# Ability of Doxorubicin-Loaded Nanoparticles to Overcome Multidrug Resistance of Tumor Cells After Their Capture by Macrophages

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***Purpose.*** Investigation of the ability of doxorubicin-loaded nanoparticles (NP/Dox) to overcome multidrug resistance (MDR) when they have first been taken up by macrophages.

***Methods.*** The growth inhibition of P388 sensitive (P388) and resistant (P388/ADR) tumor cells was evaluated in a coculture system consisting of wells with two compartments. The tumor cells were seeded into the lower compartment, the macrophages were introduced into the upper part in which the drug preparations were also added.

***Results.*** Doxorubicin exerted lower cytotoxicity on tumor cells in coculture compared with direct contact. In P388/ADR, NP/Dox cytotoxicity was far higher than that of free doxorubicin (Dox). Three different formulations of cyclosporin A (either free (CyA), loaded to nanoparticles (NP/CyA) or in a combined formulation with doxorubicin (NP/Dox-CyA)), were added to modulate doxorubicin efficacy. The addition of cyclosporin A to Dox increased drug cytotoxicity. Both CyA added to NP/Dox and NP/Dox-CyA were able to bypass drug resistance.

***Conclusions.*** Despite the barrier role of macrophages, NP/Dox remained far more cytotoxic than Dox against P388/ADR. Both NP/Dox + CyA and NP/Dox-CyA allowed to overcome MDR, but the last one should present greater advantage *in vivo* by confining both drugs in the same compartment, hence reducing the adverse effects.

**KEY WORDS:** doxorubicin; cyclosporin A; polyalkylcyanoacrylate nanoparticles; multidrug resistance; macrophages; coculture.

## INTRODUCTION

Usually, following IV administration, colloidal carriers are captured by Küpffer cells and concentrate in hepatic tissue. Such a passive drug targeting may be advantageous for the treatment of hepatic metastasis as well as for primary liver tumors. Doxorubicin-loaded polyalkylcyanoacrylate (PACA) nanoparticles or liposomes have been shown to increase the life-span of mice bearing M5076 liver metastasis (1–2). In addition, these same nanoparticles have been reported to overcome multidrug resistance (MDR) *in vitro* in many resistant cell lines (3–5).

Among the different strategies used to overcome MDR, co-administration of chemosensitizing compounds, usually acting as P-gp inhibitors, has been widely investigated (6). Cyclosporin A, a potent immunosuppressive agent, has been shown to reverse MDR in resistant cell culture studies (7) and has yielded promising results in refractory leukaemia (8). Cyclosporin A can bind directly to P-gp, inhibiting the pump efflux and leading to increased intracellular accumulation of the cytotoxic drug (9).

In order to better understand the probable effect of PACA nanoparticles *in vivo*, it is necessary to extend the studies to experimental conditions which would better represent *in vivo* behavior. Indeed, after IV administration nanoparticles are preferentially captured by the liver macrophages and not by the tumor cells themselves (10), and it may be also the case after IP administration, because of the presence of peritoneal macrophages (3). It was then considered interesting to investigate doxorubicin nanoparticles in a coculture system consisting of a macrophage-monocyte cell line (J774.A1) and a tumor cell line either sensitive or resistant to doxorubicin (P388 and P388/ADR respectively). Such an approach would contribute to a better understanding of the role of macrophages in delivering the drug to tumor cells. However, it was expected that doxorubicin would exert lower cytotoxicity on tumor cells in coculture compared with direct contact. Therefore, we decided to investigate the additional effect of a reversing agent, cyclosporin A, on the activity of doxorubicin-loaded PACA nanoparticles. An original formulation in which cyclosporin A was coencapsulated with doxorubicin within the same nanoparticle polymeric network was evaluated and compared with other nanoparticle preparations for this purpose. This combined loading should allow both drugs to be targeted to the same location in the liver tissue and would be expected to diminish the side-effects of both compounds while increasing their efficacy.

## MATERIALS AND METHODS

### Chemicals

Free doxorubicin (Adriblastine®) was a gift of Farmitalia (Carlo Erba, Italy). Cyclosporin A was kindly supplied by Novartis (Switzerland). The monomer isobutylcyanoacrylate (IBCA) and MTT were obtained from Sigma (USA). Recombinant mouse interferon-γ, $10^5$ U/vial, was obtained from Life Technologies (France). All other chemicals were purchased commercially and were of analytical grade.

### Preparation of Nanoparticles

Doxorubicin-loaded nanoparticles (NP/Dox) were prepared as described previously (5). Typically, 66.5 mg of IBCA were dropped under mechanical stirring into 6.5 ml of medium containing 5 mg of doxorubicin, 5% glucose, 1% dextran 70 and 0.5% citric acid, resulting in a theoretical payload of 0.8 mg/ml doxorubicin in the colloidal dispersion. The percentage of doxorubicin content in the nanoparticles attained 80% of the initial loading (data not shown).

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Cyclosporin A-loaded nanoparticles (NP/CyA) were also obtained as described above by emulsion polymerization. 20 μl of IBCA were dropped into 2 ml of polymerization medium. Then, cyclosporin A was added as an alcoholic solution (5 mg in 500 μl of ethanol) 1 h after the initiation of the polymerization process, resulting in a theoretical payload of 2 mg/ml cyclosporin A in the nanoparticle suspension. The incorporation efficiency was 89%.

For combined doxorubicin (Dox) and cyclosporin A (CyA) loaded PIBCA nanoparticles, Dox (0.8 mg/ml) was added in the polymerization medium at the beginning of the process as for NP/Dox preparation and CyA at a final concentration of 2 mg/ml was introduced 1 h after the polymerization process was started. The percentage of drug associated with the NP/Dox-CyA nanoparticles was 75% for doxorubicin and 86% for cyclosporin A.

The overall polymerization process lasted over 6 h. The size of the nanoparticles was determined by laser light-scattering method with photon correlation spectroscopy (Nanosizer N4 Plus, Coultronics, France) and was 139 ± 46 nm for NP/Dox, 260 ± 80 nm for NP/CyA and 288 ± 66 nm for NP/Dox-CyA. Unloaded nanoparticles (PIBCA) were obtained by the same method in the absence of drug in the polymerization medium and had a size of 240 ± 81 nm.

### Cell Lines and Culture

P388 (sensitive cells) and P388/ADR (resistant cells) were kindly supplied by the "Institut de Recherche sur le Cancer" (IRSC, France). They were grown in suspension in RPMI 1640 medium (Gibco, France) supplemented with 10% foetal calf serum (Gibco, France), penicillin-streptomycin (Eurobio, France) and 20 nM 2-mercaptoethanol (Sigma, France). The macrophage-monocyte cell line J774.A1 was obtained through the ECACC catalogue (number 91051511). It was maintained as an adherent culture under the same conditions as those described for P388 cells. The activation of J774.A1 cells was induced by adding interferon γ (IFN-γ) to the culture medium prior to the introduction of drug samples. The extent of activation was evaluated by estimating the formation of NO through the quantitative determination of nitrite produced by the activated macrophages. In order to elucidate the influence of induced NO production by activated J774.A1 cells on tumor cells, a series of experiments without IFN-γ was carried out. In such experiments, no formation of NO was detected (data not shown).

### Measurement of Nitrite

Briefly, 100 μl of the culture medium were incubated with 200 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine) in the dark at room temperature for 30 min. The absorbance was measured at 540 nm using a Labsystem microplate reader (ATGC, France). A calibration curve was prepared using sodium nitrite. The lower detection limit was 1 μM nitrite.

### Inhibition of Cell Growth

The growth inhibition of P388 sensitive and resistant cells was assessed after either direct contact between drug samples and tumor cells or coculture with J774.A1 cells, using the MTT assay (11). The samples tested were either free Dox, NP/Dox, PIBCA alone or coincubated with cyclosporin A as free (CyA) or loaded onto nanoparticles (NP/CyA and NP/Dox-CyA).

For direct contact experiments, the drug samples were simply introduced in the wells containing P388 or P388/ADR. The cells were seeded into flat-bottomed 24-well plates (Costar, USA) at $10^5$ cells/well. They were incubated for 24 h before various concentrations of drugs were added and then incubated for further 24 h with the different samples. The final cell number used as control (100%) was the number of cells found in wells at the end of the experimental time (24 + 24 h) when no drug has previously been added to the wells.

The coculture experiments were carried out in appropriate wells consisting of two compartments separated by a porous membrane (Transwell clear inserts, polyester, pore size 0.4 μm membrane diameter, Costar USA). P388 or P388/ADR cells were seeded in the lower compartment at $10^5$ cells/well, whereas J774.A1 cells were seeded in the upper part at $8.10^4$ cells/insert. The drug samples were introduced in the upper macrophage compartment only. IFN-γ was added at 100 U/ml 1 h prior to drug sample introduction in the macrophage insert. As for direct contact experiments, both P388 and J774.A1 cells were grown for 24 h in the well prior to the addition of the drugs and incubated for further 24 h with them. The preliminary growth period allowed the macrophages to become confluent while the tumor cells were in an exponential growth phase. In order to evaluate the importance of doxorubicin adsorption onto the separating membrane, growth inhibition experiments were performed after incorporation of the drug samples over the insert, but in the absence of the macrophage cells. When used, cyclosporin A was added at the concentration of 1.5 μg/ml. This dose was selected on the basis of results obtained in preliminary studies in which the cytotoxicity of different concentrations of cyclosporin A towards both tumor cells and macrophages was evaluated. Whatever the experiment, the IC50 was determined as the concentration of drug inhibiting 50% of cell growth, compared to the control.

## RESULTS

### Growth Inhibition of Tumor Cells in Direct Contact

In sensitive cells, Dox and NP/Dox exhibited the same cytotoxicity (Fig. 1a) with an IC50 value of 500 ng/ml. In contrast, in the case of resistant cells, free Dox had no inhibitory effect (IC50 value of 22,000 ng/ml), whereas NP/Dox showed a cytotoxicity close to that observed in sensitive cells, with an IC50 value of approximately 750 ng/ml (Fig. 1b). It should be noted that unloaded NP exerted a moderate cytotoxicity in both cases with IC50 values expressed as equivalent concentrations of doxorubicin of 3000 and 2000 ng/ml respectively.

### Growth Inhibition of Tumor Cells in Coculture with J774.A1 Cells

In the coculture system, NP/Dox appeared to be more cytotoxic than Dox on sensitive cells (IC50 around 2000 and 3500 ng/ml respectively) (Table I). Compared with the previous values obtained in direct contact, Dox and NP/Dox efficacy decreased 7-and 4- fold respectively in coculture. In case of resistant cells (Fig. 2a, Table I), the IC50 of NP/Dox reached

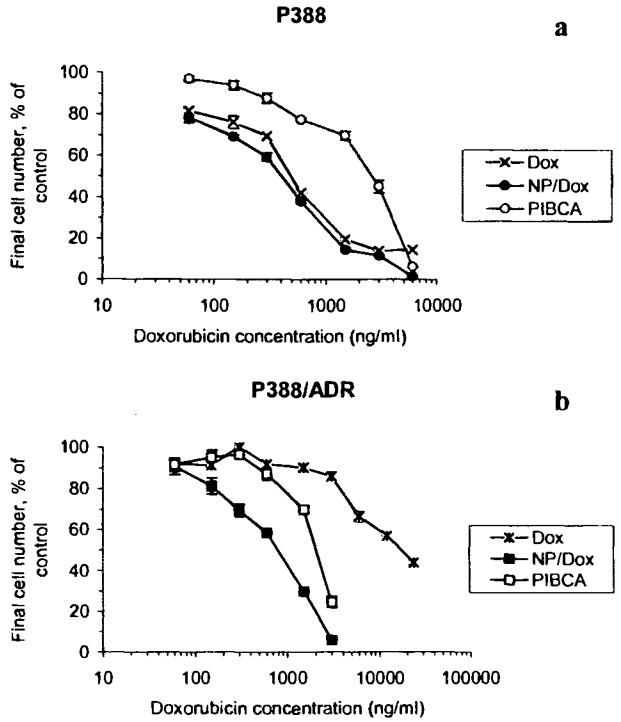


**Fig. 1.** Growth inhibition of P388 (a) and P388/ADR (b) cells by: free doxorubicin (Dox), doxorubicin-loaded nanoparticles (NP/Dox) and unloaded nanoparticles (PIBCA). Three independent experiments were performed in triplicate with less than 10% standard deviation between experiments. The results of a typical experiment are presented with error bars representing internal standard deviations.

6800 ng/ml, corresponding to a decrease in efficacy of one order of magnitude compared with direct contact experiments. The decrease of free Dox effect seemed to be even more pronounced, since no cytotoxicity could be observed for Dox, even at the highest doses tested. The addition of CyA in solution (Table I) decreased the IC50 of Dox to a value close to that of NP/Dox in sensitive cells. In the case of P388/ADR cells (Fig. 2b) both Dox and NP/Dox cytotoxicity were improved by CyA. When cyclosporin A was added as NP/CyA, very similar effects were observed, except in the case of resistant cells and NP/Dox (Fig. 2c, Table I). Indeed, the IC50 observed for NP/Dox was even higher in the presence of NP/CyA than in the absence of any cyclosporin A (9000 and 6800 ng/ml respectively). Finally, the combined formulation, NP/Dox-CyA, appeared to be the most effective on sensitive cells (Table I), together with NP/Dox + CyA, and was also the most effective on resistant cells (Fig. 2d).

The results obtained from coculture experiments in absence of IFN-γ (no macrophage activation) were similar to those observed with the activated macrophage coculture experiments (data not shown).

Finally, the cytotoxicity of doxorubicin samples in the absence of macrophages has been investigated (Table II). The IC50 values for doxorubicin and NP/Dox on P388/ADR cells were close to those obtained in direct contact (24,000 and 2500 ng/ml versus 22,000 and 750 ng/ml respectively).

### Tolerance of Drug Preparations by J774.A1

The macrophages were activated with mouse IFN-γ 100 U/ml. This dose was selected after preliminary studies and corresponded to an optimal production of NO in cell cultures in the absence of significant cytotoxicity. Dox and NP/Dox were found to be equally cytotoxic towards J774.A1 cells, with IC50 around 550 ng/ml (Table I). At the IC50 of NP/Dox, PIBCA itself induced 20% growth inhibition, but no cumulative cytotoxicity between doxorubicin and the polymer could be observed. Irrespective of the formulation (CyA, NP/CyA, NP/Dox-CyA), the addition of cyclosporin A increased the cytotoxicity of both Dox and NP/Dox on J774.A1 cells, decreasing the IC50 value to approximately 150 ng/ml (Table I), whereas by itself cyclosporin A did not exhibit any marked cytotoxicity on J774.A1 cells at concentrations up to 12 μg/ml (data not shown).

When NO production was measured as a function of doxorubicin concentration (Fig. 3), Dox and NP/Dox led to a decrease in the NO measured in the culture medium at the end of the incubation time especially at higher concentrations, probably due to the cytotoxicity (Fig. 3a). PIBCA did not exert any significant effect on NO production. In the presence of cyclosporin A, either CyA, NP/CyA or NP/Dox-CyA (Fig. 3b), the decrease in NO production was even more pronounced. This effect is consistent with the increase in cytotoxicity

**Table I.** IC 50 Values (mean +/− SD) of Dox and NP/Dox, with or Without the Different Formulations of Cyclosporine A, on P388 and P388/ADR Cells in the Coculture Experiments

| | IC 50 values (mean +/− SD), ng/ml of doxorubicin | | | | | | |
|---|---|---|---|---|---|---|---|
| | Dox | NP/Dox | Dox + CyA | NP/Dox + CyA | Dox + NP/CyA | NP/Dox + NP/CyA | NP/Dox-CyA |
| P388 | 3500 +/−200 | 2000* +/−100 | 1700 +/−100 | 1600 +/−100 | 1500 +/−100 | 1500 +/−100 | 1100* +/−100 |
| P388/ADR | — — | 6800 +/−200 | > 6000 — | 2900 +/−100 | 22,000 +/−1000 | 9000 +/−300 | 2900 +/−200 |
| J774.A1 | 550 +/−50 | 550 +/−50 | 150 +/−10 | 150 +/−10 | 150 +/−10 | 150 +/−10 | 150 +/−10 |

*Note:* Three different experiments were performed in triplicate, with less than 10% standard deviation between experiments. The results of a typical experiment are presented.
* $p < 0.05$ (NP/Dox versus NP/Dox-CyA).

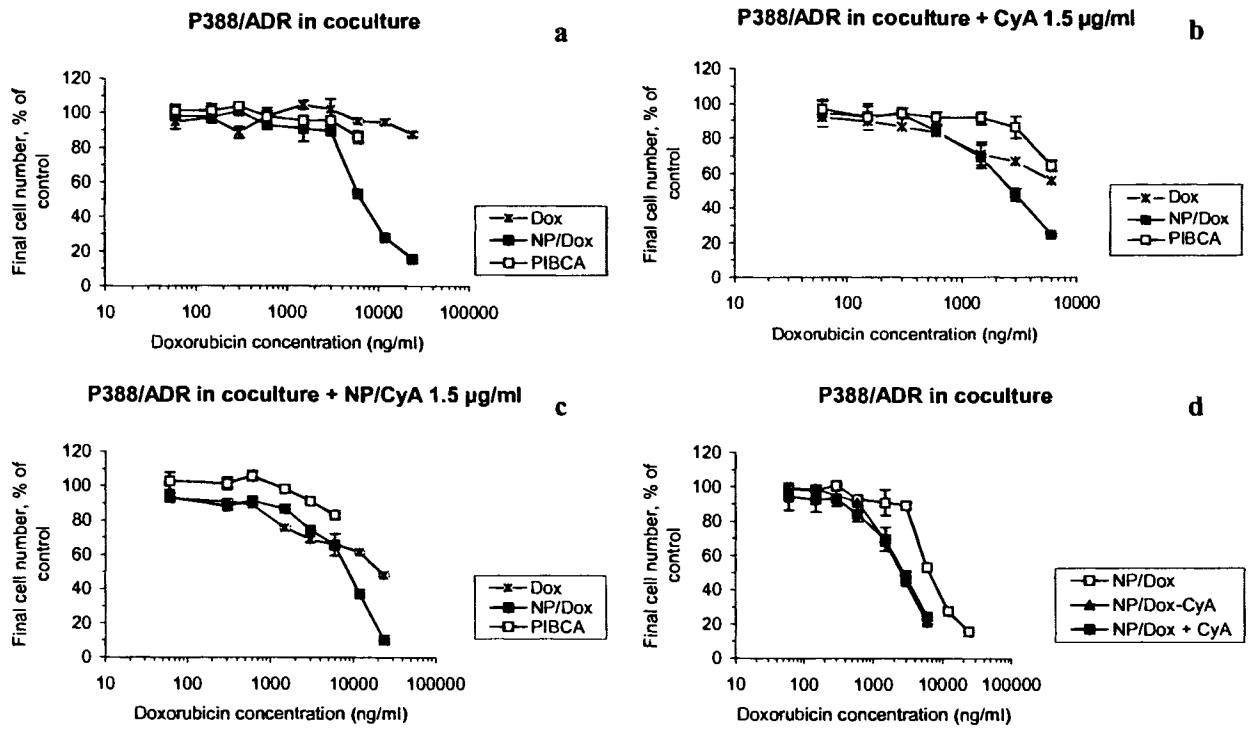


**Fig. 2.** Growth inhibition of P388/ADR cells in coculture with J774.A1 activated with IFN-γ by: Free doxorubicin (Dox), doxorubicin-loaded nanoparticles (NP/Dox) and unloaded nanoparticles (PIBCA) added to the J774.A1 compartment (a) in the presence of either free cyclosporin A (b) or cyclosporin A loaded onto nanoparticles (c) or the combined formulation of doxorubicin and cyclosporin A NP/Dox-CyA (d). Three independent experiments were performed in triplicate with less than 10% standard deviation between experiments. The results of a typical experiment are presented with error bars representing internal standard deviations.

described above. It should be mentioned that the addition of cyclosporin A alone decreased the NO production even at low concentrations, although no cytotoxicity was observed (data not shown).

## DISCUSSION

Following IV administration, PACA nanoparticles are taken up by the mononuclear phagocyte system (MPS), mainly by Küpffer cells in the liver. Even if the primary aim in developing doxorubicin nanoparticles was to reach an hepatic tumor, no direct contact between nanoparticles and tumor cells was found to occur (10). The antitumor effect was observed to be mediated by macrophages acting both as a barrier and a reservoir for the drug (10). If such a targeting strategy is valuable in the case of sensitive tumors (as the M5076 model), it is more questionable for resistant ones. Indeed, previous work (5) has shown the importance of a direct contact between the nanoparticles and the cancer cells for overcoming MDR efficiently. Therefore, it was interesting to clarify the role of the macrophages in mediating cytotoxicity to sensitive and resistant tumor cells. More precisely, two phenomena with opposite effects on drug cytotoxicity might occur: first, macrophages will certainly constitute a barrier, thus reducing the possibility of direct contact between tumor cells and nanoparticles, but secondly, doxorubicin may activate the macrophages and enhance their own tumoricidal effect (12–13). In an attempt to compensate for the possible obstacle induced by the barrier effect of macrophages, it might be useful to combine doxorubicin with cyclosporin A, a MDR-reversing agent (14).

**Table II:** IC 50 Values (mean +/− SD) of Dox and NP/Dox on P388/ADR Cells, in Free Access or in the Presence of a Membrane Insert, or After Coculture with Macrophages

| | IC50 values (mean +/− SD), ng/ml of doxorubicin | | |
|---|---|---|---|
| | Culture in free access | Culture with insert, without macrophages | Coculture with macrophages |
| Dox | 22,000 +/− 1000 | 24,000 +/− 1000 | — |
| NP/Dox | 750 +/− 50 | 2500 +/− 100 | 6800 +/− 200 |

*Note:* Three different experiments were performed in triplicate, with less than 10% standard deviation between experiments. The results of a typical experiment are presented.

- In direct contact experiments, NP/Dox overcame drug resistance.

The results in Fig. 1a and 1a′ demonstrating the ability of PACA nanoparticles to overcome drug resistance in direct contact experiments were in agreement with those published (5) except that all the IC50 values were higher. The sensitivity of both cell lines has decreased compared with our previous experiments but the resistance factor of P388/ADR remained sufficiently high to differentiate any further reversion of the resistance by the drug formulations.

- In coculture, NP/Dox partly overcame MDR and CyA still enhanced doxorubicin cytotoxicity when combined in the same nanoparticle formulation.

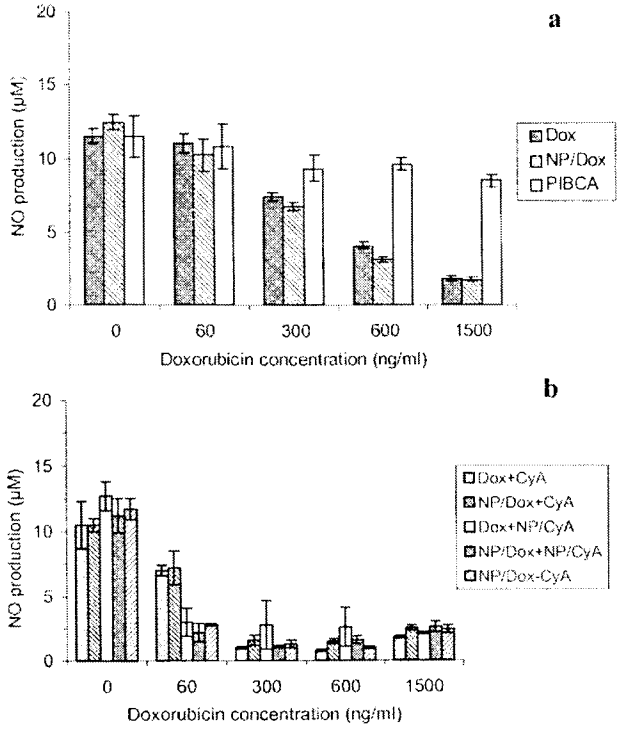


**Fig. 3.** Effect of drug preparations on NO production by J774.A1 cells: Free doxorubicin (Dox), doxorubicin loaded with nanoparticles (NP/Dox), unloaded nanoparticles (PIBCA) (a); These samples were coincubated with 1.5 µg/ml of either free cyclosporin A, or with cyclosporin A-loaded onto nanoparticles (NP/CyA), or combined doxorubicin and cyclosporin A nanoparticles (b). Three independent experiments were performed in triplicate with less than 10% standard deviation between experiments. The results of a typical experiment are presented with error bars representing internal standard deviations.

In the coculture experiments, macrophages were confluent before the drug samples were added. The resulting growth inhibition observed on P388 and P388/ADR was therefore a macrophage-mediated antitumor effect. Since the barrier role of the separating membrane was found negligible in comparison with the macrophage barrier effect (Table II) and since no particle could be found in the lower compartment when investigated by laser light-scattering (data not shown), it can be assumed that the cytotoxicity was solely due to drug released by the macrophages. In case of the sensitive cells, the IC50 values were increased compared to direct contact, but the difference observed between Dox and NP/Dox is more interesting. Indeed, whereas Dox and NP/Dox were equally cytotoxic when introduced in direct contact with P388, in coculture NP/Dox were significantly more cytotoxic than Dox. This difference, however, disappeared when cyclosporin A was coincubated with the sensitive cells, suggesting that only the effect of free doxorubicin was altered by the addition of cyclosporin A in coculture. In the case of the resistant cells, doxorubicin concentrations could not be raised sufficiently to determine the IC50 for Dox. The addition of cyclosporin A, irrespective of the formulation, increased Dox cytotoxicity, although it failed to overcome resistance. The encapsulation of doxorubicin into nanoparticles (NP/Dox) greatly improved its efficacy although it remained unable to completely reverse P388 resistance. The addition of cyclosporin A to NP/Dox had varying effects along with the formulation used. Indeed, only CyA and NP/Dox-CyA, but not NP/Dox plus NP/CyA, were able to decrease the IC50 to values close to those observed in sensitive cells. The inability of NP/CyA to potentiate the effect of NP/Dox may be explained by the assumption that the total number of nanoparticles thus added to macrophages could exceed the phagocytosis capacity of these cells. The overall doxorubicin concentration taken up by the cells would then be lower than, for example, in the case of NP/Dox-CyA, the combined formulation, which would explain the lower cytotoxicity of NP/Dox plus NP/CyA. This hypothesis is also consistent with the work of Fernandez *et al.* (15) who showed that the administration of PACA nanoparticles *in vivo* resulted in a decrease of the phagocytic function of Küpffer cells.

According to the results of coculture experiments, it would therefore be much more advantageous to combine doxorubicin and cyclosporin A in the same nanoparticle rather than in separate nanoparticle preparations. *In vitro*, these combined nanoparticles exhibited the same efficiency as that obtained by the simple mixing of NP/Dox and free CyA. However, this would not be the case *in vivo*. To attain effective concentrations of both drugs in the liver for example, cyclosporin A will need to be administered at a much higher dose when free, as compared to the dose associated with nanoparticles because of the absence of liver concentration of cyclosporin A when administered free. Thus, the overall drug doses administered *in vivo* could be lower in the case of NP/Dox-CyA, confirming the superiority of this formulation.

- How can doxorubicin and cyclosporin A encapsulated in NP/Dox-CyA, be transferred from macrophages to the tumor cells?

Previous work (5) showed that NP/Dox remained more cytotoxic than Dox against P388/ADR even when the carrier was completely biodegraded. In these conditions, it may be hypothesized that the cytotoxicity measured in resistant cells in the present coculture experiments resulted from the diffusion of doxorubicin and cyclosporin A as well as the degradation products of PACA nanoparticles from macrophages to P388 cells. It was indeed previously shown that doxorubicin was not degraded within lysosomes (16). Thus, release of intact doxorubicin from the macrophages and diffusion into tumor cells is a realistic mechanism. It is not yet known if the polycyanoacrylic acid resulting from the biodegradation of PACA nanoparticles in the lysosomes will also be released by the macrophages. Colin de Verdière (5) postulated that the efficacy of NP/Dox in resistant cells resulted from the formation of an ion-pair complex between doxorubicin and polycyanoacrylic acid, increasing the intracellular diffusion of the drug. Even if polycyanoacrylic acid is released by macrophages, it is not known whether ion-pairs with doxorubicin can still form. Nevertheless, the much greater efficacy of NP/Dox over Dox in resistant cells in coculture with macrophages tends to suggest that PACA still plays a crucial role.

- What about the possible effect of the activation of macrophages by NP/Dox-CyA?

Firstly, it was found that the different drug samples strongly inhibited the viability of J774.A1 cells: the values found for the IC50 were very low, but it must be emphasized that the drugs were added in the upper compartment of the well, in contact with J774.A1 cells. In this compartment, the volume of culture medium is 7.5 times less than the overall volume of

the well (200 μl out of 1500 μl) whereas, to facilitate comparison with the direct contact experiments, the concentrations are expressed with respect to the total cell volume. Therefore, the concentrations of drug close to the macrophages were 7.5 times higher than those close to the tumor cells. Hence, the real concentration of doxorubicin in contact with J774.A1 cells at IC50 value should be calculated to be 550 × 7.5 and not 550 ng/ml. It may be questionable if such a cytotoxicity could lead to holes in the macrophages layer thus facilitating drug samples, especially nanoparticles, to gain access to tumor cells. However, after 2 and 6h incubation (data not shown) it has been verified that 100% of J774.A1 were surviving until an overall doxorubicin concentration of 3000 ng/ml, irrespective of the formulation. Therefore, it can be ensured that the macrophage layer remains undamaged far longer after the nanoparticles degradation, thus validating the proposed coculture model.

In contrast to doxorubicin, cyclosporin A alone was not found cytotoxic against J774.A1 cells, even at 12 μg/ml (which corresponds to 7.5 times the overall cyclosporin A concentration) (data not shown). However, cyclosporin A was shown to increase Dox and NP/Dox cytotoxicity against the macrophages. At the same time, the NO production declined which may directly be related to cytotoxic effects. Nevertheless, cyclosporin A itself was shown to decrease the NO production at very low concentrations (data not shown). This is consistent with the literature since cyclosporin A has been reported to decrease both production of cytokines such as TNF-α (17–18) and NO production (19–20). In addition, Tojimbara *et al.* (21) found that cyclosporin A could inhibit Küpffer cell activation *in vitro*. Since the activation of macrophages could be advantageous in the case of resistant tumor therapy by supplying cytokines with antitumor activity, coadministration of cyclosporin A may be questioned. To address this issue, coculture experiments have been performed without IFN-γ and in the absence of cyclosporin. In contrary to previous studies with M5076 cells (22), these results clearly point out that macrophage activation does not contribute to the cytotoxicity observed on both P388 and P388/ADR giving a further argument, in favor of the interest of cyclosporin A coadministration.

In conclusion, the *in vitro* coculture test system consisting of macrophages and tumor cells developed in this study allowed to investigate the role of macrophages in mediating cytotoxicity of doxorubicin-loaded nanoparticles towards P388 sensitive and resistant cells. This model of coculture may be considered as closer than a single cell culture, to what occurs *in vivo* following IV administration of nanoparticles for the targeting of liver tumors or metastases. We have shown that NP/Dox greatly improved the efficacy of doxorubicin on resistant cells while Dox remained without effect. The association of cyclosporin A in solution with NP/Dox and the combined nanoparticle formulation of doxorubicin and cyclosporin A both allowed resistance to be overcome. NP/Dox-CyA however present a great advantage over the use of free cyclosporin A *in vivo* in confining these complementary compounds in the same compartment (for example the liver after IV administration, or the ascites after IP administration). This should allow greater efficacy to be achieved while significantly decreasing their well known adverse effects due to a large biodistribution of the free drug.

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