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Some parameters influencing cytotoxicity of free doxorubicin and doxorubicin-loaded nanoparticles in sensitive and multidrug resistant leucemic murine cells: incubation time, number of nanoparticles per cell

F. Némati [a], C. Dubernet [a], A. Colin de Verdière [a], M.F. Poupon [b], L. Treupel-Acar [c], F. Puisieux [a] and P. Couvreur [a]

[a] *Laboratoire de Pharmacie Galénique et Biopharmacie, URA CNRS 1218, Université de Paris-Sud, 92296 Chatenay Malabry (France),* [b] *URA CNRS 620, 26 rue d'Ulm, 75 231 Paris Cedex 05 (France) and* [c] *Laboratoire de Pharmacie, Faculté de Pharmacie, Avenue du Professeur Léon Bernard, 35043 Rennes Cedex (France)*

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Summary

The cytotoxic effect of polyisobutylcyanoacrylate (PIBCA) and polyisohexylcyanoacrylate (PIHCA) nanoparticles, loaded with doxorubicin, was investigated on P388 and P388/ADR cells, sensitive and resistant to doxorubicin respectively. The nanoparticles formulations were able to reverse the resistance of P388/ADR, although the polymers themselves displayed some cytotoxicity. The influence upon cytotoxicity of parameters such as incubation time, or number of nanoparticles per cell was especially investigated. From those experiments, it appeared that not only the dose of doxorubicin, but also the number of nanoparticles carrying the drug in the cell environment was of first importance: in terms of cytotoxicity, it was preferable to divide the whole dose of doxorubicin into a high number of nanoparticles, even if each nanoparticle was then carrying a lower amount of drug. Some preliminary hypotheses concerning the mechanism of action of the nanoparticles formultions are proposed.

Introduction

Multidrug resistance is an important cause of chemotherapy failure in cancer. It is often associated with the overexpression of a cell membrane glycoprotein of 170 kDa molecular mass (Chen et al., 1986; Juranka et al., 1989). This glycoprotein designed P-glycoprotein could act as an efflux pump and reject numerous drugs from the cells as shown for bacterial transport proteins (Gros et al., 1986). Indeed, multidrug resistance is associated with the low intracellular accumulation of some drugs (Gerlach et al., 1986).

Drugs, such as doxorubicin, appear to enter the cell by passive diffusion through the lipid bilayer. Upon entering the cell, these drugs bind to P-glycoprotein which forms transmembrane

Correspondence to: P. Couvreur, Laboratoire de Pharmacie Galénique et Biopharmacie, URA CNRS 1218, Université de Paris-Sud, 92296 Chatenay Malabry, France.

channels and uses the energy of ATP hydrolysis to pump these compounds out of the cell (Higgins and Gottesman, 1992).

To solve this problem, many authors have proposed the use of competitive P-glycoprotein inhibitors, such as verapamil, which are able to bind to P-glycoprotein and to overcome pleiotropic resistance (Cornwell et al., 1987). However, as the adverse effects of verapamil are serious, its clinical use to overcome multidrug resistance is limited (Ozols et al., 1987). Another strategy consisted to use colloidal particulate carriers (liposomes or nanoparticles) for delivering anticancer drugs to MDR resistant cells (Sadavisan et al., 1991; Warren et al., 1992).

Using Dox-resistant MCF7, a resistant human breast adenocarcinoma, we demonstrated very recently that a complete reversion of drug resistance to doxorubicin may be obtained after loading the drug onto biodegradable polyalkylcyanoacrylate nanoparticles (Cuvier et al., 1992). Circumvention of MDR resistance with doxorubicin-loaded nanoparticles was also confirmed with other multidrug resistant variant sublines (SKVLB1, K562-R, P388/ADR, LR 73 MDR) (Cuvier et al., 1992). Although preliminary, these results have opened interesting perspectives concerning the use of nanoparticles in delivering doxorubicin to MDR resistant cells. The mechanism by which nanoparticles were able to reverse MDR resistance is however unclear and probably, multifactorial. This paper describes the cytotoxicity of doxorubicin free and loaded into nanoparticles, in sensitive and multidrug resistant leucemic murine cells in order to highlight some important factors of the interaction of doxorubicin-loaded nanoparticles with MDR resistant cell lines.

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 does overexpress the P-glycoprotein. Suspension cultures were grown in RPMI 1640 medium (Gibco, France) supplemented with 10% foetal calf serum (Gibco, France), penicillin-streptomycin (Eurobio, France) and 2-mercaptoethanol 3 nM (Sigma, U.S.A.).

Chemicals Isohexylcyanoacrylate (IHCA) was kindly supplied by Sopar (Sopar, Belgium). Free doxorubicin (Adriblastin®) (Dox) was obtained from Farmitalia (Farmitalia, Carlo Erba, Italy). Isobutylcyanoacrylate (IBCA) was obtained from Sigma (U.S.A.). All other chemicals were obtained commercially and were of analytical grade.

Methods

Preparation of nanoparticles Nanoparticles were obtained by emulsion polymerization of a cyanoacrylic monomer as previously described by Couvreur et al. (1979). Typically, 66.5 mg of monomer (IBCA or IHCA) were dropped under mechanical stirring into 6.5 ml of a medium containing 5.1 mg of doxorubicin, 5% glucose, 1% dextran 70, and 0.5% citric acid. After 6 h (IBCA) or 20 h (IHCA) polymerization, nanoparticles were obtained, and lyophilised (Couvreur et al., 1979). The percentage of doxorubicin associated with nanoparticles was 95% for both formulations. Unloaded nanoparticles of IBCA or IHCA (NS PIBCA and NS PIHCA, respectively), were prepared in the same way, no drug being present in the polymerization medium.

Additional batches of nanoparticles were also obtained by adding varying amounts of doxorubicin (from 0.5 to 5.0 mg) in the polymerization medium, containing, after polymerization, a mass of polymer of 66.5 mg. The nanoparticles have been assayed for the amount of non-adsorbed doxorubicin, following ultracentrifugation (30000 rpm for 1 h, Beckman L7-75 Ultracentrifuge, type 70.1 Rotor). The supernatants were analysed by HPLC. Briefly, samples were injected automatically (Waters 712 Wisp, Waters, France) onto a C18 column (SFCC, France). The mobile phase was composed of methanol/sodium acetate 0.01 M/acetic acid (70:30:1.3), and used at a flow rate of 1.5 ml/min (Waters 600E, Waters, France). Doxorubicin, and daunorubicin, used as internal standard, were detected by fluorimetry (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).

Calculation of the number of nanoparticles present in the culture medium The nanoparticle density being assumed to be 1.1 (Kreuter, 1983), their number n was calculated as follows:

$$n = [\text{mass of polymer in the medium } (M)] / [\text{mass of one particle}] = (M \times 10^{12}) / (4/3)\pi r^3 \times 1.1$$

with M (in ng) and r, the radius of the nanoparticles (expressed in nm) and being determined by laser light scattering (Nanosizer N4 MD, Coultronics, France).

Drug treatment and cytotoxicity Cytotoxicity of free doxorubicin and nanoparticles doxorubicin was determined by measuring the inhibition of cell growth. Samples were free doxorubicin (Dox), doxorubicin-loaded nanoparticles (NS-Dox) made of PIHCA (NS-Dox PIHCA) or PIBCA (NS-Dox PIBCA), unloaded nanoparticles (NS) and a mixture of free doxorubicin and unloaded nanoparticles (NS + Dox), the two components being successively added to the culture medium. All treatments were performed on cells in log-phase growth seeded in 24-well plates at a cellular density of 5×10^4 cells/ml and in a humidified atmosphere (5% CO_2). Typically, cells were incubated for 48 h with various concentrations of drug. In some experiments, the incubation time was only 0.5, 1, 3 or 6 h. In these particular cases, at the end of the incubation period, the medium was removed and cells were washed twice with RPMI, resuspended and reincubated with doxorubicin-free medium for a further 48 h. At .the end of the experiment, the resultant cell number was counted in a Coulter Counter Model ZM (Coultronics, France). The survival rate of the treated cells was calculated as follows:

$$\text{percentage survival} = [(\text{number of cells in treated wells}) / (\text{number of cells in control wells})] \times 100$$

The IC_{50} (inhibition concentration) values were evaluated as the drug concentration providing a decrease of 50% of cell survival.

Results and Discussion

Growth inhibition studies

The cytotoxicity of doxorubicin on P388 (sensitive cell line) is reported in Table 1.

There was no significant difference between the IC_{50} of free doxorubicin and both NS-Dox PIBCA and NS-Dox PIHCA (25 ng/ml vs 18 and 20 ng/ml, respectively). The unloaded nanoparticles were non-cytotoxic at the doses required for the observation of a cytotoxic effect of the drug, since their IC_{50}, expressed in the equivalent dose of doxorubicin, was only 225 and 250 ng/ml for NS-PIBCA and NS-PIHCA, respectively (Table 1).

As could be expected under such conditions, the single mixture NS + Dox was as cytotoxic as the free drug (30 and 25 ng/ml for NS + Dox PIBCA and NS + Dox PIHCA, as compared to 25 ng/ml for Dox).

Concerning the P388/ADR resistant cell line (Table 2), the IC_{50} of free doxorubicin was 1500 ng/ml, 60-times higher than on the sensitive parent line. In comparison, the IC_{50} of doxorubicin-loaded nanoparticles was much lower: only 150 ng/ml for NS-Dox PIBCA, and 200 ng/ml for NS-Dox PIHCA. At the same time, the polymers themselves appeared rather cytotoxic, with an IC_{50} quite close to the previous values: 200

TABLE 1

IC_{50} (ng/ml) of doxorubicin (free, incorporated into PIHCA or PIBCA nanoparticles, or physically mixed with unloaded PIHCA or PIBCA nanoparticles) on P388 as determined by the Coulter counter® method (48 h incubation)

	PIHCA	PIBCA
NS-Dox	18	20
NS + Dox	30	25
NS	225 [a]	250 [a]

IC_{50} of free Dox, 25 ng/ml.

[a] Expressed in the equivalent dose of doxorubicin (polymer/doxorubicin ratio, 13).

TABLE 2

IC_{50} (ng/ml) of doxorubicin (free, incorporated into PIHCA or PIBCA nanoparticles, or physically mixed with unloaded PIHCA or PIBCA nanoparticles) on P388/ADR as determined by the Coulter counter® method (48 h incubation)

	PIHCA	PIBCA
NS-Dox	150	200
NS + Dox	200	350
NS	200 [a]	400 [a]

IC_{50} of free Dox, 1500 ng/ml.
[a] Expressed in the equivalent dose of doxorubicin (polymer/doxorubicin ratio, 13).

ng/ml for PIBCA and 400 ng/ml for PIHCA (expressed in the equivalent dose of doxorubicin). Finally, the NS + Dox mixtures showed intermediate cytotoxicities between NS-Dox and unloaded nanoparticles. In both cases, however, the IC_{50} tended rather toward those of the polymers themselves.

In conclusion, the use of NS-Dox on P388/ADR led to a gain in cytotoxicity as compared to the free drug. However, the reversion of cell resistance remained partial, since doxorubicin-loaded nanoparticles (NS-Dox) did not allow recovery of the level of cytotoxicity of doxorubicin on P388 sensitive cells (25 ng/ml). However, the IC_{50} of NS-Dox was 10-times lower than that of free Dox on the resistant subline P388/ADR. In comparison, this value was only 1.4 when liposome-encapsulated doxorubicin was used on the doxorubicin-resistant colon cancer cell line SW620/R (Oudard et al., 1991).

The cytotoxicity of NS-Dox could not be explained by the additional effect of the drug on the one hand, and of the polymer on the other. Indeed, although the polymers were partly cytotoxic on P388/ADR, the NS + Dox mixtures did not allow the same level of cell survival to be reached as in the case of the drug-loaded nanoparticles. However, the NS + Dox mixtures were slightly more cytotoxic than the polymers themselves. This observation could be the result of two different features. Firstly, doxorubicin could be quickly adsorbed onto the surface of the unloaded nanoparticles when those were added to the culture medium. If this were to be the case, one might expect an adsorption rate of approximately half of the encapsulation ratio obtained when doxorubicin was entrapped into the polymeric network of the particles (Bapat and Boroujerdi, 1992). If we assume that free doxorubicin could not be cytotoxic by itself at the dose used (200–350 ng/ml), then it must be considered that the enhancement of cytotoxicity of the NS + Dox mixture was due to the partial adsorption of the drug at the surface of the nanoparticles.

Secondly, the cytotoxicity of the unloaded nanoparticles on P388/ADR might be due to some metabolism or membrane disturbance, finally increasing the cytotoxicity of doxorubicin itself. Such a hypothesis has been reported with empty liposomes which were found to be able to alter the P-glycoprotein function by direct interaction (Thierry et al., 1992). If this were also the case with nanoparticles, the difference between the NS + Dox mixture and the NS-Dox drug loaded nanoparticles could then be explained by the fact that, with NS + Dox, the drug was homogeneously dissolved in the culture medium, whereas with NS-Dox, doxorubicin was immediatly concentrated close to the cell surface by membrane adhering nanoparticles. The greatest drug gradient in the second case (NS-Dox) would then favor a high drug influx into the cells, leading to a greater cytotoxicic effect.

Influence of incubation time on cytotoxicity

The two cell lines were exposed for varying incubation times to Dox, NS-Dox PIBCA and NS-Dox PIHCA, at concentrations of 30 ng/ml of doxorubicin for P388, and 300 ng/ml for P388/ADR. Unloaded nanoparticles were used as a control. Free doxorubicin was also incubated with P388/ADR at 8000 and 1500 ng/ml. Those concentrations were chosen with respect to their cytotoxic effect, after either 6 or 48 h incubation times (close to the corresponding IC_{50}). The effect of the drugs, when seen, began after 30 min for P388 (Fig. 1) and after 60 min for P388/ADR (Fig. 2). With P388, no cytotoxicity was observed with the unloaded polymer (NS PIHCA) at the concentration tested (390 ng/ml), while NS-Dox

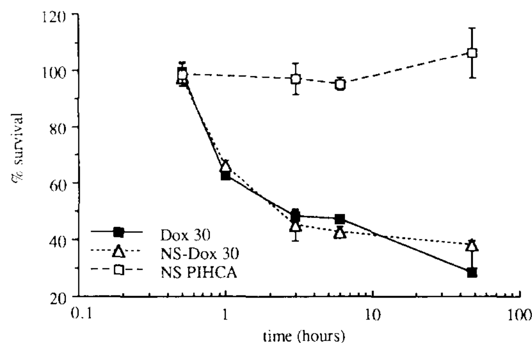

Fig. 1. Cytotoxicity of NS PIHCA (390 ng/ml PIHCA), NS-Dox PIHCA (30 ng/ml doxorubicin) and Dox (30 ng/ml) on P388 vs time of incubation.

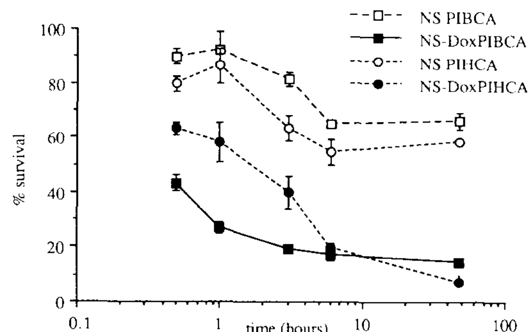

Fig. 3. Cytotoxicity of NS PIBCA, NS PIHCA, NS-Dox PIBCA and NS-Dox PIHCA on P388/ADR vs time of incubation. Polymer concentration, 3900 ng/ml; doxorubicin concentration, 300 ng/ml.

PIHCA and Dox reached a steady state in cytotoxicity after 3 h.

No difference between Dox and NS-Dox could be observed at any time of incubation.

For P388/ADR (Fig. 2), Dox had no antitumoral effect at the concentration of 300 ng/ml. This concentration was well below the IC_{50}. At 1500 ng/ml, free doxorubicin induced a regular decrease in the percentage of cell survival and the curve did not reach any plateau. In contrast, at a concentration of Dox of 8000 ng/ml, cytotoxicity reached a steady state after 3 h incubation (20% survival). NS-Dox PIHCA and NS PIHCA both reached their maximum of cytotoxicity after 6 h, with 20% survival for NS-Dox vs 60% survival for unloaded nanoparticles. It is noteworthy that the curve of NS-Dox PIHCA at 300 ng/ml had quite the same profile as Dox at 8000 ng/ml.

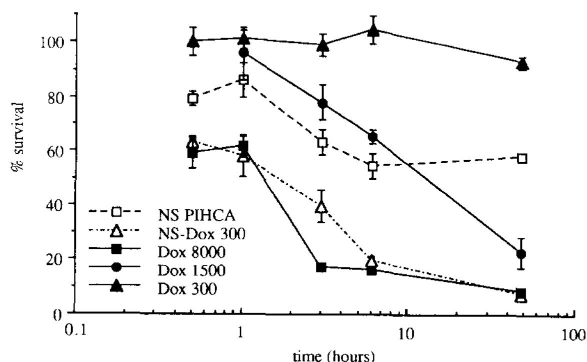

Fig. 2. Cytotoxicity of NS PIHCA (3900 ng/ml PIHCA), NS-Dox PIHCA (300 ng/ml doxorubicin) and Dox (8000 ng/ml, 1500 ng/ml and 300 ng/ml doxorubicin) on P388/ADR vs time of incubation.

Using NS-Dox PIBCA instead of NS-Dox PIHCA only modified the rate of cell survival decrease (Fig. 3). Indeed, cytotoxicity reached a steady state after 3 h incubation instead of 6 h for NS-Dox PIHCA. Consequently, during the first hours of incubation, and up to 6 h, NS-Dox PIBCA were more cytotoxic than NS-Dox PIHCA. Concerning unloaded nanoparticles, no significant difference in cytotoxicity was observed between the two polymers (PIBCA vs PIHCA).

PIBCA and PIHCA are known to differ in their erosion rate: PIHCA, with a longer alkyl chain, degrades more slowly than PIBCA (Muller et al., 1990). Slower polymer bioerosion should have implied a slower release of doxorubicin from nanoparticles, and a lower rate of appearance of the degradation products (Lherm et al., 1992), both factors being able to provide an explanation for the fact that the cytotoxicity was of the same order of magnitude but appeared later with NS-Dox PIHCA as compared to NS-Dox PIBCA.

The use of colloidal carriers to overcome multidrug resistance was based on the following hypothesis: since particulate carriers are internalized by cells, the anticancer drug would not be able to diffuse by itself through the cell membrane and, being absent from the phospholipidic membrane, it would then escape from the pump-

ing action of the P-glycoprotein. Thus, targetting would be particularly useful when P-glycoprotein is the major resistance factor. In P388/ADR cells, P-glycoprotein was largely expressed (Radel et al, 1990), but resistance was multifactorial, as assessed on the basis of the time dependence of the cytotoxicity of free doxorubicin at the dose of 1500 ng/ml (Fig. 2), probably indicating the existence of an enzymatic mechanism, saturated for high doxorubicin doses (8000 ng/ml). If this is true, it is suggested that nanoparticles should be unable to interact with resistance factors other than the P-glycoprotein.

Effect of increasing concentrations of doxorubicin associated with a constant amount of polymer

In this experiment, the doxorubicin/polymer ratio was raised. In other words, the number of nanoparticles remained constant although the dose of doxorubicin was increased. As far as P388 was concerned, the unloaded nanoparticles were not cytotoxic. The profile of cytotoxicity of NS-Dox was found to be the same for the two concentrations of polymer tested, however, up to 40 ng/ml, NS-Dox was clearly more cytotoxic than the free drug (Fig. 4).

The same experiments were carried out on P388/ADR, but on this subline, the polymer alone displayed a certain cytotoxicity at the concentration of 1000 ng/ml (data not shown).

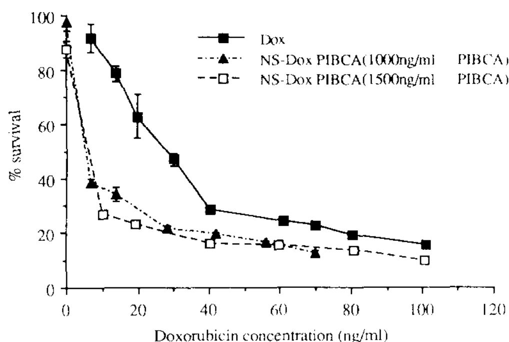

Fig. 4. Cell survival curves of P388 after a 6 h incubation time with increasing amounts of doxorubicin, free (Dox) or associated with nanoparticles (NS-Dox PIBCA). Polymer concentration was either 1000 or 1500 ng/ml for each tested concentration of doxorubicin.

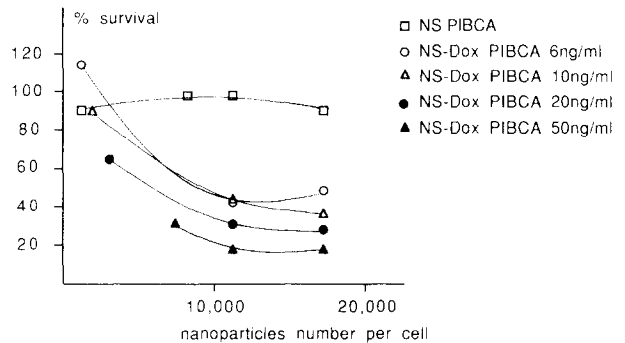

Fig. 5. Cytotoxicity of NS PIBCA and NS-Dox PIBCA on P388 after 48 h incubation vs the number of incubated nanoparticles per cell.

Cytotoxicity of NS-Dox PIBCA expressed as a function of the number of nanoparticles

Cytotoxicity data have been analysed in terms of number of nanoparticles per cell. Hence, cytotoxicity could be expressed vs the number of nanoparticles per cell, for either P388 or P388/ADR, and for four concentrations of doxorubicin (6, 10, 20 and 50 ng/ml). The number of cells seeded in each experiment was constant.

Unloaded nanoparticles were found not to be cytotoxic on P388 for a number of nanoparticles ranging up to 17 000 per cell (Fig. 5). For a given concentration of doxorubicin and up to 11 000 nanoparticles per cell, the larger the number of nanoparticles, the more cytotoxic were the formulations although each particle was then carrying a decreasing quantity of drug. In the range of 11 000–17 000 nanoparticles per cell, cytotoxicity progressively reached a steady state while cell survival, different for each drug concentration, was measured as 40–20%. Thus, doxorubicin incubated at a concentration of 6 ng/ml was more cytotoxic when the dose was divided into 11 000 nanoparticles than doxorubicin incubated at the concentration of 20 ng/ml if this dose was divided into 1500 nanoparticles. This strongly suggests that the number of nanoparticles was at least as important as the dose of doxorubicin itself.

As explained before, in the case of P388/ADR, NS PIBCA were found to be more cytotoxic. Therefore, the differential effect of doxorubicin-loaded nanoparticles only became signifi-

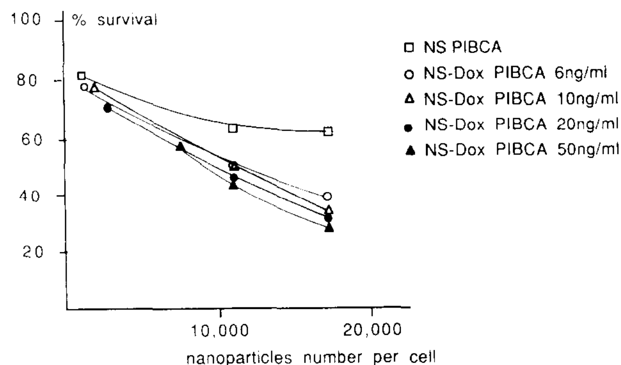

Fig. 6. Cytotoxicity of NS PIBCA and NS-Dox PIBCA on P388/ADR after 48 h incubation vs the number of incubated nanoparticles per cell.

cant for 11 000 nanoparticles per cell and more (Fig. 6). Moreover, no steady state in cytotoxicity could be observed: more than 30% of cells were still surviving in every case.

In conclusion, briefly, for a given doxorubicin dose, the greater the number of nanoparticles, the higher the cytotoxicity. On P388 particularly, it appeared that it was preferable to divide the whole dose of doxorubicin onto a large number of nanoparticles, even if each nanoparticle was then carrying a lower amount of drug. One can imagine that increasing the number of nanoparticles allowed targetting of the drug to a greater number of cells, at a dose sufficient to kill them. As previously discussed, a low amount of drug, but carried immediatly around the cells, would have the same effect as a higher doxorubicin dose but diluted in the whole culture medium (effect of doxorubicin gradient).

Sensitive and resistant cells differed slightly in the fact that a steady state of cytotoxicity was observed for 11 000 nanoparticles per cell and more in the case of sensitive cells. This was clearly not the case with the resistant subline. If nanoparticles adsorbed onto the cell membrane, or were endocytosed, a steady state would be the expression of the saturation of the interaction process between nanoparticles and cells (surface area saturated, or active endocytotic process saturated). The absence of a steady state in the case of P388/ADR could be explained by the fact that resistant cells have been shown to present a higher specific surface area than the sensitive parent (Radel et al., 1990).

In conclusion, this study has provided more information about the conditions needed for the reversion of tumor resistance by polyalkylcyanaoacrylate nanoparticles. It has been demonstrated that unloaded nanoparticles probably play a role in sensitizing cells to doxorubicin, even at non-cytotoxic doses of polymer.

Acknowledgement

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