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DOXORUBICIN-LOADED NANOSPHERES BYPASS TUMOR CELL MULTIDRUG RESISTANCE

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Abstract—We have demonstrated that *in vitro* resistance of tumor cells to doxorubicin (Dox) can be fully circumvented by using doxorubicin-loaded nanospheres (Dox-NS), consisting of biodegradable polyisohexylcyanoacrylate polymers of 300 nm diameter and containing 2.83 mg of Dox per 31.5 mg of polymer. Five different multidrug-resistant cell lines, characterized by *mdr*1 amplification, were used in this study: Dox-R-MCF7, a human breast adenocarcinoma; SKVBL1, a human ovarian adenocarcinoma; K562-R, a human erythroleukemia; and two murine lines: P388-Adr-R, a monocytic leukemia of DBA2 mouse, and LR73MDR, a Chinese hamster ovarian cell line. These lines were 38.7, 210, 232, 143 and 20 times more resistant than their corresponding sensitive counterparts, respectively. Using Dox-NS, we obtained complete reversion of drug resistance *in vitro*, i.e. cell growth inhibition comparable with that obtained with sensitive cells exposed to free Dox. *In vivo*, we significantly prolonged the survival of DBA2 mice which had previously received P388-Adr-R cells by i.p. injections of Dox-NS, while free Dox injection was ineffective toward this rapidly growing tumor. (Prolongation of survival time: 115% vs 167% after Dox vs Dox-NS treatment, respectively.) Using the MCF7 cell line and its resistant variant, we studied the intracellular concentration and the cytoplasmic and nuclear distribution of Dox by laser microspectrofluorometry (LMSF). In sensitive cells, we observed a similar accumulation and distribution of Dox whatever the form of Dox delivery, i.e. whether free or carried by nanospheres. Analysis by LMSF showed that 99% of intranuclear Dox was bound to DNA after treatment with both forms of Dox. Of Dox, 81 and 83% were found in the intranuclear compartment of sensitive cells incubated with free Dox and Dox-NS, respectively. Resistant cells incubated with Dox-NS accumulated the same amount of Dox as sensitive cells incubated with free Dox or with Dox-NS. Dox, when loaded in nanospheres, bypasses the efflux mechanism responsible for multidrug resistance. LMSF analysis showed that Dox, transported and released by nanospheres, interacts with DNA identically in sensitive and resistant cells.

Chemoresistance of cancer cells, either primary or secondary to treatment, is a frequent cause of treatment failure. Resistance to anthracyclines is often associated with multidrug resistance; such resistance is explained by a decreased drug accumulation in tumor cells [2–6]. A membrane glycoprotein which acts as an energy-dependent pump, designated as P-glycoprotein [7, 8], may be responsible for accelerated drug efflux. Other workers, including ourselves, had previously developed drug carriers in order to protect the drug against rapid degradation or to slow down drug release, thereby reducing the general toxicity to patients [9, 11]. Such benefits have been obtained using doxorubicin (Dox‖) embedded in nanospheres of polyisohexylcyanoacrylate [12]. The nanospheres are colloidal, particulate biodegradable carriers [13–15]. Their goal is to allow the intracellular capture of associated drugs by an endocytotic process [11, 16] which is very active in tumor cells and also in some specialized normal cells, while Dox freely enters both normal and tumoral cells. It has been shown previously that nanospheres loaded with propidium iodide can enter cells by endocytosis and are detectable as fluorescent granulations surrounding the nucleus [17]. In addition, it was observed that Dox-loaded nanospheres (Dox-NS) can significantly reduce the toxicity of Dox after intravenous administration into mice [12]. Moreover, the *in vivo* antitumoral efficiency of Dox was investigated and found to be improved [18]. We hypothesize that Dox, delivered to resistant cells as Dox-NS, could escape the P-glycoprotein-dependent accelerated efflux process and could assist circumvention of multidrug resistance.

Here we compare the toxicity of free Dox and Dox-NS to cell lines that differ in their drug sensitivity. We correlate these effects with localization and concentration kinetics by measuring Dox intracellular concentrations by laser microspectrofluorometry (LMSF).

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‖ Abbreviations: Dox, doxorubicin; NS, nanospheres; Dox-NS, Dox-loaded NS; FITC, fluoresceine; LMSF, laser microspectrofluorometry; PBS, phosphate-buffered saline; MSF, mean specific fluorescence.

MATERIALS AND METHODS

Cells. Five pairs of parental sensitive cell lines and their multidrug-resistant variant sublines have been

Table 1. Origin and characteristics of cell lines studied

Cell line	Given by	Histologic type; origin	Type of selection; selecting drug
MCF7	K. Cowan	Breast cancer;	*In vitro* culture;
Dox-R-MCF7	[4]	human	Dox
SKOV3	V. Ling	Ovarian cancer;	*In vitro* culture;
SKVLB1	[20]	human	Vinblastine
K562	M. Manfait	Erythroblastic leukemia;	*In vitro* culture;
K562-R	[19]	human	Adriamycin
P388	T. Tsuruo	Monocytic leukemia;	*In vivo* selection;
P388-Adr-R	[6]	DBA2 mouse	Adriamycin
LR73	P. Gros	Ovarian cancer;	*mdr*1 transfection
LR73MDR	[21]	Chinese hamster	

studied: MCF7, a human breast adenocarcinoma [4]; K562, a human erythroleukemia [19]; SKOV3, a human ovarian cell line [20]; P388, a murine monocytic leukemia [6] and LR73, a Chinese hamster ovarian cell line [21]. Three of these resistant variants were obtained by selection *in vitro* with increasing concentrations of Adriamycin® (Dox-R-MCF7, K562-R) or with Vinblastine (SKVLB1); one line was obtained by treating DBA2 syngeneic mice, bearing the P388 ascitis, with adriamycin; and another by transfection of Chinese hamster ovarian cancer cells with the *mdr*1 gene (LR73MDR) [21]. Cells were routinely cultured according to their own culture conditions. Table 1 shows tissue and species origin of the lines.

Flow cytometry analysis of P-glycoprotein expression. The murine monoclonal antibody C219 (ORIS), an IgG2a antibody coupled to fluoresceine (FITC) and specific for P-glycoprotein, was used for direct immunofluorescence staining. It recognizes a highly conserved cytoplasmic epitope found in all P-glycoprotein isoforms and represents a universal probe for P-glycoprotein expression [22].

The same immunostaining protocol was used for all the cell lines studied. When adherent cells were tested, a cell suspension was obtained by scraping cells from the plates and pipetting them up and down in cold PBS (phosphate-buffered saline; Flow, Les Ulis, France; pH 7.2, without Ca^{2+} and Mg^{2+}, at 4°). The cells were then permeabilized for 5 min at 4° with methanol 70% (v/v) and washed twice in PBS. For each assay, a suspension of 10^6 cells was treated with the FITC-conjugated C219 antibody, diluted at 1:20 in PBS containing 1% bovine serum albumin. An identical cell suspension was treated with a murine FITC-conjugated IgG2a antibody, at the same dilution, to determine the non-specific binding. After 30 min incubation in the dark at 4°, the cells were washed twice in 1% PBS containing bovine serum albumin and the cell suspensions were adjusted to 10^6 cells/mL.

Flow cytometry analyses were performed on a FACSCAN flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.) where the excitation source was an argon ion laser emitting a 488 nm beam at 15 mW. The green fluorescence of FITC (1 Ex max: 494 nm; 1 Em max: 517 nm) was collected through a 530/30 nm band pass filter and 10^4 cells/sample were analysed. The green fluorescence, related to P-glycoprotein expression, was measured on a logarithmic scale. P-glycoprotein fluorescence values were converted to linear values and expressed as arbitrary fluorescence units. For all cell lines tested, the mean linear fluorescence of the isotypic control was set at 100. Under these conditions the mean specific fluorescence (msf) for each cell type was calculated as follows: msf = (mean linear antibody fluorescence) − 100, where 100 corresponds to the mean linear fluorescence of the isotypic control. This mode of evaluation allows comparison of the level of P-glycoprotein expression between one cell line and another [23].

Drugs and polymers. Doxorubicin chlorohydrate (Dox) was provided by Roger Bellon (Mons, France). Dox-NS and unloaded nanospheres (NS) were supplied by Sopar (Bruxelles, Belgium). The Dox-NS suspension was prepared as a clinical batch (Dox: 2.83 mg; polyisohexylcyanoacrylate: 31.5 mg) as described previously [24], freeze dried and rehydrated with PBS before use (Flow) in order to obtain the required Dox concentrations. The mean diameter of these particles was about 300 nm. In the reconstituted solution, 96% (±2%) of Dox was loaded into the nanospheres.

Molar concentrations of Dox as indicated in this paper were calculated taking into account only the concentration of Dox, whether it was free or loaded. When NS suspensions were used, dilutions were prepared in the same way as for NS-Dox, so that the polymer concentrations were always the same.

Cell survival assay. Cells, growing as monolayers, were seeded at 5×10^4 cells/culture dish (35-mm diameter, Falcon) in 2 mL of RPMI 1640 medium (Gibco, Uxbridge, U.K.), supplemented with 10% fetal calf serum (FCS) and incubated at 37° in a 5% CO_2 atmosphere for 24 hr. Free Dox solution, Dox-NS, unloaded NS suspension and a mixture of unloaded NS and free Dox (NS + Dox; corresponding to the respective amounts of NS and Dox in Dox-NS) were prepared immediately before use, added to the cell monolayers at different concentrations, and usually incubated for 6 hr or other time intervals as indicated in Figs 1 and 2. Each experiment was carried out in triplicate. After

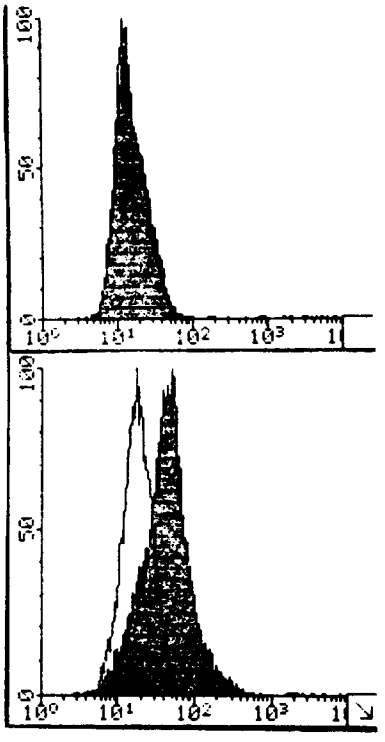

Fig. 1. Analysis of P-glycoprotein expression in MCF7 and Dox-R-MCF7 by flow cytometry (clear area: isotypic control; grey area: assay with C219 antibody). Upper part: in the MCF7 cell line, the two histograms overlapped, indicating the lack of P-glycoprotein expression. Lower part: in the Dox-R-MCF7 cell line, the histogram pattern of the assay, using the C219 antibody and the isotypic control, was representative of cells with high expression of P-glycoprotein: 78% of the cells were positive, with a msf of 30.

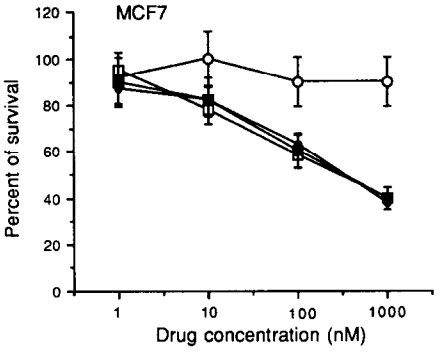

Fig. 2. Cell survival curves of the human adenocarcinoma MCF7 cell line after 6 hr incubation with increasing concentrations of free Dox, Dox-NS, NS and NS + Dox, followed by 72 hr of culture in fresh medium. Means of percentages ± SD are shown. Free Dox (■), Dox-NS (●), NS (○), NS + Dox (hatched line).

incubation in the presence of drug, the cells were washed three times with PBS and 2 mL of drug-free medium were then added. The cells were then incubated for an additional 2-day period.

Cells growing in suspension, such as P388 and K562, were seeded in 15-mL tubes suitable for cell culture (Falcon) at a density of 5×10^4 cells/mL, and drug was immediately added to the culture medium. After 6 hr incubation, the cultures were centrifuged and the medium containing the drugs was removed. Cells were washed three times with PBS and fresh, drug-free medium was added. The cell suspensions, containing 5×10^4 cells, were distributed in 35-mm culture dishes and incubated for 2–3 additional days.

In all cases, controls comprised the same number of cells cultivated in drug-free medium. Cells were counted in a Coulter Counter (Coultronics, Margency-France), following trypsinization in the case of adherent cells.

Drug toxicity was evaluated by its inhibitory effect on cell proliferation. The survival rate of treated cells was calculated and results are expressed as follows: Percent of survival = 100 × (number of treated cells surviving) ÷ the total number of untreated cells.

The mean percentages of cell survival and the corresponding standard deviations were calculated. Curves of percentages of cell survival were established and the ID_{50} values, doses capable of reducing the cell proliferation by 50%, were deduced from them.

In vivo *assays*. At day 0, 8-week-old female DBA2 syngeneic mice, bred and maintained in specific pathogen-free conditions at the animal house of Villejuif (France) received an i.p. injection of 1×10^6 P388-Adr-R cells. Before injection, these cells have been continuously cultivated in the presence of 1 μM Dox, and were washed and suspended in 0.1 mL of free serum culture medium. After injection, mice were then divided randomly into groups of 10. At days 1, 2, 3 and 4, NS-Dox or free Dox, diluted in a final volume of 0.1 mL of PBS, was injected by i.p. route at 2 mg/kg body weight. Mice were observed daily and the deaths were due to the proliferation of P388-Adr-R cells. Without any treatment, all the mice died with tumoral ascitis between days 11 and 15. The survival times of mice were registered and curves of survival were established. Fifty per cent survival time is the delay corresponding to the death of 50% of the mice per group (in this case, five mice). This experiment was repeated four times.

LMSF. Fluorescent emission spectra from a volume within a living cell were recorded with a microspectrofluorometer (modified Raman spectrometer OMARS 89, DILOR, Lille, France) as already described [19, 25]. By means of an optical microscope (Olympus BH2) equipped with a 100X water phase contrast immersion objective (Leitz Fluotar), a laser beam was focused on a spot of less than 1 μm in diameter. Sample observation and collection of fluorescence emission were obtained through the same optics. The actual fluorescence sampling was restricted by means of a suitable pinhole diaphragm on the image plane of the microscope objective. The emission light signal,

spectrally dispersed by a diffraction grating, was detected with an optical multichannel analyser, made of a cooled 512-diode array and optically coupled to an image intensifier. Data were collected locally, and then transferred to a computer for analysis with specially developed software. Laser power and instrumental response were controlled by the daily use of rhodamine B as an external standard.

Following different treatments, including with free Dox or Dox-NS, sensitive MCF7 and Dox-R-MCF7 cells were scraped from the culture dish and seeded in a dish containing PBS. At least 20 spectra from the same intracellular location were accumulated in order to obtain a signal to noise ratio of about 30. The reported data were collected from 20 different cell nuclei within the first 15 min following transfer of the cells into PBS. During this time, the intranuclear concentrations of Dox remained unaffected. Sample heating, photoleaching and photodamage were checked and found to be negligible under our experimental conditions. The laser power on the sample was 4 μW and the illumination time 1 sec. Phase contrast microscopy was used to show that cells remained viable after repeated fluorescence determinations.

The fluorescence emission arising from the nucleus of a cell treated with Dox or NS-Dox was expressed as the sum of spectral contributions of free Dox, DNA-bound Dox and intranuclear autofluorescence. According to the studies performed in aqueous solutions, we showed recently [19, 26] that each of these contributions has a characteristic spectral shape and the fluorescence yield of the free form of Dox was 48 times higher than that of the DNA-bound form. Thus, Dox concentrations in the living cell nucleus were obtained from the determined spectral surface contributions and by means of the corresponding fluorescence yields. Intranuclear concentrations of Dox are expressed as micromolar concentrations.

Table 2. Concentrations of Dox or Dox-NS that inhibit the proliferation of parental line cells and of their resistant variants by 50%

Cell line	Dox ID_{50} (μM) (% of resistance)	Dox-NS ID_{50} (μM)
MCF7	0.3	0.3
Dox-R-MCF7	11.6 (38.7)	0.4
SKOV3	0.3	0.3
SKVLB1	63.1 (210)	0.4
K562	0.05	0.05
K562-R	11.6 (232)	0.4
P388	0.03	0.04
P388-Adr-R	4.3 (143)	0.08
LR73	0.04	0.03
LR73MDR	0.8 (20)	0.05

* % of resistance was calculated as:

$$100 \times \frac{\text{Dox } ID_{50} \text{ for resistant cells}}{\text{Dox } ID_{50} \text{ for sensitive cells}}.$$

Table 3. Quantitative evaluation by flow cytometry of P-glycoprotein expression in the different cell lines studied, using a fluorescent C219 antibody

Cell line	msf	% of positive cells
MCF7	0	0
Dox-R-MCF7	30	78
SKVOV3	0	0
SKVLB1	11	23
K562	0	0
K562R	12	53
P388	0	0
P388-Adr-R	41	68
LR73	0	0
LR73MDR	43	80

RESULTS

Dox-induced inhibition of cell proliferation

The two cell lines originated from human adenocarcinomas, MCF7 and SKOV3, were 6–10 times more spontaneously resistant to Dox than K562, P388 or LR73. Table 2 reports the inhibiting doses (ID_{50}) of Dox, capable of reducing the cell proliferation by 50%. After drug selection, all cell lines became resistant to very high concentrations of Dox. For the six Dox-resistant cell lines, the ID_{50} of free Dox was 20–232 times higher than that of the corresponding sensitive parental line.

P-glycoprotein expression of sensitive and resistant cell lines

The different cell lines analysed by flow cytometry can be regrouped into three families (Table 3). The first, illustrated by the SKVLB1 cell line, was characterized by a modest percentage of cells expressing P-glycoprotein (23%) at a low level as indicated by a low msf (msf = 11). The second family, illustrated by the K562-R cells, was characterized by a relatively high percentage of cells expressing P-glycoprotein (53%) at a low level (msf = 12). The third family comprises the Dox-R-MCF7 (Fig. 1), P388-Adr-R and LR73MDR cell lines and was characterized by a high percentage of positive cells (68–80%) expressing P-glycoprotein at a high level (msf = 30–43).

Sensitivity to Dox-NS

Proliferation inhibition of sensitive parental cells was similar after treatment with either free Dox or the mixture of free Dox and NS (NS + Dox). Figure 2 shows the results obtained with MCF7 cells, as an example. Unloaded NS were not toxic at the concentration used in Dox-NS. Proliferation of resistant cells, incubated for 6 hr in the presence of Dox-NS and then subcultivated for 3 days, was strongly inhibited. A decrease in ID_{50} from 30–250 times was measured and compared with that of free Dox (Table 2). This inhibiting effect of Dox-NS was dose dependent as shown by the drug-resistant LR73MDR cell line (*mdr*1 transfected) and the Adriamycin-resistant mammary adenocarcinoma

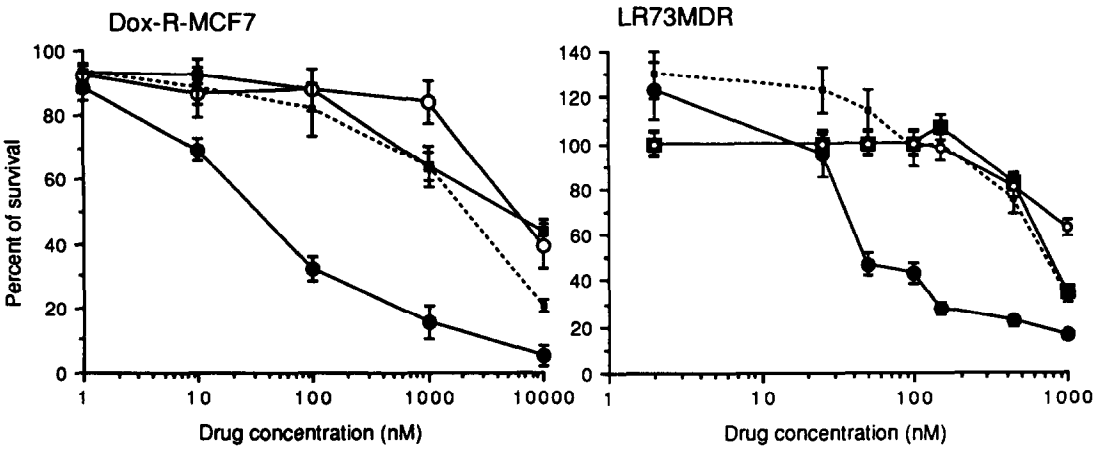

Fig. 3. Cell survival curves of a human adenocarcinoma Dox-R-MCF7 variant subline and of LR73MDR. Each cell line was selected for resistance to Dox, or transfected with the *mdr*1 cDNA and selected in increasing concentrations of free Dox (■), Dox-NS (●), NS (○), NS + Dox (hatched line). Means of percentages ± SD are reported.

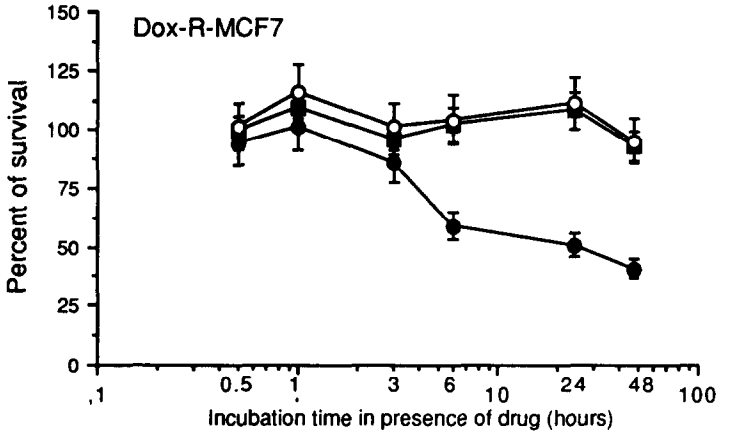

Fig. 4. Cell survival curves of a human adenocarcinoma Dox-R-MCF7 variant subline cultivated in the presence of free Dox, Dox-NS, NS and NS + Dox. The concentration of Dox was 0.4 μM of Dox whether free or loaded in NS, and the concentration of unloaded NS corresponded to the polymer concentration of Dox-loaded nanospheres. Times correspond to incubation times with the drugs, which were added to the culture medium 24 hr after the cells were seeded. In all cases, cells were cultivated in fresh medium after incubation with drugs and maintained until a total culture duration of 96 hr was reached. Free Dox (■), Dox-NS (●), NS (○), NS + Dox (hatched line). Means of percentages ± SD are reported.

Dox-R-MCF7 (Fig. 3). We noticed that the reversion to drug sensitivity when using Dox-NS was almost complete, as evidenced by the quite similar ID_{50} observed for the resistant variants of the MCF7 and SKOV3 lines treated with Dox-NS and the ID_{50} of the corresponding sensitive parental cells treated with free Dox. Circumvention of resistance was also obtained with resistant variants of the K562 and P388 lines: from 232 times more resistant with free Dox to eight times with Dox-NS, and from 143 times more resistant with free Dox to 2.6 times with Dox-NS, respectively. Using LR73 cells, we observed that Dox-NS was much more toxic than free Dox, leading to an improvement of the ID_{50} by approximately one log. Resistance of LR73MDR cells was circumvented as the ID_{50} of free Dox was 0.8 μM whereas it was 0.05 μM using Dox-NS.

Time kinetics of cell proliferation inhibition

The toxicities of free Dox, Dox-NS, NS and NS + Dox were studied after incubation for various periods of time at a given concentration of drug (0.4 μM for Dox-R-MCF7). Twenty four hours after seeding, the cells were incubated for 0.5, 1, 3, 6, 24 or 48 hr. The cytotoxicity of Dox-NS increased with incubation time for up to 6 hr (Fig. 4) and continued

Table 4. Effects of free Dox or Dox-NS on the survival of DBA2 mice bearing P388-Adr-R ascitis (i.p. injection of 10^6 P388-Adr-R cells at day 0)

	50% survival time (days)			
Groups of 10 mice	Non-treated (*C*)	Dox (*T*)	Dox-NS (*T*)	NS
1	11	14	21	
2	14	15	21	14
3	11	11	17	
4	11	14	18	
Mean	11.7	13.5	20.2	
T/C		115%	164%	
P (*T* vs *C*)		ns	0.003	

Dox or Dox-NS was given daily by i.p. route at days 1–4 at a dose of 2 mg/kg body weight/day.

P is the probability that *T* is statistically different from *C*, using the Student's *t*-test.

ns, not significant.

to increase slightly after this delay. Free Dox, NS and NS + Dox were not toxic to Dox-R-MCF7 cells, whatever the incubation time.

In vivo *efficacy of Dox-NS*

Table 4 shows the survival of mice bearing a P388-Adr-R tumoral ascitis. At day 0, DBA2 mice received an intraperitoneal injection of 10^6 cells. This leukemia killed the recipient mice in 11–14 days (11.7 days, mean). Mice (10 per group) were treated with an i.p. injection of 2 mg/kg body weight of free Dox for 4 consecutive days (day 1–4; the total amount of Dox being 8 mg/kg). A short but constant prolongation of survival was obtained (mean survival 13.5 days; this is not statistically significant from the survival of untreated mice). Treatment with Dox-NS significantly improved the Dox efficacy. The *T/C* ratios (50% survival time of treated mice divided by that of control mice) varied between 154 and 190% (mean $T/C = 164\%$, $P = 0.003$). Treatment with NS did not modify the survival time of the mice. These preliminary results show that a significant improvement in Dox efficacy toward resistant tumors may be obtained by its linkage with nanospheres.

Intranuclear and intracellular concentration of Dox studied by LMSF

We initially studied the spectrum of Dox when it was loaded into nanospheres (Dox-NS) and suspended in PBS. The spectrum of Dox-NS appeared to be different from that of free Dox (Fig. 5).

The intranuclear uptake of Dox was determined after incubation of cells with free Dox or with Dox-NS at identical concentrations and for various incubation times. MCF7 cells were preincubated in culture medium containing 166 nM of Dox or Dox-NS culture medium. Dox-R-MCF7 cells were incubated in culture medium containing 995 nM of Dox or Dox-NS. Cells were studied for Dox content 1 hr after the addition of drugs to the medium. The intranuclear spectrum of Dox appeared to be

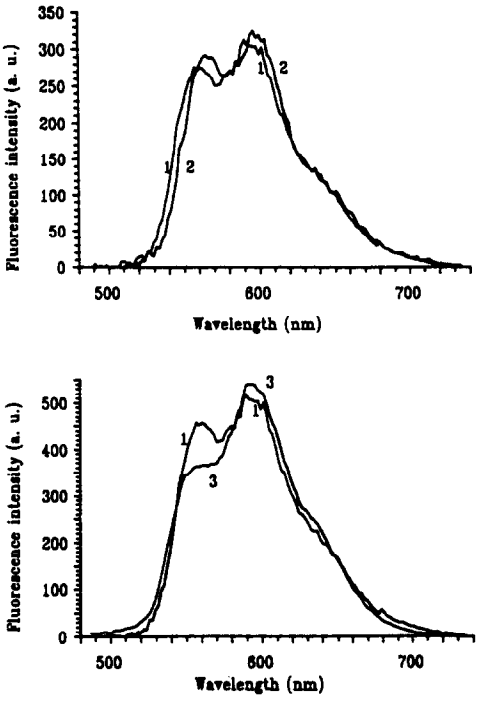

Fig. 5. Fluorescence emission spectra of (1) free Dox in PBS, (2) DNA-bound Dox in PBS and (3) emission spectra from nanospheres coupled with Dox. Spectra (1) and (2), and (1) and (3) are normalized in integrated surfaces.

identical for sensitive and resistant cells and no difference in the Dox spectrum was observed, whatever the form of Dox used (i.e. whether loaded or not). This observation proves that Dox reaches the nuclear compartment as a free compound, even when it enters the cells loaded into nanospheres. The fraction of intranuclear Dox bound to DNA was almost 99% in both cases.

In MCF7 cells, the intranuclear uptake of Dox was quite similar, whatever the form of Dox used (free Dox or Dox-NS), and increased as a function of the incubation time (from 30 min to 6 hr, Fig. 5). Detectable amounts of Dox were also measured in resistant cells incubated with free Dox. Intracellular Dox levels increased with the incubation time. In Dox-R-MCF7 cells, incubated with Dox-NS, the intranuclear uptake of Dox was 3–5 times lower, compared with the same cells incubated with free Dox (after six hr of incubation). In fact, with Dox-NS no significant increase in intranuclear Dox concentration was observed between 30 min and 6 hr of incubation time, while a clear increase was observed in the sensitive cells as a function of the incubation time. The intranuclear uptake of Dox, free or loaded, was also studied after prolonged incubation times (24 and 48 hr for resistant cells, and 24 hr for sensitive cells) and at an identical concentration of 0.4 μM (data not shown). As expected, the concentration of Dox in resistant cells incubated with free Dox was lower than in sensitive cells. At a free Dox concentration of 12 μM (ID_{50}),

Table 5. Intranuclear concentrations of Dox after incubation with Dox or Dox-NS, at their respective ID_{50} values, in the MCF7 cell line and in its resistant variant, Dox-R-MCF7

	Intranuclear concentrations of Dox (μM)		
	Dox-R-MCF7 incubation time		MCF7 incubation time
	24 hr	48 hr	24 hr
Dox-NS	8.0 ± 3	20.0 ± 7	38 ± 11
Dox	12.2 ± 5	21.1 ± 7	33 ± 9

Free Dox was at 12 μM for Dox-R-MCF7 and 0.4 μM for MCF7; Dox-NS was at 0.4 μM for both cell lines. Detection was by LMSF.

12.2 μM of Dox were detected in the nuclei of resistant cells after 24 hr (compared with 33 μM in sensitive cells); this rose to 21 μM after 48 hr (Table 5). A significant uptake of Dox was detected when cells were incubated with Dox-NS, with uptake being lower in resistant cells than in sensitive cells (8 μM vs 38 μM, respectively, after 24 hr incubation).

DISCUSSION

Multidrug resistance is the main cause of chemotherapy failure in cancer. It is often associated with the overexpression of a cell membrane glycoprotein of 170 kD molecular mass [7, 8]. This glycoprotein, designated P-glycoprotein, could act as an efflux pump and reject numerous drugs from the cells as shown for bacterial transport proteins [27]. Indeed, multidrug resistance is associated with a low intracellular accumulation of some drugs.

Our interest focused on drug carriers as agents capable of circumventing multidrug resistance. Contradictory results have been obtained using drug-loaded liposomes [5, 28–30], probably because these phospholipidic vesicles are of poor stability, especially in medium containing serum. Thus, polymeric polyisohexylcyanoacrylate nanoparticles of small size (200 nm) and biocompatibility, which are more stable although biodegradable, were considered as good candidates for targeting Dox with a view to circumventing multidrug resistance. Nanospheres enter the cells by endocytosis [15] and deliver embedded Dox to the lysosomes. Thus, Dox release can take place close to the nuclear membrane and Dox is therefore likely to bind immediately to DNA, as observed in our series of experiments using the LSMF technique.

We demonstrate here that Dox-NS circumvents the resistance of tumor cells selected in two different ways: either by drug exposure, or by stable *mdr*1 transfection. On a panel of multidrug-resistant cells, the ID_{50} of Dox-NS was found to be close to that obtained with free Dox on sensitive parental cells. In parallel, we have shown that unloaded NS and NS + Dox were not toxic to resistant cells. Cytotoxicity of Dox-NS on sensitive cells was usually equivalent to that of free Dox. The cell line LR73 was an exception: Dox-NS were more toxic than the same Dox concentration of free Dox, and these cells did not express any detectable level of P-glycoprotein.

Dox-NS, injected by i.p. route *in vivo*, allowed a significant prolongation of the survival time of mice bearing P388-Adr-R-resistant leukemia cells grafted as an ascitis, while free Dox had a very poor efficacy. Despite these positive results, no cure of the mice treated with Dox-NS was obtained, while *in vitro* a very strong inhibition of cell proliferation was observed. This difference between the *in vivo* and *in vitro* results could be due to several factors, the main one probably being related to the rapid clearance of Dox-NS *in vivo* by physiological processes. These include active peritoneal macrophages, capable of capturing Dox-NS. *In vitro*, quite stable concentrations of drugs are maintained in the culture medium. *In vitro* kinetics experiments have shown that a minimal 3-hr contact was necessary in order to obtain a detectable effect of Dox-NS. Therefore, it is likely that prolonged and repeated infusions will be necessary to improve the Dox-NS efficacy *in vivo*. Different schedules of administration are being tested to investigate this point. Nevertheless, these *in vivo* assays demonstrate that the inefficiency of Dox for treating resistant tumors could be significantly improved by nanosphere loading.

How do resistant cells circumvent their multidrug resistance when treated with Dox-NS? P-glycoprotein acts as a pump excluding toxic compounds, such as Dox, from the resistant cells. There is evidence to suggest that: (i) multidrug-resistance is related to a decreased drug accumulation in the resistant cells [2, 4], (ii) P-glycoprotein is overexpressed and sometimes the corresponding *mdr*1 gene is amplified in resistant cells [5], (iii) inhibition of P-glycoprotein function, by inhibitors of calcium channels, is accompanied by a decreased resistance [6] and (iv) transfection of sensitive cells with *mdr*1 confers multidrug resistance [21]. The simplest model for explaining this assembly of findings is that P-glycoprotein directly excludes drugs from cells. This implies drug recognition by the P-glycoprotein. Our results suggest that Dox-NS are not recognized as a drug by P-glycoprotein, perhaps due to the molecular structure or the ionic charge of the complex.

Detection of Dox by LMSF was extremely useful showing three different spectra of Dox, depending on whether it was bound to DNA or to the nanospheres, or free. LMSF is a non-destructive technique which allows the study of the Dox content in a single living cell compartment, namely the nucleus, and also assists in the definition of molecular interactions between Dox and DNA [25]. LMSF studies of resistant cells, treated with Dox-NS, showed that Dox was bound to nanospheres in the cytoplasm. However, even when incubated in the form of Dox-NS, the drug bound to its DNA target in its free form as efficiently as after the treatment of sensitive cells with free Dox.

In testing sensitive MCF7 cells at ID_{50}, we showed that intracellular and intranuclear Dox concentrations were identical, whatever the form of Dox used. In both cases, 99% of intranuclear Dox was bound to DNA. Kinetics of Dox intranuclear

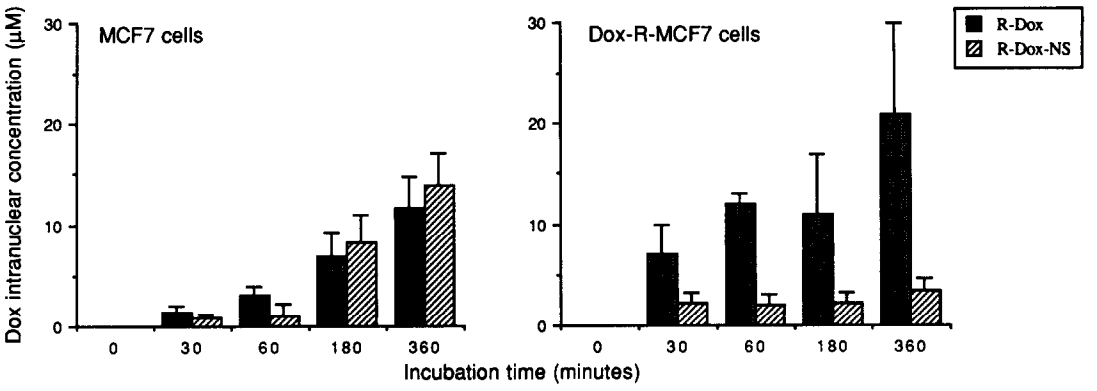

Fig. 6. Intranuclear concentrations of Dox (μM) measured by LMSF in MCF7 and Dox-R-MCF7 cells incubated with free Dox (black bars), or with Dox-NS (striated bars). The means of four measurements ± SD are shown. The concentration of Dox in the culture medium was 0.1 μM for MCF7 and 0.6 μM for Dox-R-MCF7 cells.

uptake were identical when Dox was given as free Dox or as Dox-NS (Fig. 6) and the intranuclear concentrations of drug were identical at 24 hr for both formulations. These results suggest that the mechanisms of action of free and carried Dox are identical in sensitive cells. Surprisingly, endocytosis of Dox-NS did not delay Dox uptake in the nucleus, whereas it delayed the cytotoxicity of Dox-NS. It is noteworthy that similar kinetics of cytotoxicity were observed when sensitive cells were incubated with Dox-NS, indicating that the delay of 3–6 hr, necessary to obtain a significant effect, was dependent on the form of Dox used and not on the sensitivity or resistance of the cells.

In the sensitive cells, an increase in Dox accumulation was observed whatever the form of Dox used. However, incubation for 1 hr was enough to kill a significant number of cells, while 3–6 hr incubation time was necessary to obtain the same efficiency with Dox-NS. This observation suggests that the amount of Dox detected at a given time does not predict toxicity and that toxicity results from several parameters, including the delay in Dox–DNA interaction. Furthermore, no spontaneous release of Dox from Dox-NS in the culture medium has been observed.

Dox-NS inhibited identically the proliferation of MCF7 cells as well as that of Dox-R-MCF7 cells. However, the intranuclear content of Dox was significantly lower in resistant cells than in sensitive cells during the first 3–6 hr of incubation. This difference was decreased after 24 hr of incubation. This delay is reflected by the survival curves and also by the prolongation of incubation time acquired by Dox-R-MCF7 cells with Dox-NS, and leads to a reinforcement of the inhibition of cell proliferation which was not observed with sensitive cells (results not shown). In addition, a plateau of toxicity at the highest doses of Dox-NS was observed in resistant cells. This could be due either to a residual population of cells resistant to Dox-NS, because of a weak endocytosis, or to a saturation level of the endocytosis process.

A low capacity of endocytosis in resistant cells could explain the delay in Dox incorporation, compared with the incorporation kinetics in sensitive cells. Fluorescence observations (Dox is spontaneously fluorescent) did not show any particular accumulation of Dox-NS at the cell membrane or in the cytoplasm of resistant cells. The main point is that reversion of resistance was quite complete with Dox-NS. As soon as an identical intranuclear threshold of Dox was reached a 50% growth inhibition was obtained with similar doses of Dox-NS in incubation medium. This was about 20 μM for both cell lines. This excludes Dox prenuclear mechanisms of action, which have been suggested by some authors [27].

P-glycoprotein drug exclusion is not the unique mechanism of multidrug resistance. Multifactorial resistance has been illustrated by the studies of Schott Robert [31] and Deffie *et al.* [32]. Indeed, forms of resistance dependent on either cellular or on cytoplasmic targets [1, 4, 5] have been described. Resistance could also be ascribed, although not exclusively, to an accelerated repair of DNA lesion [32] with an altered efficiency ot topoisomerase II [1, 33], to an accelerated efflux of Dox from the nucleus, to increasing free radical detoxification or to protection against these radicals [34]. Therefore, it is exciting to observe that the resistance of some cells, such as Dox-R-MCF7 [34] and P388-Adr-R [32], known to be multifactorial, can be circumvented by loading the drug into nanospheres, just as that of the purely multidrug-resistant cells LR73MDR which have been transfected with the *mdr*1 cDNA. These results suggest that, whatever the complexity of resistance mechanisms (as observed in the clinical situation), drug loading might be a means of improving the therapeutic index of drugs. In addition, Dox-NS was found to be much less toxic, namely to cardiac tissue, than free Dox [11, 12].

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