell death. Moreover, lipoplatin produced a higher percentage of apoptotic cells than either untreated or cisplatin-treated cells in all of the tumor cell lines. Among NSCLC cell lines it was significantly higher in ChaGo-K1 and ChaGo-CPL, whereas in the RCC cell lines, the highest apoptosis was observed in CAKI-1.

In this study, we also investigated the baseline expression level of MDR-1 and LRP efflux proteins, which have been reported to be linked to platinum compound resistance [11,27,28]. Basal MDR-1 expression was similar in all cell lines, independently of drug resistance, indicating the lack of effect of this marker on both cisplatin, as reported in other studies [29], and lipoplatin

Fig. 4



TUNEL assay. Percentages (median values) of apoptotic cells after exposure to 10 μmol/l of cisplatin for 6 h or to 20 μmol/l of lipoplatin for 72 h followed by a 72-h washout. The experiments were performed at least three times, and the results of one representative experiment are shown.

resistance. Conversely, LRP expression progressively and significantly increased in NSCLC cell lines as resistance to the drugs increased. Furthermore, in RCC cell lines, LRP expression was about 3-fold higher in the most resistant CAKI-2 than in the most sensitive CAKI-1 cell line.

ERCC1, the most important enzyme in the NER system, has been shown to play a role in repairing a variety of distorting lesions, such as platinum-induced DNA adducts [11]. Although studies on the influence of ERCC1 on drug resistance have not always produced concordant results, the majority suggest that ERCC1 is a marker of cisplatin resistance [11,30–34]. In our study, ERCC1 expression levels in NSCLC were higher in the three cell lines most resistant to cisplatin and lipoplatin.

On the basis of data available in the literature, lipoplatin [14,15] would seem to have fewer adverse side effects than cisplatin. In this study, we confirmed the lower toxicity of lipoplatin with respect to cisplatin using hematopoietic precursors as normal cells. In fact, cisplatin strongly inhibited the formation of GM-CFU at a concentration 100-fold lower than that of the peak plasma level. Conversely, lipoplatin caused only low toxicity and at the highest concentration tested. This was confirmed by the extent of DNA damage detected by the Comet assay.

In conclusion, the results from the present preclinical study show that lipoplatin produces a stronger cytotoxic effect in NSCLC and RCC cell lines and a lower toxicity than cisplatin in normal bone marrow stem cells. These findings would seem to indicate the potential usefulness of lipoplatin in the clinical treatment of these tumor histotypes. Moreover, ERCC1 and LRP expression seem to be predictors of resistance to cisplatin and lipoplatin and could therefore be used to identify subsets of patients who could benefit from treatment with these drugs.

Acknowledgements

The authors thank Professor Rosella Silvestrini for her invaluable scientific contribution, Marco Rosetti for data collection and technical assistance, and Gráinne Tierney for editing the manuscript. This study was supported by Istituto Oncologico Romagnolo.

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