Cancer Chemother Pharmacol (1994) 33: 504–508

Cancer Chemotherapy and Pharmacology

© Springer-Verlag 1994

Uptake of doxorubicin from loaded nanoparticles in multidrug-resistant leukemic murine cells

A. Colin de Verdière[1], C. Dubernet[1], F. Nemati[1], M. F. Poupon[2], F. Puisieux[1], P. Couvreur[1]

[1] Laboratoire de Pharmacie Galénique et Biopharmacie, URA CNRS 1218, Université de Paris-Sud, F-92296 Chatenay Malabry, France
[2] URA CNRS 620, 26 rue d'Ulm, F-75231 Paris Cedex 05, France

Received: 27 April 1993/Accepted: 25 October 1993

Abstract. Previous studies have clearly demonstrated that polyisobutylcyanoacrylate (PIBCA) doxorubicin-loaded nanoparticles (NS-Dox PIBCA) can overcome the resistance of P388/ADR cells. In the present paper, we found that overcoming multidrug resistance with the aid of doxorubicin (Dox) loaded onto these nanoparticles was associated with an increased intracellular drug content. Indeed, after a 6-h incubation period, the amount of cell-associated drug was 5 times higher when the cells were incubated with NS-Dox PIBCA as compared with free Dox. Further experiments, such as uptake studies in the presence of cytochalasin B or efflux studies, indicated a possible mechanism of nanoparticle/cell interaction. These results suggested that nanoparticles did not enter the cells by an endocytic process, in contrast to a previous hypothesis.

Introduction

Drug resistance is an important aspect of treatment failure in patients with cancer. The mechanism by which tumor cells become resistant to multiple chemotherapeutic agents is poorly understood, although it has been the subject of extensive investigations. Multidrug resistance (MDR) has been correlated with the overexpression of a 170,000-Da glycoprotein, the P-glycoprotein (Pgp) [2, 9]. This protein is believed to participate in the active pumping of drugs out of the cells. Other mechanisms have been described, including a decrease in topoisomerase II activity and an increase in glutathione-S-transferase activity, allowing a more efficient free-radical detoxification of doxorubicin (Dox) in Dox-resistant cells [8, 10].

Correspondence to: P. Couvreur

Using Dox-resistant MCF7, a resistant human breast adenocarcinoma with Pgp overexpression, we recently demonstrated that a complete reversion of resistance to doxorubicin could be obtained after loading of the drug onto biodegradable polyisohexylcyanoacrylate nanoparticles [5]. Identical results were obtained using a leukemic murine line (P388) with polyisohexyl- (PIHCA) and polyisobutylcyanoacrylate (PIBCA) nanoparticles [6]. Since multidrug resistance is generally related to decreased drug accumulation, it was hypothesized that nanoparticles could increase the intracellular concentration of Dox and/or protect it from the P-glycoprotein-dependent accelerated efflux process. However, this was never demonstrated. Thus, the aim of this work was to provide information on the kinetics of drug uptake by P388/ADR cells so as to clarify the mechanism by which nanoparticles were capable of reversing multidrug resistance.

Materials and methods

Materials

Cell lines and culture. P388 (sensitive cell line) and P388/ADR (resistant subline) were kindly supplied by the Institut de Recherche sur le Cancer (IRSC, France). The resistant subline overexpresses Pgp. Suspension cultures were grown in RPMI 1640 medium (Gibco, France) supplemented with 10% fetal calf serum (Gibco, France), penicillin-streptomycin (Eurobio, France), and 3 n*M* 2-mercaptoethanol (Sigma, USA).

Chemicals. Free Dox (Adriblastin) was obtained from Farmitalia (Carlo Erba, Italy). [^{14}C]-Dox was provided by Amersham (UK). Isobutylcyanoacrylate (IBCA) was obtained from Sigma (USA). All other chemicals were obtained commercially and were of analytical grade.

Methods

Preparation of nanoparticles. Nanoparticles were prepared by anionic polymerization of the monomer in aqueous medium [4]. Typically, 66.5 mg of monomer (IBCA) was dropped under mechanical stirring

into 6.5 ml of a medium containing 5 mg of Dox, 5% glucose, 1% dextran 70, and 0.5% citric acid. After a 6-h polymerization, nanoparticles were obtained and lyophilized. The percentage of Dox associated with the nanoparticles was 95%. Lyophilized Dox nanoparticles (NS-Dox PIBCA) were resuspended in sterile distilled water before their use. Unloaded nanoparticles of IBCA (NS PIBCA) were prepared in the same way, with no drug being present in the medium. The mean particle diameter of the nanoparticles was estimated using a Nanosizer N4MD (Coultronics, France). The mean particle diameters of NS-Dox PIBCA and NS PIBCA were 186±31 and 250±42 nm, respectively. The preparation of [^{14}C]-Dox loaded onto polyisobutylcyanoacrylate nanoparticles (NS-[^{14}C]-Dox PIBCA) was carried using the different components in the same proportion described above but in a total volume of 1.3 ml instead of 6.5 ml. The specific activity of the Dox used was 20 μCi/mg. To avoid evaporation, the preparation was carried out in a cold room and stirred for 24 h. The mean diameter of the particles was 170±20 nm.

Studies of drug-uptake rates by cells in culture. For these experiments, cells were seeded at a density of 5×10^5 cells/ml. After 22 h, free Dox, Dox-loaded nanoparticles (NS-Dox), or an extemporaneous mixture of free Dox and unloaded nanospheres (NS+Dox) were added to the incubation medium at Dox concentrations of 100 ng/ml (corresponding to 1,300 ng/ml of polymer) for the P388 cell line and 2,000 ng/ml (corresponding to a concentration of 26,000 ng/ml of polymer) for the resistant subline. Cells were then incubated for varying periods (0, 15 min, 30 min, 1 h, 2 h, 4 h, and 6 h). In the experiments with cytochalasin B, the latter was added to the culture medium at a final concentration of 20 μg/ml at 10 minutes before drug addition. Using a macrophage cell line, we verified that under these conditions, cytochalasin B did inhibit the phagocytosis of fluorescent latex nanoparticles of approximately the same size (data not shown). Since cytochalasin B obviously modified the growth rate of P388/ADR, the uptake study was limited to 240 min. During this short period, treatment with cytochalasin B had no influence on the number of cells present in the wells. At each time point, the contents of three wells were harvested separately by centrifugation and washed three times with ice-cold phosphate-buffered saline (PBS).

Cell-associated Dox was determined after extraction, according to the method of Baurain et al. [1] and measured by high-performance liquid chromatography (HPLC). Briefly, the cell pellets were suspended in 0.1 ml of PBS, and 0.1 ml of borate buffer (pH 9.7) containing 3 μg of daunorubicin/ml as an internal standard was added. After the addition of 0.5 ml of chloroform/methanol (4:1, v/v), the mixture was centrifuged and the organic phase was evaporated to dryness and assayed. Samples were dissolved in the mobile phase and injected automatically (Waters 712 Wisp; Waters, France) onto a C18 column (SFCC, France). The mobile phase was composed of methanol/0.01 *M* sodium acetate/acetic acid (65/35/1.3, by vol.) and used at a flow rate of 1.5 ml/min (Waters 600E; Waters, France). Dox and daunorubicin were detected spectrofluorometrically (Waters 470; Waters, France) at an excitation wavelength of 470 nm and an emission wavelength of 550 nm. Peak areas were integrated using a Waters 412 data module (Waters, France). For generation of the standard curve, the whole extraction procedure was performed on a solution of free Dox and on a suspension of NS-Dox PIBCA in the culture medium. The curves obtained were the same.

Studies of doxorubicin efflux from cells. Cells were prepared as described for the uptake studies (at a density of 5×10^5 cells/ml). They were incubated with free [^{14}C]-Dox or NS-[^{14}C]-Dox PIBCA at a Dox concentration of either 2,000 or 4,000 ng/ml for 1 h or at a concentration of 2,000 ng/ml for 4 h. At the end of these influx periods, the 24-well plates were centrifuged, the supernatants were discarded, and 1 ml of the drug-free culture medium was added to each well. At regular intervals (at first, every 15 min; later, every 30 min), the content of each well was harvested separately by centrifugation and washed three times with ice-cold PBS. Each pellet was then dissolved in a 0.5% aqueous solution of Triton X-100. A volume of 0.5 ml was assayed for radioactivity (LS 6000 TA; Beckman, USA), and a 0.5-ml volume was assayed for protein content according to the method of Lowry et al. [12].

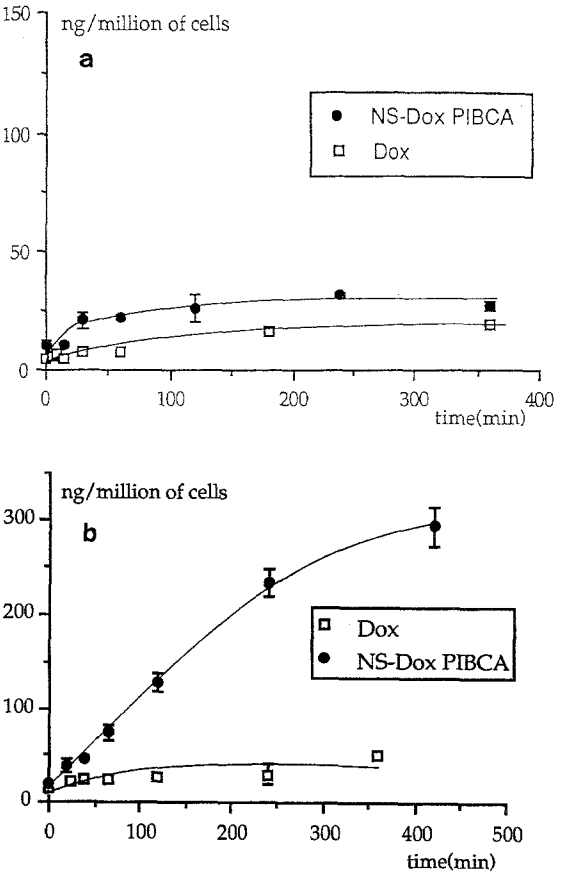

Fig. 1a, b. Amount of Dox associated with leukemic murine cells versus time of incubation. The drug was added either as free Dox or associated with nanoparticles (*NS-Dox PIBCA*). **a** Sensitive cells (P388); Dox concentration, 100 ng/ml. **b** Resistant subline (P388/ADR); Dox concentration, 2,000 ng/ml

In vitro release of Dox. The rate of Dox release from NS-Dox PIBCA was measured in vitro. The drug-loaded nanoparticles were incubated in the culture medium at a Dox concentration of 2,000 ng/ml at 37° C in a humidified atmosphere containing 5% CO_2. Free Dox was incubated under the same conditions as a control. Samples were taken after various incubation periods and nanoparticles were separated from the released drug by ultrafiltration on a polysulfone membrane (300,000-Da molecular-weight cut-off; Millipore, France) at 2,000 *g* for 5 min. The filtrate was assayed by HPLC as described above. The results are expressed as the percentage of drug released relative to control values.

Each experiment was done two to four times and the results were reproducible. Curves represent single typical experiments; on the curves, the error bars represent the standard deviation.

Results

Drug uptake by cells in culture

Cells were incubated with Dox at 100 ng/ml for the sensitive line and at 2,000 ng/ml for the resistant one. Using the trypan blue exclusion test, we verified that for all the formulations tested, no cytotoxicity occurred during the course of the experiment.

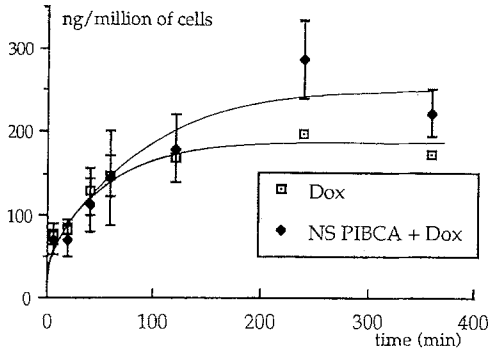

Fig. 2. Amount of Dox associated with P388/ADR versus time of incubation. The drug was added either as free Dox or as a mixture with nanoparticles (*NS PIBCA+Dox*). Concentration, 2,000 ng/ml

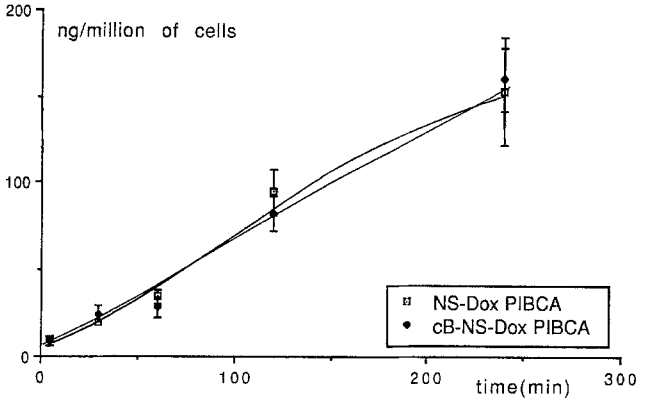

Fig. 3. Amount of Dox associated with P388/ADR versus time of incubation. Dox was added associated with nanoparticles (*NS-Dox PIBCA*) at a concentration of 2,000 ng/ml in the presence (*cB-NS-Dox PIBCA*) or absence (*NS-Dox PIBCA*) of 20 μg cytochalasin B/ml

When P388 cells were incubated with 100 ng Dox/ml the amount of cell-associated Dox slowly increased for both NS-Dox PIBCA and Dox, reaching a plateau after a 120-min incubation (Fig. 1a). The calculated amount of cell-associated Dox was found to be close to 15 ng per million cells for Dox and 25 ng per million cells for NS-Dox PIBCA. This observation was consistent with the cytotoxicity data previously published [5], showing very similar LD_{50} values (the dose resulting in 50% cell mortality) for Dox and NS-Dox.

In the case of P388/ADR cells incubated with free Dox at a concentration of 2,000 ng/ml, the uptake quickly reached a plateau after 30 min, corresponding to a concentration of 20 ng per million cells (Fig. 1b). When PIBCA nanoparticles were used, the uptake of Dox was dramatically increased to 300 ng per million cells and no plateau was observed, even after a 6-h incubation period. Thus, in this case, the amount of cell-associated Dox represented about 15% of the quantity of Dox added to the culture medium at the beginning of the experiment, in contrast to a value of about 1% when Dox was added in the free state.

Finally, when P388/ADR cells were incubated with the mixture NS+Dox, the uptake of the drug, although significantly different ($P < 1\%$) over 240 min, was only slightly increased as compared with the free drug (Fig. 2).

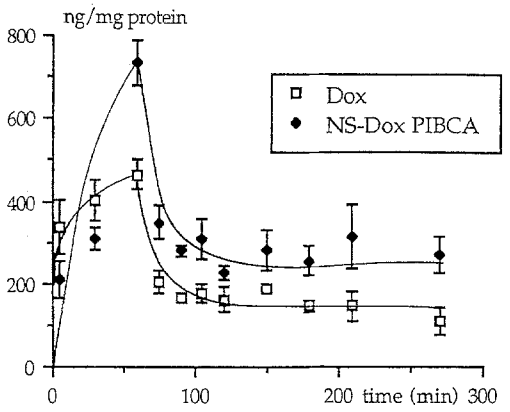

Fig. 4. Amount of Dox associated with P388/ADR versus time of incubation. The drug was loaded either as free Dox or as NS-Dox PIBCA at a concentration of 4,000 ng/ml. Washing was performed after a 1-h incubation

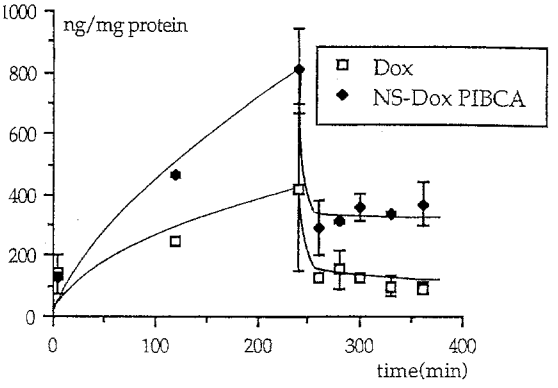

Fig. 5. Amount of Dox associated with P388/ADR versus time of incubation. The drug was loaded either as free Dox or as NS-Dox PIBCA at a concentration of 2,000 ng/ml. Washing was performed after a 4-h incubation

When cytochalasin B was added to the culture medium before the drug, the cell incorporation of Dox was unchanged, regardless of the form in which it was added (Dox or NS-Dox PIBCA; Fig. 3).

Efflux studies

The efflux studies were initially performed at a Dox concentration of 2,000 ng/ml. When the drug was allowed to enter the cells for only 1 h, the levels of cell-associated drug were not significantly different between Dox and NS-Dox PIBCA, such that the difference in drug concentration after efflux was even less marked (data not shown). This result suggested that NS-Dox behaved similarly to the free drug under efflux conditions. However, to differentiate Dox from NS-Dox PIBCA more clearly, the efflux studies were then performed after loading for 1 h at a Dox concentration of 4,000 ng/ml (Fig. 4) or for 4 h at a Dox concentration of 2,000 ng/ml (Fig. 5).

In both cases and for both formulations, the drug levels in the cells immediately dropped after washing and stabilized after less than 20 min. The proportion of drug retained in the cells after the efflux period was quite similar in each

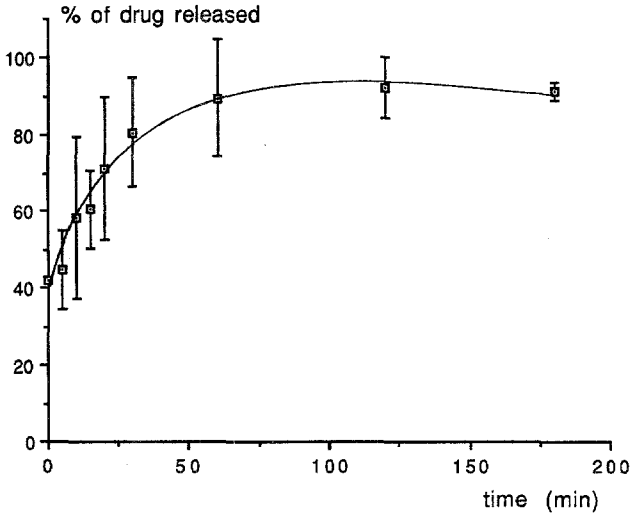

Fig. 6. In vitro Dox release from NS-Dox PIBCA in cell-culture medium. The values represent the percentage of total Dox occurring free in the medium at each time point

case. For example, in the case of NS-Dox PIBCA, 40% of the drug present in the cells (loading time of 1 h; Fig. 4) persisted 4 h later. This value was 33% in the case of free Dox. When the influx time was prolonged to 4 h (Fig. 5) at a Dox concentration of 2,000 ng/ml, the proportion of drug retained in the cells after efflux corresponded to 40% and 28% for NS-Dox and free Dox, respectively. However, in both cases – 1-h and 4-h loading followed by washing – much higher absolute levels were retained in the cells after efflux in the case of nanoparticles.

In vitro release studies

NS-Dox PIBCA were incubated with the cell-culture medium under the same conditions used for the drug-uptake experiments. NS-Dox PIBCA were found to release Dox quickly; after 1 h, the major part of the drug associated with nanoparticles had been released in the culture medium (Fig. 6).

Discussion

The strategy of using colloidal carriers to deliver anticancer drugs to multidrug-resistant (MDR) cells in generally based on the hypothesis that encapsulated drugs might escape from the pumping action of Pgp after entering the cells by endocytosis [5]. Hence, for a given dose of nanoparticle-associated drug, the intracellular level might be increased, leading to the reappearance of a cytotoxic effect toward resistant cells. To verify this hypothesis, drug-uptake studies were carried out with Dox-loaded polyalkylcyanoacrylate nanoparticles. The efficacy of this form has previously been shown on P388/ADR cells [5]. Whereas the level of Dox incorporation was similar for Dox and NS-Dox PIBCA in the case of sensitive P388 cells, a very marked difference could be observed with P388/ADR cells. With this latter cell line, PIBCA nanoparticles allowed higher intracellular concentrations of Dox to be reached, which was correlated with a higher degree of cytotoxicity as compared with that of the free drug [6].

That drug incorporation by the cells was not influenced by cytochalasin B suggested that endocytosis was not the main mechanism of nanoparticle-cell association, although this was the most likely mechanism of interaction between such colloidal carriers and cells. Finally, efflux studies revealed that nanoparticles could not retain the drug inside the cells for significantly longer periods than in the case of the free drug. Thus, it is probable that intracellular Dox was no longer associated with the polymer.

These data strongly suggest that nanoparticles did not enter the cells. It is not out of the question that they could have adsorbed onto the cell membrane. Lherm et al. [11] put forward the hypothesis that the particles could adhere to the cells and be degraded close to the cell membrane. As a consequence, the drug would be released from the nanoparticles, entering the cells by passive diffusion, as does free Dox. It is noteworthy that the rate of release of Dox from nanoparticles in the culture medium was very fast. Thus, the rapid drug release from nanoparticles adhering to the cell membrane should lead to a large amount of drug diffusing in the intracellular compartment, as was observed in the uptake studies. Such a massive diffusion of Dox from PIBCA nanoparticles could saturate Pgp. In other words, drug would be partly pumped out of the cells, but most of it would remain in the cells and would be capable of exerting cytotoxicity.

Furthermore, taking into account the recent observation of Thierry et al. [13] that empty liposomes could interact directly with Pgp, it is possible that PIBCA – probably through its degradation products – might modify cell membrane properties. Indeed, Lherm et al. [11] stated that a possible explanation of the toxicity observed on fibroblasts is that nanoparticles could adhere to the cells and create a high local concentration of degradation products leading to cell membrane damage. Nevertheless, levels of Dox in MDR cells continued for many hours to rise more rapidly when the drug was used in particle form than when it was applied as free Dox, even if by that time the Dox had been released from the nanoparticles. This apparent contradiction is probably not the result of simple polymer interactions with the membranes and may reflect a complex damaging effect on the membranes resulting from clusters of Dox molecules presenting simultaneously on the polymer. In addition, this pattern of continued rapid accumulation seems to be restricted to MDR cells. It is thus conceivable that Pgp could be involved directly in binding clusters of particle-associated Dox. The increased cytotoxicity observed for NS-Dox PIBCA as compared with free Dox might also result solely from the direct cytotoxic effect of the polymer (or its degradation products) on the plasma membrane, which could lead to a higher permeability of the membrane to Dox. However, we cannot exclude the possibility of a direct membrane cytotoxic effect of Dox [14, 15]; this would result from the adsorption of NS-Dox PIBCA particles on the cells, leading to higher concentrations of Dox near the membrane as compared with treatment with free Dox.

To date, the use of particulate carriers has always been limited to cells capable of endocytosing particulates [3, 7]. Surprisingly, our data showed that NS-Dox PIBCA were cytotoxic to P388/ADR without being endocytosed. Consequently, further investigations are needed to clarify the exact mechanism by which the drug is more cytotoxicic to MDR cells when it is associated with nanoparticles. Therefore, the possibility of a direct cytotoxic effect on cell membranes should be investigated.

Acknowledgements. This study was supported by a grant accorded to the Groupement de Recherche CNRS (number 9,650). The authors are grateful to L. Treupel-Acar for helpful discussion.

References

1. Baurain R, Zenebergh A, Trouet A (1979) Cellular uptake and metabolism of daunorubicin as determined by high-performance liquid chromatography. Anal Biochem 94: 112–116
2. Chen CJ, Chin JE, Ueda K, Clark DF, Pastan I, Gottesman MM, Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell 47: 381–389
3. Couvreur P, Tulkens P, Roland M, Trouet A, Speiser P (1977) Nanocapsules: a new type of lysosomotropic carrier. FEBS Lett 84: 323–326
4. Couvreur P, Kante B, Rolland M, Guiot P, Bauduin P, Speiser P, et al (1979) Polyalkylcyanoacrylate nanocapsules as potential lysomotropic carriers: preparation, morphological and sorptive properties. J Pharm Pharmacol 31: 331–332
5. Cuvier C, Roblot-Treupel L, Chevillard S, Millot JM, Mainfait M, Bastian G, Couvreur P, Poupon M-F (1992) Doxorubicin loaded nanospheres bypass tumor cell multidrug resistance. Biochem Pharmacol 44: 509–517
6. Dubernet C, Nemati F, Colin De Verdière A, Poupon MF, Couvreur P (1992) Efficacy of polyalkylcyanoacrylate nanospheres loaded with doxorubicin in the reversion of multidrug resistant cell lines. Clin Exp Metastasis 10 [Suppl]: 105
7. Guise V, Jaffray P, Delattre J, Puisieux F, Adolphe M, Couvreur P (1987) Comparative cell uptake of propidium iodide associated with liposomes or nanoparticles. Cell Mol Biol 33: 397–405
8. Isabella P de, Capracino G, Zinino F (1991) The role of topoisomerase II in drug resistance. Life Sci 48: 2195–2205
9. Juranka PF, Zastawany RL, Ling V (1989) P-glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. FASEB 3: 2583–2592
10. Lee FYF, Sciandra J, Siemann DW (1989) A study of the mechanism of resistance of Adriamycin in vivo. Biochem Pharmacol 38: 3697–3705
11. Lherm C, Muller RH, Puisieux F, Couvreur P (1992) Alkylcyanoacrylate drug carriers. II. Cytotoxicity of cyanoacrylate nanoparticles with different alkyl chain length. Int J Pharm 84: 13–22
12. Lowry OH, Rosenbrough NJ, Foarr AL, Randal RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265
13. Thierry AR, Dritschilo A, Rahman A (1992) Effect of liposomes on P-glycoprotein function in multidrug resistant cells. Biochem Biophys Res Commun 187: 1098–1105
14. Tritton TR, Yee G (1982) The anticancer agent Adriamycin can be actively cytotoxic without entering cells. Science 217: 248–250
15. Tritton TR, Yee G, Wingard LB (1983) Immobilized Adriamycin: a tool for separating cell surface from intracellular mechanisms. Fed Proc 42: 284–287