Cancer Chemother Pharmacol (1996) 37: 556–560

ORIGINAL ARTICLE

Yan-Ping Hu · Sophie Jarillon · Catherine Dubernet
Patrick Couvreur · Jacques Robert

On the mechanism of action of doxorubicin encapsulation in nanospheres for the reversal of multidrug resistance

Received: 8 March 1995/Accepted: 6 June 1995

Abstract We had previously shown that doxorubicin encapsulation in polyisohexylcyanocrylate nanospheres could circumvent the P-glycoprotein-mediated multidrug resistance (MDR) exhibited by C6 rat glioblastoma in culture. We then investigated what could be the mechanism of such a circumvention. The cytotoxicity of free and encapsulated doxorubicin was evaluated in two MDR variants of the C6 cell line in a device allowing the separation of cells from drugs by a polycarbonate membrane of 0.2-μm pore size. We observed that the progressive disruption of the nanospheres allowed their doxorubicin content to reach the cell monolayer and exert its cytotoxicity in a fashion similar to that exhibited by free doxorubicin. However, no circumvention of MDR is obtained by doxorubicin encapsulation when drug-containing nanospheres are separated from the cells by the polycarbonate membrane. In addition, no effect on azidopine binding to P-glycoprotein-enriched membranes is exerted by unloaded nanospheres, even after their spontaneous degradation in cell-culture medium. Taken together, these results suggest that a physical contact between doxorubicin-containing nanospheres and the cells is required for the circumvention of MDR. The role of degradation products from the nanospheres as modulators of P-glycoprotein activity can be ruled out.

Key words Anthracyclines · Drug encapsulation · Multidrug resistance

Y.-P. Hu · S. Jarillon · J. Robert (✉)
Institut Bergonié and Université de Bordeaux II, 180 rue de Saint-Genès, F-33076 Bordeaux Cedex, France

C. Dubernet · P. Couvreur
Centre d'Études Pharmaceutiques, CNRS URA 1218, 5 rue Jean-Baptiste-Clément, F-92296 Châtenay-Malabry Cedex, France

Introduction

There is a considerable interest for drug encapsulation in cancer treatment. It has long ago been shown that doxorubicin encapsulated in liposomes presents original pharmacokinetic features, especially being characterized by a prolonged half-life improving drug disposition [6, 13]. There has been a constant effort aimed at improving the stability of liposomes so as to divert them from reticuloendothelial cells, where most of them are rapidly sequestrated [5]. Other systems of drug encapsulation in microparticles have also been developed and tested with and without a molecular targeting of the vehicle to the cancer cell (for a review, see [15]).

It has more recently been discovered that doxorubicin encapsulation could represent a valuable means of circumventing multidrug resistance. In several studies the group of Rahman [14, 18] and Warren et al. [20] have shown that doxorubicin embedded in liposomes retains significant activity against multidrug-resistant cells. Similar results have been obtained with doxorubicin encapsulated in polyisohexylcyanoacrylate (PIHCA) nanospheres by Cuvier et al. [3] and ourselves [1]. Multidrug resistance is a general mechanism of tumor-cell resistance to anticancer drugs, involving the overexpression of an adenosine triphosphate (ATP)-driven membrane pump called P-glycoprotein (for a review, see [8]). This protein is capable of extruding a variety of lipophilic natural products with an apparently low specificity. Its overexpression in in vitro models selected with such drugs is very frequent and has also been found in human tumors, with the frequency being higher in previously treated cancers [7, 12]. Several works have clearly shown the potential clinical interest of circumventing multidrug resistance to improve response rates to chemotherapeutic regimens [4, 17]. Although this circumvention can be obtained with various chemicals, the interest of other means such as drug encapsulation cannot be minimized.

However, the mechanism by which drug encapsulation allows the bypass of multidrug resistance (MDR) is not fully understood. It appeared from the work of Rahman et al. [14] and Warren et al. [20] that the presence of liposomes, simply added to free doxorubicin, could enhance doxorubicin cytotoxicity and cellular accumulation, similarly to drug encapsulation. In addition, it was observed that unloaded liposomes as well as doxorubicin-loaded liposomes could inhibit azidopine binding to P-glycoprotein [14], a good way of indicating that these preparations directly interfered with the pump, just as do verapamil and other chemicals. Therefore, it seems that circumvention of drug resistance in these conditions is due not to drug encapsulation itself but rather to direct P-glycoprotein inhibition by some components of the liposomes. In the case of PIHCA nanospheres, the mechanism of resistance reversal is not as clear. We have shown [1] that this reversal can be obtained without any restoration of drug accumulation and that the addition of blank nanospheres to free doxorubicin has no effect on doxorubicin cytotoxicity. The results we describe herein suggest that a direct contact of doxorubicin-containing nanospheres with the cells is a requisite for circumventing drug resistance and that no direct interference of PIHCA nanospheres with P-glycoprotein is detectable.

Materials and methods

Cell culture

In this study we used the C6 rat glioblastoma cell lines we had previously characterized [9, 16] and employed in studying the reversal of MDR by various chemicals [10] and by doxorubicin encapsulation in PIHCA nanospheres [1]. The C6 0.001 variant continuously grows with 0.001 μg doxorubicin/ml medium and presents a purely typical MDR phenotype and 3- to 6-fold resistance to doxorubicin. The C6 0.5 variant currently grows with 0.5 μg doxorubicin/ml medium and presents MDR features clearly associated with other doxorubicin-specific resistance mechanisms; its factor of resistance to doxorubicin is around 400–600. Cells were grown with Dulbecco's modified minimal Eagle's medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (Seromed) in a humidified atmosphere containing 5% CO_2. They were replicated each week and the medium was changed every 2 days. Cells were freed of drug at 4 days before experiments.

Drugs and supplies

Doxorubicin was kindly provided by Pharmacia (Rueil-Malmaison, France). It was used either free or after encapsulation in PIHCA nanospheres. This particulate form was prepared by polymerization of isohexylcyanoacrylate in the presence of doxorubicin as previously described [19]. It has been shown by fluorescence spectroscopy that 10–12% of doxorubicin is recovered in the supernatant after centrifugation, indicating that only this proportion of the drug is not embedded inside the polymer but remains either free or adsorbed at the surface of the nanospheres. The mean diameter of these particles was 342 ± 120 nm when they contained doxorubicin and 169 ± 23 nm when they were prepared in the absence of drug (unloaded nanospheres). To study whether a close contact of cells with the nanospheres was required for their cytotoxic effect, we performed certain incubations in the presence of a polycarbonate membrane inserted between the cells and the drug-containing medium. These membranes (pore size 200 nm) were obtained from Nunc (catalog number 1-62243, supplied by Poly-Labo, Strasbourg, France); they were mounted on cupules measuring 1 cm in diameter, which were inserted in the wells of 24-well cell-culture plates (Nunc) as shown in Fig. 1. When incubations with drugs were to be performed, 500 μl drug-free medium was first added to the cells; a cupule was then inserted in each well, and 500 μl drug-containing medium was added, the concentration of drug being calculated for the total 1,000 μl of the well. For validation of the system, preliminary experiments were performed in the absence of cells, and doxorubicin concentrations were estimated in the two compartments (above and beneath the polycarbonate membrane) by fluorometry using a spectrofluorometer (Kontron Instruments, model SFM-25), with excitation and emission wavelengths being set at 480 and 590 nm, respectively.

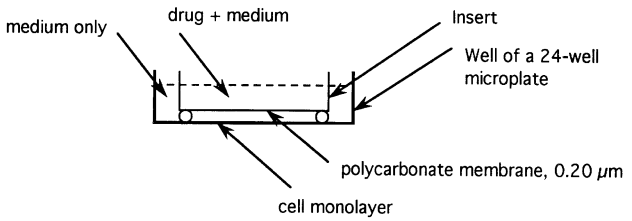


Fig. 1 Schematic representation of the experimental device used for separating cell and drug compartments by a polycarbonate membrane (pore size 0.20 μm)

Cytotoxicity

Growth inhibition was measured with the tetrazolium dye (MTT) assay during the exponential phase of growth of the cells. Since cells had different growth-doubling times, it was necessary to adapt experimental procedures to growth conditions so as to optimize the MTT assay as previously described [1]. Briefly, an adequate number of cells were seeded in 24-well microplates; one cell cycle later, the medium was changed for drug-containing medium, which was added either directly to the cell monolayer or into the cupule inserted in the wells (with a double drug concentration). Doxorubicin was present either in the free soluble form or in the particulate form and was incubated with the cells for 24 h. Cupules were then removed, cell monolayers were rinsed, and cells were allowed to grow for two further cell cycles with fresh medium, after which the MTT assay was performed. Concentrations of doxorubicin ranged between 0.01 and 30 μg/ml; unloaded nanospheres were used under the same conditions described for doxorubicin-containing nanospheres and at equivalent concentrations. IC_{50} values were estimated as the drug concentrations giving 50% inhibition of cell growth as compared with that of untreated cultures.

Azidopine binding

Azidopine labeling of P-glycoprotein and its inhibition by free and particulate doxorubicin and by unloaded nanospheres were evaluated on membrane preparations of C6 0.5 cells. Membranes were obtained from 1.5×10^8 cells by homogenization with a Dounce homogenizer in 25 ml 0.01 *M* TRIS-HCl buffer (pH 7.5) containing 0.25 *M* sucrose and 0.2 m*M* $CaCl_2$. After the addition of ethylenediaminetetraacetic acid (EDTA) to a final concentration of

1 m*M* and of 100 ml 0.01 *M* TRIS-HCl buffer (pH 7.5) containing 0.025 *M* sucrose, homogenates were centrifuged at 1,000 *g* for 10 min. The supernatant was distributed in ultracentrifuge tubes on to a layer made of 0.01 *M* TRIS-HCl buffer (pH 7.5) containing 1 *M* sucrose and 1 m*M* EDTA and then centrifuged at 11,000 *g* for 30 min. The interfacial cloudy layer was recovered in 0.01 *M* TRIS-HCl buffer containing 0.25 *M* sucrose and pelleted at 76,000 *g* for 1.25 h. This pellet was recovered in 4 ml of the last buffer, distributed into aliquots containing 1–2 mg protein, and kept frozen at − 70° C.

To aliquots of these membrane preparations containing 20 μg protein, either free or particulate doxorubicin or unloaded nanospheres were added at various final concentrations (12.5–100 μ*M* in doxorubicin); then, 5 μCi [^{3}H]-azidopine (sp. act., 50 Ci/mmol; Amersham, Les Ulis, France) and 0.01 *M* TRIS-HCl buffer (pH 7.5) containing 0.25 *M* sucrose were added to a final volume of 100 μl. This mixture was first incubated in a dark environment for 30 min at room temperature and then irradiated under UV light (350 nm) for 30 min. Each preparation was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [11]. Gels were incubated in an Amplify solution (Amersham) and dried under vacuum for 1 h. Autoradiography was performed by 11-day periods of exposure at − 70° C to MP-hyperfilm (Amersham). Spots were quantified on the autoradiograms using a Hoefer densitometer (Bioblock, Strasbourg, France).

Results and discussion

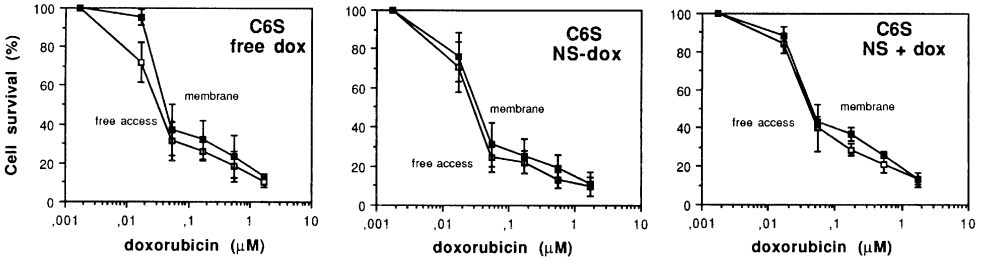
Preliminary experiments showed that there was a progressive equilibration of free doxorubicin concentrations in both compartments of the wells, above and beneath the membrane, for both formulations of doxorubicin (free or encapsulated in nanospheres). Equal concentrations of free doxorubicin were obtained in both compartments within 24 h. Assuming that free doxorubicin could freely cross the polycarbonate membrane and that the doxorubicin-loaded nanospheres could not, it can be concluded that there was a spontaneous disruption of the polymer, as has been shown to occur progressively [2], allowing free doxorubicin liberated from the particulate form to cross the polycarbonate membrane.

When C6 sensitive cells were exposed to free doxorubicin, to doxorubicin-loaded nanospheres, or to free doxorubicin plus unloaded nanospheres, the same cytotoxicity was observed with and without the membrane between the cell compartment and the drug compartment (Fig. 2). With C6 0.001 cells we observed similar cytotoxicity for free doxorubicin and for free doxorubicin plus unloaded nanospheres in the presence or absence of the membrane between cells and drug. In contrast, whereas doxorubicin-loaded nanospheres could bypass the resistance of this line when cells had free access to the drug, as previously shown [1], the 4-fold resistance was maintained when the membrane was inserted between the drug and cell compartments (Fig. 3). Similar conclusions can be drawn from the results obtained with C6 0.5 cells (Fig. 4); free access of the drug conjugate with cells resulted in a 5-fold reduction in the resistance factor, whereas the presence of a membrane insert in the well prevented any significant reversal of drug resistance. We have gathered in Table 1 the IC_{50} values obtained under the various conditions presented in Figs. 2–4.

It can be concluded that a contact between loaded nanospheres and cells is necessary for observal of the MDR-reversing properties of this drug formulation. It is possible to exclude the hypothesis that degradation products of the nanospheres, even if they are capable of crossing the polycarbonate membrane, could be responsible for the reversal of doxorubicin resistance. If this were the case, the addition of unloaded nanospheres to free doxorubicin, in direct contact with the cells, would have also had a reversing effect.

To explore the possible interference of PIHCA nanospheres or their degradation products with P-glycoprotein, we evaluated the inhibition by unloaded nanospheres of tritiated azidopine binding to P-glycoprotein-enriched membranes of C6 0.5 cells. Unloaded nanospheres were used either immediately after reconstitution (intact nanospheres) or at 24 h after reconstitution in culture medium (degradation products). It appears that no inhibition of azidopine binding by intact or degraded unloaded nanospheres was detectable, whereas doxorubicin itself as well as doxorubicin-loaded nanospheres decreased azidopine binding to P-glycoprotein-enriched membranes (Fig. 5). Therefore, it appears that no component of PIHCA nanospheres that could be in contact with the cells has a direct inhibitory effect on P-glycoprotein. The resistance-reversing effect of PIHCA nanospheres is therefore associated with drug encapsulation itself. Work is

Fig. 2 Cytotoxicity of free doxorubicin, doxorubicin-loaded PIHCA nanospheres, and free doxorubicin combined with unloaded PIHCA nanospheres in C6 sensitive cells after free contact of the drug formulations with the cell monolayer (—□—) and in the presence of a polycarbonate membrane between cell and drug compartments (—■—). The results represent mean values ± SD of 2 independent experiments performed in triplicate. The percentage of cell survival was estimated by the MTT assay



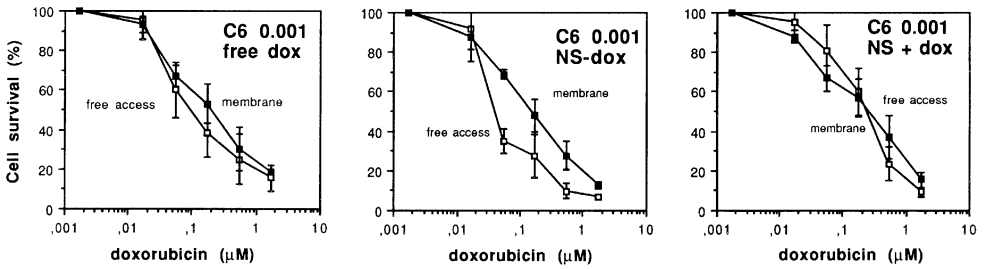


Fig. 3 Cytotoxicity of free doxorubicin, doxorubicin-loaded PIHCA nanospheres, and free doxorubicin combined with unloaded PIHCA nanospheres in C6 0.001 doxorubicin-resistant cells. Symbols are the same as those in Fig. 2

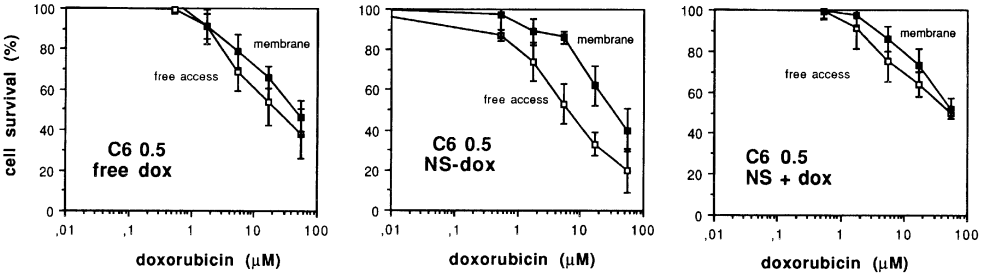
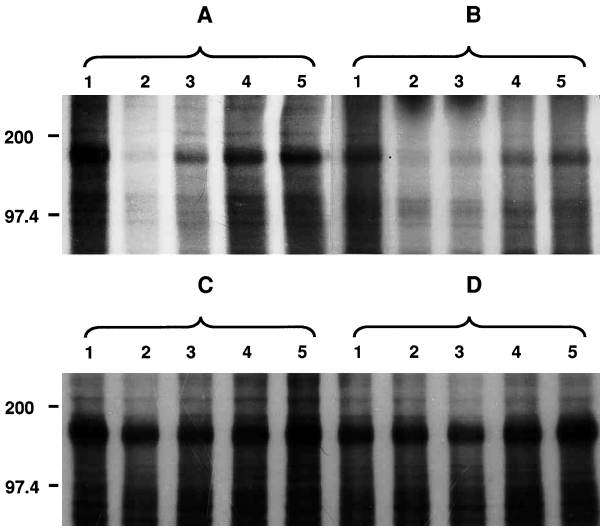


Fig. 4 Cytotoxicity of free doxorubicin, doxorubicin-loaded PIHCA nanospheres, and free doxorubicin combined with unloaded PIHCA nanospheres in C6 0.5 doxorubicin-resistant cells. Symbols are the same as those in Fig. 2

Table 1 Doxorubicin IC_{50} values obtained in the three cell lines according to the drug formulation used. IC_{50} values were evaluated by the MTT assay after 24 h of exposure of cells to the drug and 2 cycles of cell regrowth. The results represent mean values ± SD for 2 experiments performed in triplicate (*Dox* Free doxorubicin, *NS-dox* doxorubicin-loaded PIHCA nanospheres, *NS + dox* Dox combined with unloaded PIHCA nanospheres, *free access* after free contact of drug formulations with cell monolayers, *membrane* in the presence of a polycarbonate membrane between cell and drug compartments)

Drug formulation and incubation conditions	Doxorubicin IC_{50} (μM)		
	C6	C6 0.001	C6 0.5
Dox (free access)	0.034 ± 0.008	0.103 ± 0.015	22.4 ± 3.4
Dox (membrane)	0.041 ± 0.008	0.198 ± 0.060	43.0 ± 8.3
NS-dox (free access)	0.031 ± 0.006	0.039 ± 0.005**	6.45 ± 0.89**
NS-dox (membrane)	0.033 ± 0.008	0.144 ± 0.034	31.0 ± 5.5
NS + dox (free access)	0.043 ± 0.008	0.232 ± 0.050	≈ 55.2
NS + dox (membrane)	0.046 ± 0.003	0.258 ± 0.060	≈ 55.2

**$P < 0.01$ (free access *vs* membrane)



in progress to identify an internalization process of doxorubicin-loaded nanospheres that could explain the bypass of P-glycoprotein.

Acknowledgements This work was supported by grants from the Centre National de la Recherche Scientifique (GDR 0965). We are grateful to Mrs. C. Chapey for excellent technical assistance.

Fig. 5A–D Autoradiograms of SDS-PAGE electrophoreses of membrane preparations of C6 0.5 cells after incubation with 5 μCi [^{3}H] azidopine and photolabelling in the presence of **A** free doxorubicin or of **B** doxorubicin-loaded nanospheres at the following concentrations: and *lanes 1* no drug, *lanes 2* 100 μM, *lanes 3* 50 μM, and *lanes 4* 25 μM, *lanes 5* 12.5 μM. Similar autoradiograms were obtained after incubation of membranes in the presence of **C** unloaded intact nanospheres or **D** degraded unloaded nanospheres, which were used at the same concentrations as doxorubicin-loaded nanospheres in the corresponding lanes

References

1. Bennis S, Chapey C, Couvreur P, Robert J (1994) Enhanced cytotoxicity of doxorubicin encapsulated in polyisohexylcyanoacrylate nanospheres against multidrug-resistant tumour cells in culture. Eur J Cancer 30A : 89
2. Colin de Verdière A, Dubernet C, Nemati F, Poupon MF, Puisieux F, Couvreur P (1994) Uptake of doxorubicin from loaded nanoparticles in multidrug-resistant leukemic murine cells. Cancer Chemother Pharmacol 33 : 504
3. Cuvier C, Roblot-Treupel L, Millot JM, Lizard G, Chevillard S, Manfait M, Couvreur P, Poupon MF (1992) Doxorubicin-loaded nanospheres bypass tumor cell multidrug resistance. Biochem Pharmacol 44 : 509
4. Dalton WS, Grogan TM, Meltzer PS, Scheper RJ, Salmon SE (1989) Drug resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. J Clin Oncol 7 : 415
5. Gabizon AA (1992) Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. Cancer Res 52 : 891
6. Gabizon A, Shiota R, Papahadjopoulos D (1989) Pharmacokinetics and tissue distribution of doxorubicin encapsulated in stable liposomes with long circulation times. J Natl Cancer Inst 81 : 1484
7. Goldstein LJ, Galski H, Fojo A, Willingham M, Lai SL, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM, Lieber M, Cossman J, Gottesman MM, Pastan I (1989) Expression of a multidrug resistance gene in human cancers. J. Natl Cancer Inst 8 : 116
8. Gottesman MM, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62 : 385
9. Huet S, Schott B, Robert J (1992) P-glycoprotein overexpression cannot explain the complete doxorubicin-resistance phenotype in rat glioblastoma cell lines. Br J Cancer 65 : 538
10. Huet S, Chapey C, Robert J (1993) Reversal of multidrug resistance by a new lipophilic cationic molecule, S9788. Comparison with 11 other MDR-modulating agents in a model of doxorubicin-resistant rat glioblastoma cells. Eur J cancer 29A : 1377
11. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 : 680
12. Marie JP, Zittoun R, Sikic BI (1991) Multidrug resistance (MDR1) gene expression in adult acute leukemias. Correlations with treatment outcome and in vitro drug sensitivity. Blood 78 : 586
13. Rahman A, Carmichael D, Harris M, Roh JK (1986) Comparative pharmacokinetics of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes. Cancer Res 46 : 2295
14. Rahman A, Husain SR, Siddiqui J, Verma M, Agresti M, Center M, Safa AR, Glazer RI (1992) Liposome-mediated modulation of multidrug resistance in human HL-60 leukemia cells. J Natl Cancer Inst 84 : 1909
15. Robert J, Gianni L (1993) Pharmacokinetics and metabolism of anthracyclines. Cancer Surv 17 : 219
16. Schott B, Bennis S, Pourquier P, Ries C, Londos-Gagliardi D, Robert J (1993) Differential overexpression of mdr1 genes in multidrug-resistant rat glioblastoma cell lines selected with doxorubicin or vincristine. Int J Cancer 55 : 115
17. Sonneveld P, Durie BGM, Lockhorst HM, Marie JP, Solbu G, Suciu S, Zittoun R, Lowenberg B, Nooter K (1992) Modulation of multidrug resistant multiple myeloma by cyclosporin. Lancet 340 : 255
18. Thierry AR, Vige D, Coughlin SS, Belli JA, Dritschilo A, Rahman A (1993) Modulation of doxorubicin resistance in multidrug resistant cells by liposomes. FASEB J 7 : 572
19. Verdun C, Couvreur P, Vranckx H, Lenaerts V, Roland M (1986) Development of a nanoparticle release formulation for human use. J Control Rel 3 : 205
20. Warren L, Jardiller JC, Malarska A, Akeli MG (1992) Increased accumulation of drugs in multidrug-resistant cells induced by liposomes. Cancer Res 52 : 3241