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Original article

Cationic lipid:DNA complexes allow bleomycin uptake by melanoma cells

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ABSTRACT

Bleomycin is a chemotherapeutic agent barely diffusible through the plasmatic membrane. We evaluated DNA/cationic lipids complexes (lipoplexes) as mediators of its uptake in four spontaneous canine melanoma derived cell lines (Ak, Bk, Br and Rkb). Cell survival after lipofection plus or minus bleomycin was determined by the acid phosphatase method and the cellular uptake of lipoplexes, carrying the *E. coli* β-galactosidase gene, was evidenced by SYBR Green I staining. The four cell lines resulted sensitive to the bleomycin/lipoplexes system in both spatial configurations. Survival rates values were lower than 20% in monolayers of the four tested lines and lower than 30% in three lines (Ak, Bk and Rkb) when grown as spheroids. The sensitization to bleomycin depended on lipoplexes in Ak and Rkb while Bk (in both spatial configurations) and Br (as monolayers) were sensitive to bleomycin alone. Although some degree of sensitivity to bleomycin was induced by cationic lipids alone in Ak and Rkb monolayers, the maximal bleomycin effects appeared in the presence of lipoplexes. The sensitization was independent of transcriptional activity. The co-administration of lipoplexes diminished bleomycin IC_{50}: 10-fold in Ak and Rkb monolayers; and sensitized the Ak and Rkb resistant spheroids. The bleomycin cytotoxic effects depended on lipoplexes concentration and diminished when cells were incubated at 8 °C. Our results suggest that lipoplexes sensitize cells to bleomycin, increasing its uptake by an active transport mechanism, such as endocytosis. The bleomycin/lipoplexes system appears as a promising combination of chemotherapy and non-viral cancer gene therapy.

1. Introduction

Bleomycin (BLM) is a glycopeptide antibiotic with antineoplastic activity due to its endonuclease activity [1]. It has been proposed as a chemotherapeutic agent for melanoma since the skin cells do not express a hydrolase capable of inactivating the drug [1]. The cytotoxicity of BLM to certain tumor types might be related to the efficiency of drug uptake. However, BLMs are large (molecular weight of 1.5 kDa), hydrophilic peptides, barely capable of diffusing through the plasmatic membrane. Moreover, different strategies have been developed to bypass the cytoplasmic membrane barrier: Bleosome™ [2], Sendai virus envelope conjugated with cationic gelatin (CG-HVJ-E) [3] and cell electroporation [4,5]. The first two strategies deliver the BLM, using vehicles as ultra-deformable liposomes (Bleosome™) [2] and CG-HVJ-E [3]. The third strategy, cell electroporation, sensitizes the cells to the cytotoxic effects of BLM by increasing the amount of BLM molecules in the cytoplasm [4,5]. This approach was successfully applied to dog patients as monotherapy for melanoma [6] or combined with immunogene therapy for recurrent squamous cell carcinoma [7] as well as for human melanoma patients [8].

Canine malignant melanoma is a spontaneous tumor displaying histopathological features and biological behavior similar to human melanoma, but with a faster progression and an extremely poor prognosis. This highly aggressive canine tumor is too invasive to be cured only by surgical resection and is frequently resistant to current therapies [9,10]. This has prompted investigations to define new treatment strategies.

Here, we are presenting data indicating that cationic lipids, especially when complexed to DNA (lipoplexes, LPX), can efficiently facilitate the delivery of BLM into melanoma tumor cells. Therefore, the co-administration of BLM and LPX would be a viable strategy to enhance the BLM effects by enhancing the BLM assimilation inside the cell via endocytosis.

2. Materials and methods

2.1. Cell cultures

Cultured cells derived from four surgically excised oral (Br, Bk, Rkb) and ocular (Ak) canine melanomas [11] were obtained by enzymatic digestion of tumor fragments with 0.01% Pronase (Sigma, St. Louis, MO) and 0.035% DNase (Sigma) or by mechanical

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disruption in serum free culture medium [12]. They were cultured as monolayers and spheroids at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen), 10 mM HEPES (pH 7.4) and antibiotics. Serial passages were done by trypsinization (0.25% trypsin and 0.02% EDTA in PBS) of sub-confluent monolayers.

2.2. Plasmids

Plasmid psCMVβ carrying the *E. coli* β-galactosidase gene [12] was amplified in *E. coli* DH5α (Invitrogen), grown in LB medium containing 100 μg/ml neomycin and purified using an ion-exchange chromatographic method (Qiagen, Valencia, CA).

2.3. Liposome preparation and in vitro lipofection

DC-Chol (3β[N-(N′,N′-dimethylaminoethane)-carbamoyl cholesterol) and DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethilammonium bromide) were synthesized and kindly provided by BioSidus (Buenos Aires, Argentina). DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphatidyl ethanolamine) was purchased from Sigma. Liposomes were prepared at lipid/co-lipid molar ratios of 3:2 (DC-Chol:DOPE) or 1:1 (DMRIE:DOPE) by sonication as described [13,14]. Optimal DNA:lipid ratios and lipid mixtures were determined for every cell line [12].

Cultured cells at a 5×10^4 cells/cm^2 density (about 40% confluence) were exposed to LPX (0.5 μg plasmid DNA or polydIdC/cm^2) during 3–5 h in culture media without serum. PolydIdC was purchased from Sigma.

2.4. Sensitivity assays

Twenty-four hours after transfection, the βgal-transiently expressing cells were seeded on 96-wells plates as monolayers (4×10^3 cells per well) or on top of 1.5% solidified agar to form spheroids (Ak: 0.5×10^3 cells per well; Bk, Br and Rkb: 5×10^3 cells per well). Unless otherwise indicated, BLM (kindly provided by Gador S.A. Buenos Aires, Argentina) was added to a 3 μg/ml final concentration immediately after the corresponding LPX were added. After 5 days as monolayers or after 12 days as spheroids, cell viability was quantified with acid phosphatase assay (APH) [15]. The cell survival rate was calculated as the ratio of the absorbances between the cells treated and the control cells without BLM.

2.5. Lipoplexes curve and lipofection at low temperature

Dilutions were made from the 1X β-gal LPX described above for dose-response studies. Cells were incubated at 8 °C from 1 hour before lipofection in a box equilibrated with 5% CO_2 inside a refrigerator. LPX and BLM were removed by three washes (5 min each) with cold or pre-warmed DMEM-F12, containing 10% FBS. After 5 days, cell viability of monolayers was determined by the APH assay as described above.

2.6. SYBR green staining of lipoplexes

Plasmid DNA was incubated for 5 min with a 9X concentration of SYBR® Green I Nucleic Acid Gel Stain (Invitrogen) before the DNA/cationic lipids complex formation. Then, lipofection was carried out as described above. Optimal conditions for counting were found at 120 min of incubation. Once finished, cells were rinsed with culture media and fixed sequentially with a solution of 0.05% glutaraldehyde and 1% formaldehyde in PBS.

Photographs were taken using the inverted fluorescent microscope Eclipse TE2000-S (Nikon, Japan) at 200X. The percentage of cells that incorporated LPX was determined by counting the cells with at least one LPX dot over the total of the cells in the visual field photographed. Three different photograph fields were taken per experiment; each experiment was repeated three times.

2.7. K^+ depletion

Potassium was removed as reported in [16,17]. A K^+-free buffer (140 mM NaCl, 20 mM Hepes pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mg/ml D-glucose) was used. Cells were incubated for 5 min with hypotonic buffer (K^+-free buffer/H_2O 1:1) and then washed three times for 5 min with K^+-free buffer. Control cells were subjected to the same treatment in the presence of 10 mM KCl. Finally, in a minimum volume of buffer (with or without K^+), LPX and BLM were added and were incubated for 30 or 60 min and cell survival at day 5 were determined by the APH method as described above.

2.8. Statistics

Results were expressed as mean ± standard error of the mean (SEM) (*n*: number of experiments corresponding to independent assays). Differences between groups were determined by two-way analysis of variance (ANOVA). The difference between means was determined with the Bonferroni post-test.

3. Results

3.1. The presence of lipoplexes overcame the resistance to bleomycin

We assayed the cytotoxic effects of BLM on four canine melanoma cell lines (Ak, Bk, Br and Rkb) previously obtained and characterized in our laboratory [12]. The assay was performed in both spatial configurations: as standard bi-dimensional monolayers and as tri-dimensional spheroids. Ak and Br mono-spheroids were very compact with a spherical symmetry (Fig. 1a) whereas Bk and Rkb spheroids were looser: Bk displaying a big central nucleus surrounded by a close cloud of cells and Rkb a small nucleus surrounded by a large cloud of cells (Fig. 1b).

We tested the sensitivity to the chemotherapeutic agent BLM when it was added to the culture media immediately after the addition of LPX. We chose the pharmacologically relevant BLM concentration (3 μg/ml) reported in previous works [18,19].

As displayed on Fig. 1a,b while Ak and Rkb were not sensitive to BLM alone in any spatial configuration, Bk was sensitive in both configurations and Br only when cultured as monolayers. Lipofection with the reporter gene β-galactosidase (βgal) rendered Ak cells sensitive to BLM (about 80% of cells dying in both spatial configurations). While no significant changes were observed for sensitive Bk. In the case of Br and Rkb, βgal lipofection increased BLM cytotoxicity both in monolayers and spheroids. Compared to their respective monolayers, Br spheroids were relatively less sensitive to BLM, displaying a multicellular resistance (MCR) phenotype. Microscopic monitoring of treated spheroids (Fig. 1a,b) paralleled to the results obtained by the APH assay (Materials and methods). The degree of spheroids compactness did not appear to be related to the resistance to BLM since three out of four spheroids (compact Ak and Br, and loose Rkb) were resistant to BLM alone. Conversely, the four assayed cell lines were sensitive to the drug in the presence of LPX in both spatial configurations.

Rkb monolayers transiently expressing βgal and monolayers and spheroids treated with polydIdC lipoplexes in the absence of BLM, showed significantly lower cells survival when compared to

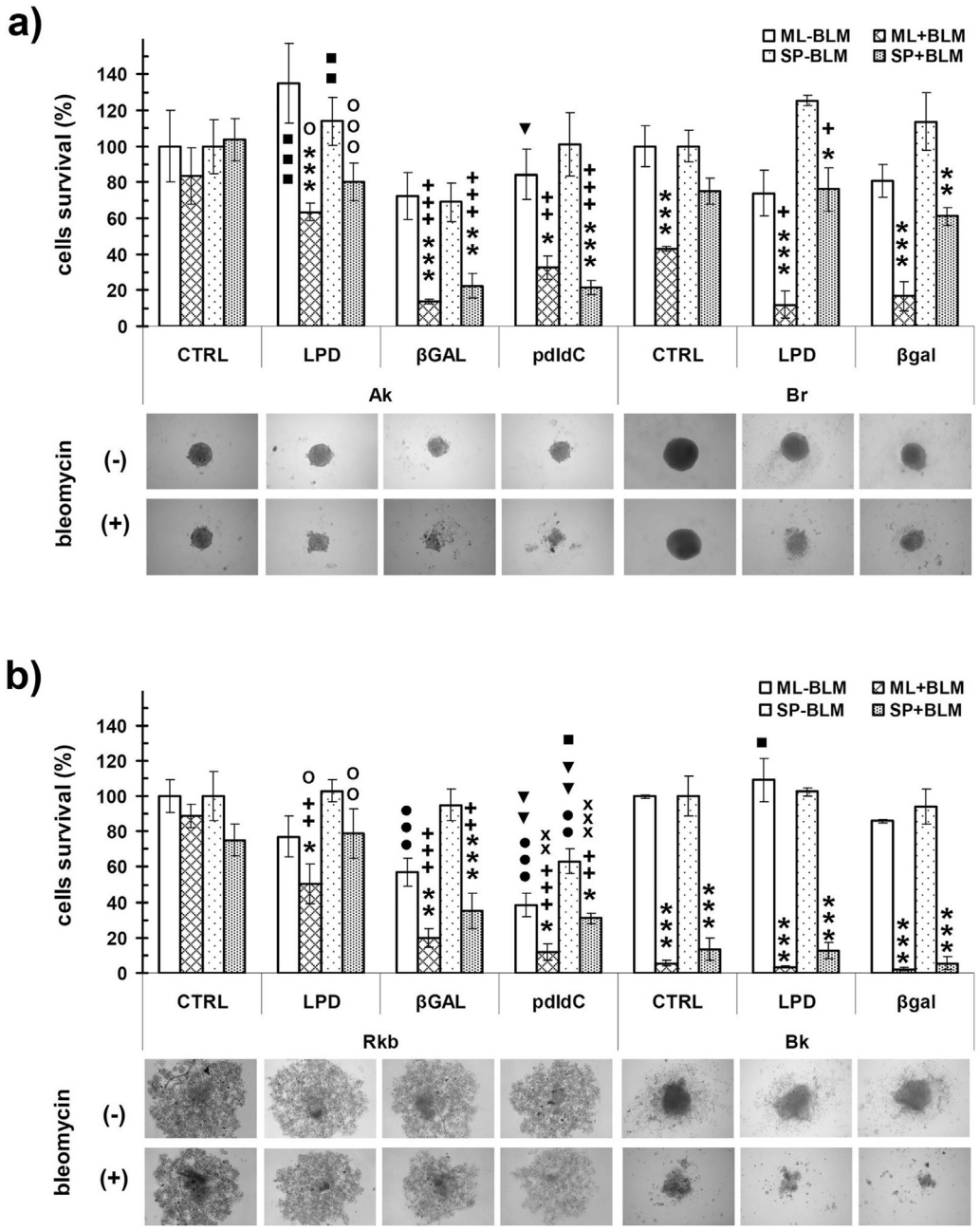

Fig. 1. Cytotoxic effects of bleomycin (BLM, 3 μg/ml) and lipofection on monolayers (ML) and spheroids (SP). Untreated (control, CTRL), cationic lipid only (LPD) or transiently βgal- or polydIdC-lipofected Ak, Br, Rkb and Bk canine melanoma cells were cultured as monolayers or spheroids and cell viability was quantified by APH on day 5 for monolayers and on day 12 for spheroids as described in Materials and methods. Results are expressed as means ± SEM of $n > 4$ independent experiments. Images represent individual spheroids ($n > 4$) growing in 96-well plates treated as described in the bars plot. BLM/LPX effects did not depend on spatial configuration for Ak (interaction $P = 0.5695$) and Bk (interaction $P = 0.6593$) and depended on it for Rkb (interaction $P = 0.0475$) and Br (interaction $P = 0.0194$). Compared to the respective: (✱: treatment without BLM), (+: control), (**x**: LPD), (**o**: βgal with BLM), (●: control), (▼: LPD), (■: βgal without BLM).
One symbol: $P < 0.05$; two symbols: $P < 0.01$; three symbols: $P < 0.001$.

control cells probably because of interferon-β release as described elsewhere [20].

To determine if the separate components of LPX could have effects by themselves, the action of DNA and cationic lipids (LPD) was tested separately. No significant differences on cell survival were found when BLM was added with naked plasmid DNA compared to BLM alone (data not shown). As depicted on Fig. 1a,b, both Br monolayers and spheroids displayed the same pattern of sensitization either with the addition of free LPD or LPX. It is worth to note that the addition of free LPD was able of sensitizing Ak and Rkb monolayers and Br spheroids to BLM, as compared to untreated controls (Fig. 1a,b). Nevertheless, Ak and Rkb spheroids survival was not affected by this condition, diverging from the case of LPX added together with the chemotherapeutic agent. Both Ak and Rkb spheroids required LPX for being sensitized to BLM.

3.2. The effects of lipoplexes on bleomycin cytoxicity did not rely on the transcriptional activity

The high efficiency of the BLM/LPX system encouraged us to study more deeply its mechanism of action. Fig. 1a,b show that similar sensitivity to BLM of Ak and Rkb was reached when the βgal carrying plasmid was replaced by a non-coding unspecific sequence polydIdC in the LPX, indicating that the sensitization was

independent of any transgene transcriptional activity and/or binding sequences for regulatory factors carrying nuclear localization sequences (NLS).

3.3. Bleomycin dose-response curves shifted leftwards in the presence of lipoplexes

To quantify the effects of LPXs on those cells whose sensitivity to BLM was significantly increased, dose-response curves for the drug were assayed and IC_{50} calculated (Fig. 2). The presence of LPX shifted the curves leftwards indicating a 10-fold increase of sensitivity for both Ak and Rkb monolayers; and > 4-fold and > 6-fold for their respective spheroids. The differences in the survival rates between both curves (control and βgal LPX) started to be significant at 1 μg/ml BLM in Ak and Rkb monolayers and 0.1 μg/ml BLM in Ak spheroids. In Rkb, the differences were significant only between 3 and 10 μg/ml BLM. Again, the spheroids response pattern was very similar between both cell lines even though their spheroids were morphologically different (Fig. 1c).

3.4. The sensitivity to bleomycin depended on the lipoplexes concentration

Once it was determined that LPX were responsible of the enhanced BLM cytotoxicity, the effects of increasing concentrations of LPX for a fixed amount of BLM were assayed. Ak and Rkb monolayers were incubated with 3 μg/ml BLM and serial LPX dilutions (0,1X; 0,2X; 0,5X; 0,75X and 1X). Concentrations greater than 1X standard were highly toxic for cells. At normal cells incubation temperature (37 °C), the dose-response curve showed that cell death was proportionally correlated with the increasing amount of added LPX (Fig. 3). Both curves correlated with a dose-response curve (Ak: $R^2 = 0.80$ and Rkb: $R^2 = 0.73$) with IC_{50} of 0.14X for Ak and 0.28X for Rkb. The higher determination coefficient and the lower IC_{50} seen in Ak suggest that Ak was more sensitive to the variations of LPX concentrations than Rkb.

3.5. The cytotoxic effects of the bleomycin/lipoplexes combination was temperature sensitive

The cytotoxic effect of BLM varied according to the LPX concentration (Fig. 3a) and cell mediated endocytosis is the main studied transport mechanism by which in vitro cultured cells incorporate LPX [14,17,21,22]. So, it could be inferred that the endocytic activity of the cell would be involved in BLM/*LPX* transport into the cytoplasm. If this was the case, it should be a temperature-dependent cellular process.

Consequently, a new dose-response curve where BLM and LPX were added at 8 °C was assayed (Fig 3a). Survival of Ak and Rkb monolayers resulted significantly higher starting at 0.2X and 0.5X lipoplexes concentrations, respectively. In the case of Rkb, the dose-response curve was barely noted, and the correlation coefficient very low (0.29).

The strong dependence of the BLM/LPX system on the incubation temperature suggests the intervention of an active transport mechanism in the incorporation of the drug by the cells during the co-administration.

3.6. The cellular uptake of lipoplexes required K^+

Clathrin-mediated endocytosis of LPX is inhibited by K^+ depletion [17]. To ascertain the possible involvement of K^+, Ak and Rkb cells were incubated with LPX in the absence or presence of K^+ for 30 or 60 min (Fig. 3b). In the presence of K^+, both cell lines were significantly sensitive to BLM/LPX: Ak starting at 30 min and Rkb at 60 min. In the absence of K^+, none of the cell lines were inhibited by BLM/LPX at both times, suggesting the key involvement of clathrin-mediated endocytosis for BLM uptake.

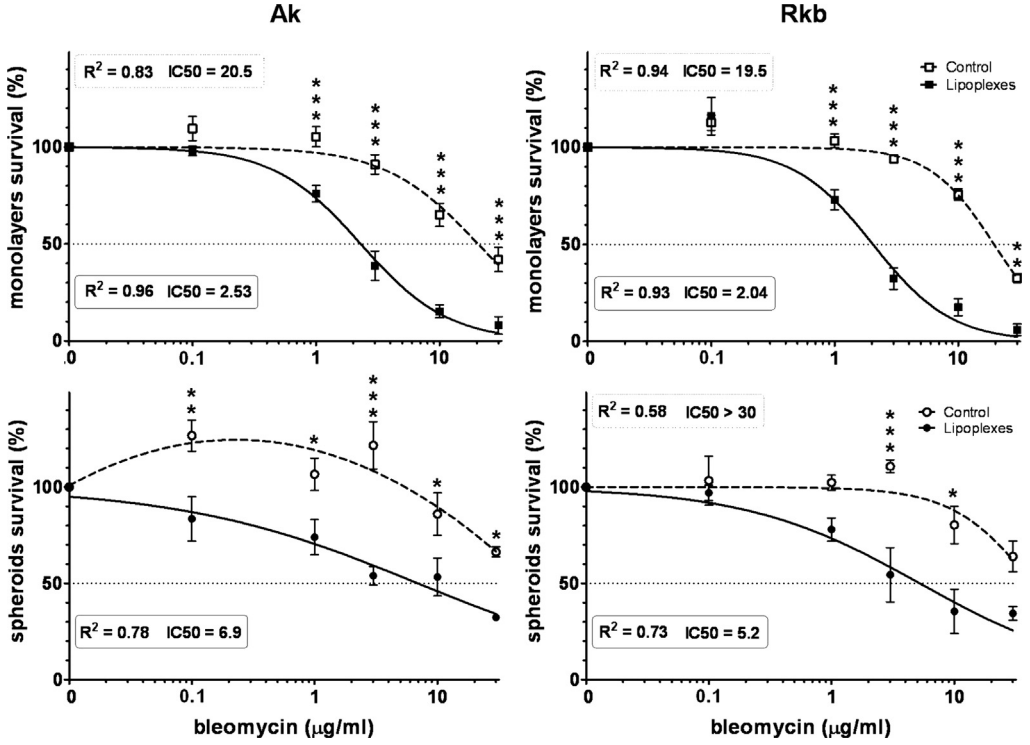

Fig. 2. Bleomycin effect on cells survival: dose-response curves for untreated (open symbols) and βgal-lipofected (full symbols) Ak and Rkb monolayers. Lipofection was performed at 37 °C in the absence or increasing amounts of bleomycin as described in Materials and methods. Results were expressed as mean ± SEM of $n > 4$ independent experiments. Each panel displays the results as percentage of the respective untreated condition (no drug). Compared to the respective lipofected condition: ✱.
One symbol: $P < 0.05$; two symbols: $P < 0.01$; three symbols: $P < 0.001$.

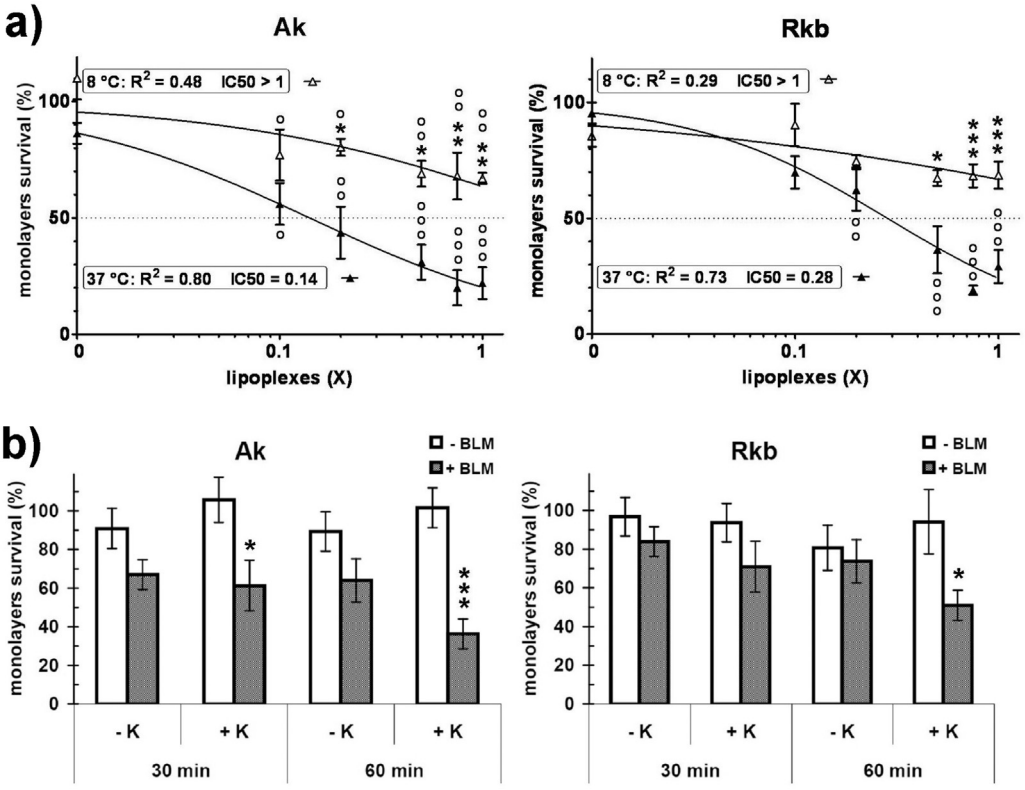

Fig. 3. Effects of lipoplexes (a) and K^+ depletion (b) on cells survival. **(a)** Dose-response curves for untreated (open symbols) and βgal-lipofected (full symbols) Ak and Rkb monolayers at different temperatures. Lipofection was performed at 8 °C or 37 °C in the presence of increasing amounts of lipoplexes as described in Materials and methods. Results were expressed as mean ± SEM of $n > 4$ independent experiments. Each panel displays the results as percentage of the respective untreated condition (no lipoplexes). Compared to: ✱(37 °C), **o** (non-lipofected). **(b)** K^+ depletion and bleomycin effects on βgal-lipofected Ak and Rkb monolayers survival. The assay was performed at 37 °C as described in Materials and methods. Results were expressed as mean ± SEM of $n > 4$ independent experiments. Compared to: ✱ (non K^+ depleted condition). One symbol: $P < 0.05$; two symbols: $P < 0.01$; three symbols: $P < 0.001$.

3.7. The cellular uptake of lipoplexes was concentration and temperature sensitive

To test the effect of the incubation temperature on the uptake of LPX, they were stained with SYBR Green I (Fig. 4a, left) and evaluated by epifluorescence microscopy. LPX were seen as cytoplasmic discrete green dots surrounding strongly stained cell nuclei (Fig. 4a, right). The incorporation of LPX was evaluated by counting the number of cells with at least 1 LPX green dot within the totality of the cells in the optical field. When the cells were incubated with stained free plasmid DNA, no cytoplasmic dots appeared (Fig. 4a, center). In this case, Ak nuclei appeared stained because of the presence of free dye.

In agreement with the survival rates obtained on Fig. 3 the incorporation of LPX decreased when the LPX were diluted (Fig. 4b). Furthermore, when incubated at 8 °C a significant decrease of LPX uptake was found in both Ak and Rkb monolayers at concentrations ranging from 0.25 to 0.75X and 0.50 to 0.75X, respectively (Fig. 4b). It is noteworthy to mention that no differences in the incorporation of LPX were seen at 1X LPX concentration in both cell lines. This fact implies that even though the sensitivity to the BLM/LPX system decreased at 8 °C, an additional temperature independent path (evidenced at higher LPX concentration) played an important role in the incorporation of the LPX, but this alternative did not involve a concomitant BLM uptake.

4. Discussion

As far as we know, our work is the first study reporting the sensitization to the cytotoxic effects of BLM by DNA/cationic lipids complexes (Figs. 1 and 2). The physicochemical properties of BLM molecules and the negative impact of low temperatures on the BLM cytotoxicity induced by LPX strongly suggest the involvement of an active transport mechanism (Fig. 3). This hypothesis is supported by the evidence of the SYBR Green LPX staining experiments (Fig. 4).

Our findings strongly suggest that the cytotoxic effect of the BLM/LPX system described above is due to an augmented incorporation of BLM inside the cells facilitated by a temperature dependant incorporation of LPX, such as endocytosis.

The addition of cationic lipids was able of partially improving the efficacy of bleomycin in the case of Ak and Rkb monolayers as well as Br monolayers and spheroids, probably because the interaction of the lipids with negatively charged macromolecules present in the culture media. These complexes would also be internalized by endocytosis. Nevertheless in BLM resistant Ak and Rkb, the survival rates to the drug in the presence of LPX (either βgal –19 and 35%– or polydIdC –39 and 31%) were always lower than in the presence of the corresponding lipids (47 and 66% respectively), indicating that the effects of LPX were stronger than those of LPD.

Although incubation at low temperatures inhibits the invagination of the cell membrane because it is an energy-dependant process, the lipofection efficiency of cells incubated at 8 °C did not significantly differ from that obtained when incubated at 37 °C (data not shown). Then, our results also support the presence of a temperature independent mechanisms, incorporating LPX inside the cells as lipid mixing [21], inter-membrane fusion [23], or union of the LPX to cell receptors or to cell membranes specific sites [21]. These additional mechanisms would not contribute to BLM uptake.

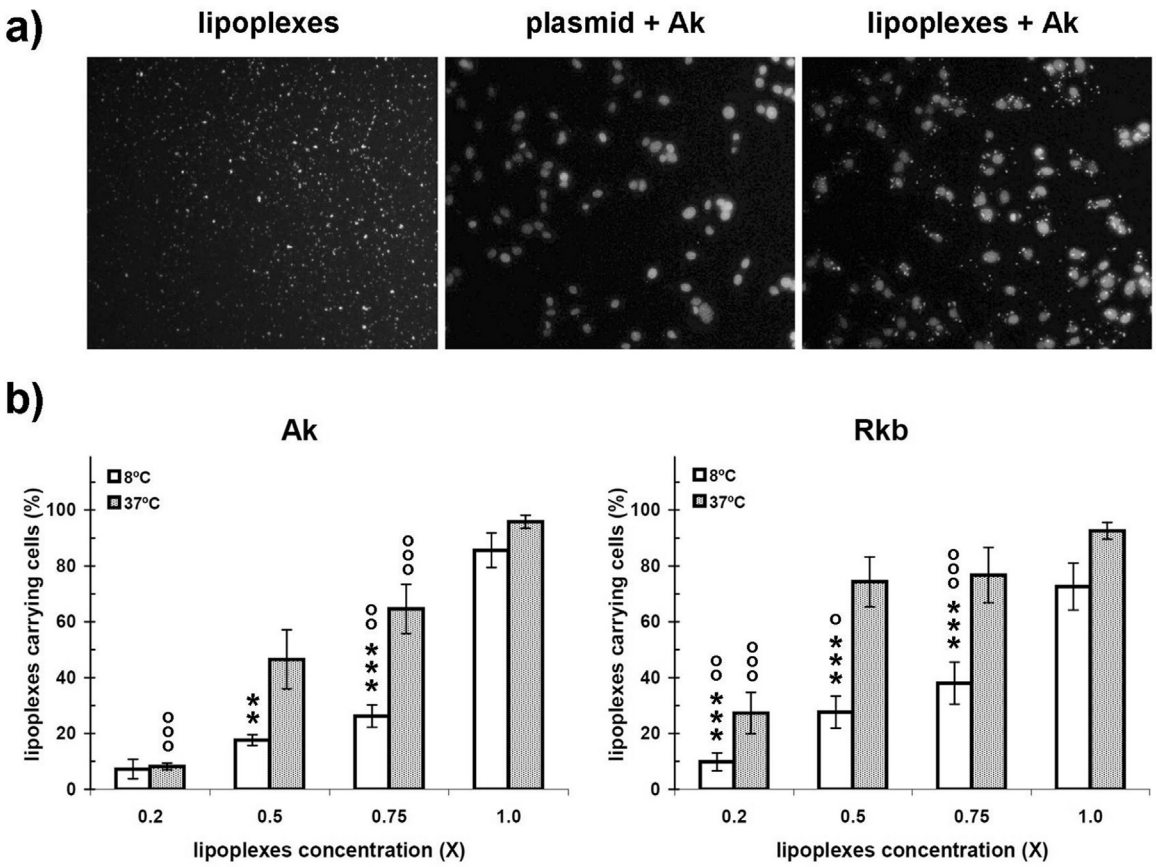

Fig. 4. Temperature effect on lipoplexes uptake for untreated (control) and βgal-lipofected Ak and Rkb monolayers. **(a)** SYBR green stained lipoplexes (left), unlipofected Ak cells incubated with free stained βgal plasmid DNA (center) and lipofected Ak cells (right). The assay was performed as described in Materials and methods. Epifluorescence micrographs were taken at 200×. **(b)** Proportion of lipoplexes carrying as a function of lipoplexes concentrations at 8 °C and 37 °C. The assay was performed as described in Materials and methods. Results were expressed as mean ± SEM of $n > 4$ independent experiments. Compared to: ✱ (37 °C), **o** (the previous dilution on its right). One symbol: $P < 0.05$; two symbols: $P < 0.01$; three symbols: $P < 0.001$.

So, even though LPX could enter cells using both temperature-dependent, and -independent uptake mechanisms, BLM/LPX combination were predominantly incorporated via temperature-dependent endocytosis. Anyhow, BLM possesses a very high intrinsic cytotoxicity, allowing that as few as some hundreds of BLM molecules internalized in a cell are sufficient to kill that cell [5].

The combination of BLM and LPX was able of overcoming the spheroids resistance phenotype in the three out of four cells lines evaluated in vitro (Bk excluded because its intrinsic sensitivity to BLM, Fig. 1), thus, it could constitute the base for a highly effective therapeutic strategy in vivo. It is worth to note that LPX did not display any BLM antagonistic effects in sensitive cells (Br monolayers and Bk in both spatial configurations).

The results presented here strongly encourage further research on the BLM/LPX system as an enhancer of non-viral LPX-mediated cancer gene therapy taking into account that:

- BLM is a chemotherapeutic agent commonly used in human [24] and veterinary oncology [6];
- the BLM/LPX system effectiveness was maintained when cells were grown as multicellular spheroids;
- in vivo tumors and their respective spheroids in vitro show a very high similarity in the response to the non-viral gene transfer [12]; the cytotoxic effects of human interferon-β gene lipofection were strongly enhanced by BLM in multicellular spheroids derived from human melanoma [25].

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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References

[1] Jingyang C, Stubbe J. Bleomycins: towards better therapeutics. Nat Rev Cancer 2005;5:102–12.
[2] Lau KG, Hattori Y, Chopra S, O'Toole EA, Storey A, Nagai T, et al. Ultradeformable liposomes containing bleomycin: in vitro stability and toxicity on human cutaneous keratinocyte cell lines. Int J Pharm 2005;300:4–12.
[3] Mima H, Yamamoto S, Ito M, Tomoshige R, Tabata Y, Tamai K, et al. Targeted chemotherapy against intraperitoneally disseminated colon carcinoma using a cationized gelatin-conjugated HVJ envelope vector. Mol Cancer Ther 2006;5:1021–8.
[4] Orlowsky S, Belehradek Jr J, Paoletti C, Mir LM. Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs. Biochem Pharmacol 1988;37:4727–33.

[5] Poddevin B, Orlowsky S, Belehradek Jr J, Mir LM. Very high cytotoxicity of bleomycin introduced into the cytosol of cells in culture. Biochem Pharmacol 1991;42:S62–75.
[6] Spugnini EP, Dragonetti E, Vincenzi B, Onori N, Citro G, Baldi A. Pulse-mediated chemotherapy enhances local control and survival in a spontaneous canine model of primary mucosal melanoma. Melanoma Res 2006;16:23–7.
[7] Reed SD, Fulmer A, Buckholz J, Zhang B, Cutrera J, Shiomitsu K, et al. Bleomycin/interleukin-12 electrochemogene therapy for treating naturally occurring spontaneous neoplasms in dogs. Cancer Gene Ther 2010;17:457–64.
[8] Testori A, Rossi CR, Tosti G. Utility of electrochemotherapy in melanoma treatment. Curr Opin Oncol 2012;24:155–61.
[9] Ramos-Vara JA, Beissenherz ME, Miller MA, Johnson GC, Pace LW, Fard A, et al. Retrospective study of 338 canine oral melanomas with clinical, histologic, and immunohistochemical review of 129 cases. Vet Pathol 2000;37:597–608.
[10] Smith SH, Goldschmidt MH, McManus PM. A comparative review of melanocytic neoplasms. Vet Pathol 2002;39:651–78.
[11] Finocchiaro LME, Glikin GC. Cytokine-enhanced vaccine and suicide gene therapy as surgery adjuvant treatments for spontaneous canine melanoma. Gene Ther 2008;15:267–76.
[12] Gil-Cardeza ML, Villaverde MS, Fiszman GL, Altamirano NA, Cwirenbaum RA, Glikin GC, et al. Suicide gene therapy on spontaneous canine melanoma: correlations between in vivo tumors and their derived multicell spheroids in vitro. Gene Ther 2010;17:26–36.
[13] Felgner JH, Kumar R, Sridhar CN, Wheeler CJ, Tsai YJ, Border R, et al. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J Biol Chem 1994;269:2550–61.
[14] Gao X, Huang L. Cationic liposome-mediated gene transfer. Gene Ther 1995;2:10–22.
[15] Friedrich J, Eder W, Castaneda J, Doss M, Huber E, Ebner R, et al. A reliable tool to determine cell viability in complex 3-D culture: the acid phosphatase assay. J Biomol Screen 2007;12:1115–9 [Erratum in: J Biomol Screen.12 (2007) 1115–1119].
[16] Larkin JM, Donzell WC, Anderson RG. Potassium-dependent assembly of coated pits: new coated pits form as planar clathrin lattices. J Cell Biol 1986;103:2619–27.
[17] Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolea-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther 2005;12:468–74.
[18] Pron G, Mahrour N, Orlowski S, Tounekti O, Poddevin B, Belehradek Jr J, et al. Internalization of the bleomycin molecules responsible for bleomycin: a receptor-mediated endocytosis mechanism. Biochem Pharmacol 1999;75:46–56.
[19] Tounekti O, Pron G, Belehradek Jr J, Mir LM. Bleomycin, an apoptosis-mimetic drug that induces two types of cell death depending on the number of molecules internalized. Cancer Res 1993;53:5462–9.
[20] Li S, Wilkinson M, Xia X, David M, Xu L, Purkel-Sutton A, et al. Induction of IFN-regulated factors and antitumoral surveillance by transfected placebo plasmid DNA. Mol Ther 2005;11:112–9.
[21] Resina S, Prevot P, Thierry AR. Physico-chemical characteristics of lipoplexes influence cell uptake mechanisms and transfection efficacy. PLoS One 2009;4(6):e6058.
[22] Vaughan EE, DeGiulio JV, Dean DA. Intracellular trafficking of plasmids for gene therapy: mechanisms of cytoplasmic movement and nuclear import. Curr Gene Ther 2006;6:671–81.
[23] Marchini C, Pozzi D, Montani M, Alfonsi C, Amici A, Candeloro De Sanctis S, et al. Role of temperature-independent lipoplex-cell membrane interactions in the efficiency boost of multicomponent lipoplexes. Cancer Gene Ther 2011;18:543–52.
[24] Mir LM, Tounekti O, Orlowski S. Bleomycin: revival of an old drug. Gen Pharmacol 1996;27:745–8.
[25] Villaverde MS, Gil-Cardeza ML, Glikin GC, Finocchiaro LM. Interferon-β lipofection I. Increased efficacy of chemotherapeutic drugs on human tumor cells derived monolayers and spheroids. Cancer Gene Ther 2012;19:508–16.