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Enhanced Cytotoxicity of Doxorubicin Encapsulated in Polyisohexylcyanoacrylate Nanospheres Against Multidrug-resistant Tumour Cells in Culture

Sanae Bennis, Christiane Chapey, Patrick Couvreur and Jacques Robert

We have studied the cytotoxicity and accumulation of doxorubicin encapsulated in polyisohexylcyanoacrylate nanospheres in a model of doxorubicin-resistant rat glioblastoma variants differing by their degree of resistance to this drug. We observed that the particulate form of doxorubicin was always more cytotoxic than free doxorubicin, whereas coadministration of drug-unloaded nanospheres with free doxorubicin did not modify significantly doxorubicin cytotoxicity. In C6 0.001 cells, which were 6-fold resistant and present a pure multidrug-resistant phenotype, the reversal of doxorubicin resistance was complete. In C6 0.1 cells, which were 60-fold resistant, as with C6 1V cells (selected with vincristine), the reversal of doxorubicin resistance was almost complete, with a residual resistance factor of 2–3. In C6 0.5 cells, which were 600-fold resistant to doxorubicin, the reversal of resistance was only partial and, in all cases, not above the expected participation of P-glycoprotein to the phenotype of resistance. Intracellular drug accumulation after 2-h exposure to 17.2 μmol/l doxorubicin was systematically reduced by a factor of 2–3 when doxorubicin was incubated under the form of nanospheres; doxorubicin accumulation after a 2-h exposure to IC_{50} was also highly reduced in all cell lines for doxorubicin-loaded nanospheres. This work shows that association of doxorubicin with nanoparticles could provide a useful tool for circumventing multidrug resistance, probably by a bypass of P-glycoprotein rather than by an inhibition of this pump.
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INTRODUCTION

ANTICANCER DRUGS, despite the important progress they have brought in the treatment of cancer, still remain of limited efficacy in numerous solid tumour types, especially because cancer cells can develop mechanisms of resistance allowing them to evade chemotherapy. Multidrug resistance (MDR) is probably the best known of all, since its molecular and genetic support has been identified [1, 2] and because its clinical relevance has been proved [3]. MDR is due to the overexpression of a plasma membrane high molecular weight glycoprotein, P-glycoprotein (Pgp), which is able to extrude various xenobiotics, including some anticancer drugs, out of the cells.

Anthracyclines, vinca alkaloids, epipodophyllotoxins, acridines and taxol are the best known of the drugs against which cancer cells can resist through Pgp overexpression. Cells can also develop more specific mechanisms allowing them to escape the action of these drugs, and these mechanisms can be added to the MDR mechanism, rendering the resistance characteristics "multifactorial" in several *in vitro* models [4]. Despite the fact that the mechanism by which Pgp extrudes drugs out of the cells has not been fully elucidated, the inhibition of its function is the subject of intensive research aiming at the circumvention of MDR. Since the original discovery of the MDR reversing properties of verapamil [5], numerous pharmacological agents have been identified as potential modulators of anticancer drug action through Pgp inhibition (see for review [6]). Some of them have entered clinical trials and are currently being evaluated in association with doxorubicin, etoposide or vincristine for the reversal of MDR. Other means of Pgp inhibition can be found by the use of neutralising antibodies such as MRK 16 [7] or UIC 2 [8] which can be more specific and, therefore, less toxic than the low molecular weight inhibitors. A completely different approach for circumvention of Pgp action is the encapsulation of drugs in liposomes or nanospheres; only a few studies have been undertaken with encapsulated drugs. The rationale for the development of drug carriers results from the fact that Pgp probably recognises the drugs to be effluxed while they are present in the plasma membrane, and not when they are dissolved in the cytoplasm: there is increasing evidence that Pgp acts as a phase separator rather than as a transmembrane transporter [9]. Therefore, delivering drugs directly to the cytoplasm or to the lysosomes, after endocytosis of "drug quanta" which cannot be recognised as such by Pgp, could prove an efficient means of circumventing MDR.

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The general aim of drug carrier development has been to protect the drug against degradation, to slow down drug release in the blood stream, rather than to circumvent resistance. However, some recent *in vitro* data have suggested the validity of this approach [10, 11]. We have tested in this study the circumvention of doxorubicin resistance by the use of doxorubicin encapsulated in polyisohexylcyanoacrylate (PIHCA) nanospheres [12]. These polymeric colloidal carriers are biodegradable and when loaded with doxorubicin, they were found to dramatically decrease the overall toxicity of that drug *in vivo* [13]. This paper shows that nanospheres can selectively enhance doxorubicin cytotoxicity in a model of doxorubicin-resistant C6 rat glioblastoma lines differing by their degree and mechanism of resistance [14].

MATERIALS AND METHODS

Cell culture

Rat glioblastoma cells (clone C6) originated from a *N*-nitrosomethylurea-induced tumour obtained by Benda *et al.* [15] and subsequently grown in culture. Several doxorubicin- and vincristine-resistant lines were obtained by stepwise exposure to progressively increasing concentrations of the drug [14, 16]. Three different sublines resistant to doxorubicin were obtained: C6 0.001, C6 0.1 and C6 0.5; they routinely grow with, respectively, 0.001, 0.1 and 0.5 μg of doxorubicin per ml culture medium and are 6-, 60- and 600-fold resistant to doxorubicin. A vincristine-resistant line was also obtained, C6 1V, which grows with 1 μg/ml vincristine. The pharmacological characteristics of the lines have already been presented [14, 17]. Briefly, the C6 0.001 line presents a pure MDR phenotype, with a resistance only due to Pgp overexpression. On the contrary, C6 0.5 line has developed a supplementary mechanism of doxorubicin resistance, presently under investigation, which is added to the MDR mechanism. The C6 0.1 and C6 1V lines have a predominantly MDR mechanism, but also certain features being the consequence of some doxorubicin- or vincristine-specific mechanisms of resistance.

Cells were routinely grown in Dulbecco's modified minimum Eagle's medium supplemented with 10% fetal calf serum and an antibiotic cocktail, all supplied by Seromed (Berlin, Germany). They grew at 37°C in 64 cm^2 Petri dishes from Nunc (Copenhagen, Denmark) in a humidified atmosphere containing 5% CO_2. The cell layers were replicated each week and the medium was changed each 2 or 3 days.

Doxorubicin nanospheres

Doxorubicin hydrochloride (dox) was obtained from Laboratoire Roger Bellon (Neuilly, France), and isohexylcyanoacrylate (IHCA) was obtained from Sopar (Bruxelles, Belgium). Doxorubicin-loaded nanospheres (dox-NS) were prepared by emulsion polymerisation of IHCA in the presence of doxorubicin in well-defined conditions, as already described [18]. The nanospheres obtained were 200–300 nm in diameter. The weight ratio polymer/doxorubicin was about 12 in these conditions. Despite the fact that the mechanism of internalisation has never been tested with the C6 lines, the nanospheres are expected to be degraded by intracellular esterases, then releasing the drug embedded in the polymeric network [19]. Doxorubicin-loaded and unloaded nanospheres were lyophilised and reconstituted with phosphate-buffered saline just before use, and diluted in culture medium at the doxorubicin concentration required for growth inhibition curves. Unloaded nanospheres were used as controls at the same concentration as loaded nanospheres. A review of the preparation and physico-chemical properties of PIHCA nanospheres has been published elsewhere [12]. A recent account on the human pharmacokinetics of dox-NS was recently published [20].

Cytotoxicity evaluation

A colorimetric assay using the tetrazolium salt, MTT [21], was used to assess cytotoxicity of doxorubicin, either free or encapsulated in nanospheres. The conditions of the assay were chosen so as to allow exponential growth throughout the evaluation period, and the assay gave with doxorubicin similar IC_{50} as a clonogenic assay (Huet and Robert, unpublished results). Cells were seeded as follows in 96-well plates in a volume of 200 μl : 500 cells per well for C6 S, 800 cells for C6 0.001, 1500 cells for C6 0.1 and C6 1V, 2000 cells for C6 0.5. The plates were incubated in the routine culture conditions for 1 day for C6 S and C6 0.001, and 2 days for C6 0.1, C6 1V and C6 0.5. Culture medium was then replaced by fresh medium containing doxorubicin at appropriate concentrations, either in free form or encapsulated in nanospheres. Controls containing unloaded nanospheres at the same amount as in the tests were always run simultaneously. Incubations were performed for 2, 6 and 24 h in three independent sets of experiments. After drug exposure, monolayers were washed twice with fresh culture medium and the cultures allowed to grow further for 4 days (C6 S and C6 0.001 cells) or 5 days (C6 0.1, C6 1V and C6 0.5 cells). At this time, 300 μl of medium containing 0.5 mg/ml MTT were added in each well and the plates incubated at 37°C for 4 h. Medium was then removed and 200 μl of DMSO were added to dissolve the formazan crystals. Absorbance was immediately determined on a two-wavelength Microplate Auto Reader (Biotek Instruments, EL 311) at test and reference wavelengths of 570 and 630 nm, respectively. Blank controls without cells were substracted from sample absorbance values. Triplicate determinations gave a coefficient of variation of less than 10%. Cytotoxicity was expressed as IC_{50}, the concentration giving a 50% decrease of absorbance as compared to controls incubated simultaneously without doxorubicin.

Doxorubicin accumulation

For these determinations, 80 000 C6 S or C6 0.001 cells were seeded in 10 cm^2 Petri dishes with 3 ml medium, or 150 000 C6 0.1 or C6 1V cells, or 500 000 C6 0.5 cells were seeded in 20 cm^2 Petri dishes with 5 ml drug-free medium. The medium was changed 3 days later; on the fourth day, the number of cells was approximately 2 000 000 cells per dish in all cases. Drug accumulation was then evaluated after 2-h exposures to various drug concentrations. The cell layers were rinsed, harvested by gentle stirring and pelleted rapidly at 4°C; 0.5 ml water and 0.5 ml 40% trichloroacetic acid were successively added and the samples kept at 4°C overnight to ensure protein precipitation. The acid-soluble fraction was used to evaluate doxorubicin concentration by fluorometry using a SFM25 Kontron spectrofluorometer with excitation and emission wavelengths set at 480 and 592 nm, respectively. Proteins were assayed in the acid-insoluble pellet by the technique of Lowry *et al.* [22].

RESULTS

The antiproliferative activity of free doxorubicin (dox) and doxorubicin entrapped into nanospheres (dox-NS) has been studied in the C6 variants after a 2-h exposure to the drug. It has been found that dox-NS was, in all cases, more cytotoxic than dox when incubated with the cells on an equimolar basis,

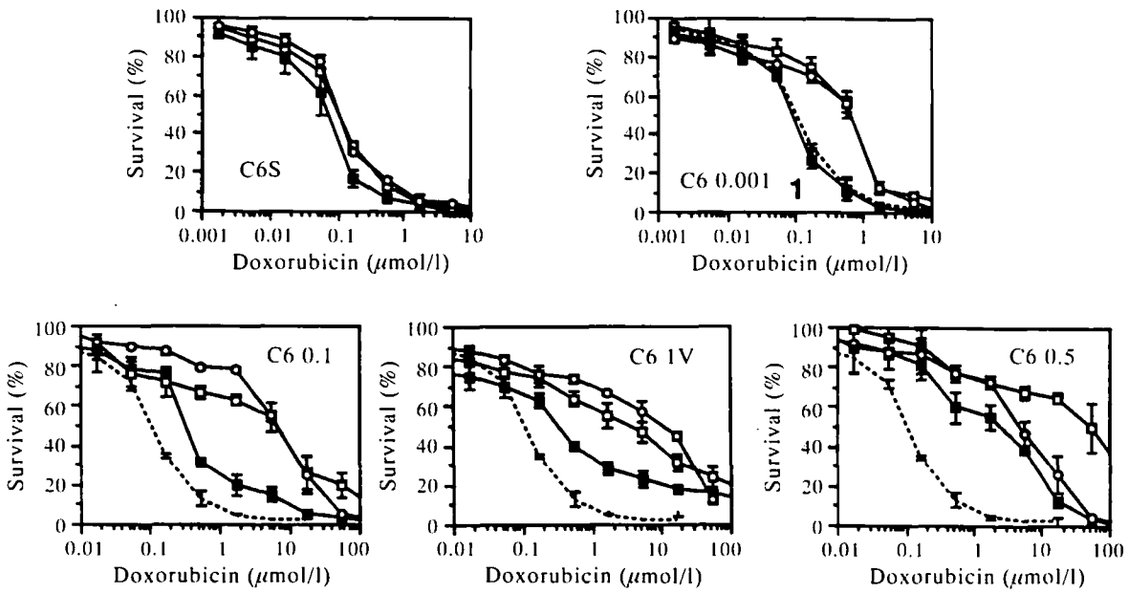

Fig. 1. Growth inhibition curves of the five cell lines after a 2-h incubation with free dox (-□-), dox-NS (-■-) or free dox + NS (-○-). In the charts concerning the resistant lines, dox cytotoxicity measured in sensitive cells is plotted in dotted lines for comparison.

whereas coadministration of drug-unloaded nanospheres with dox (dox + NS) did not modify significantly the cytotoxicity of dox. Figure 1 shows the growth inhibition curves obtained in the five cell lines after a 2-h exposure to dox, dox-NS and dox + NS. Table 1 presents the IC_{50} observed in the five cell lines, as well as the degree of sensitisation (reversal factor) to doxorubicin which was obtained through the association of the drug with nanospheres. In C6-sensitive cells, dox-NS provided a 30% enhancement of dox cytotoxicity; in C6 0.001 cells, doxorubicin resistance was completely overcome by the use of dox-NS; in C6 0.1 and C6 1V cells, the circumvention of doxorubicin resistance was almost complete, the IC_{50} of dox-NS in these lines being two to three times higher than the IC_{50} of dox in the corresponding sensitive line. In contrast, resistance of C6 0.5 cells was never completely overcome by dox-NS; the reversal of resistance was about 20-fold, but a 30-fold resistance factor remained with this cell line. The benefit of using dox-NS was maintained when drug exposures were increased to 6 h and 24 h. Increasing exposure times from 2 to 6 and 24 h allowed, respectively, a 2-fold and a 4-fold reduction of the IC_{50} of both dox and dox-NS in all cell lines, except for the C6 0.5 line in which dox IC_{50} reduction was higher (8–10 fold). Figure 2 presents typical results (C6 0.1 cells) obtained when varying exposure time.

Table 1. Comparative cytotoxicity of free and encapsulated doxorubicin

Cell line	IC_{50}* Free dox	IC_{50}* dox-NS	Reversal factor†	Residual resistance factor‡
C6	0.102 ± 0.003	0.077 ± 0.009	1.3	< 1
C6 0.001	0.60 ± 0.09	0.091 ± 0.005	6.7	< 1
C6 0.1	6.02 ± 1.00	0.29 ± 0.03	21	2.8
C6 0.5	56.1 ± 8.6	2.75 ± 0.49	20	27.0
C6 1V	3.27 ± 0.17	0.34 ± 0.07	9.6	3.3

* IC_{50} were determined after 2-h exposure to various concentrations of the drug. Means ± S.D. of three to five independent experiments performed in triplicate are expressed in μmol/l. † Reversal factor was estimated by dividing the IC_{50} obtained with free dox by the IC_{50} obtained with dox-NS. ‡ Residual resistance factor was calculated by dividing the IC_{50} obtained with dox-NS by the IC_{50} obtained with free dox in the C6 sensitive line.

On the other hand, there was a clear cytotoxicity of drug-unloaded nanospheres themselves when used alone at high polymer concentrations; this cytotoxicity was more pronounced for the C6 sensitive cells than for the resistant cells. The cytotoxic equivalent concentrations of NS were, however, far above the cytotoxic concentration of dox-NS, except in the C6 0.5 line, for which they were of the same magnitude, suggesting that the cytotoxicity evaluated in this line with dox-NS could be due to the single additional effect of both doxorubicin and nanospheres. In all other lines, cell survival in the presence of drug-unloaded nanospheres was > 90% at the IC_{50} values of dox-NS.

The intracellular accumulation of doxorubicin after 2-h exposures to dox or dox-NS has been estimated. Two concentrations of doxorubicin were tested: 17.2 μmol/l or a concentration corresponding to the IC_{50} for each cell line and for each tested formulation. At the concentration of 17.2 μmol/l, accumulation of dox was reduced in the resistant sublines depending on their degree of resistance; interestingly, there was a 2–3-fold reduction of dose accumulation when doxorubicin was presented to the cells associated with nanospheres (dox-NS). It was noteworthy, when considering the intracellular IC_{50} (i.e. the intracellular accumulation of doxorubicin measured after a 2-h exposure at IC_{50}), that C6 0.1, C6 1V and C6 0.5 cells could tolerate high intracellular concentrations of doxorubicin after incubation with free dox, but that this tolerance was abolished or highly reduced after incubation with dox-NS. In other words, the same intracellular amount of drug was responsible for the same cytotoxicity only when the drug was presented to the cell as nanospheres, but not when it was presented as a solution.

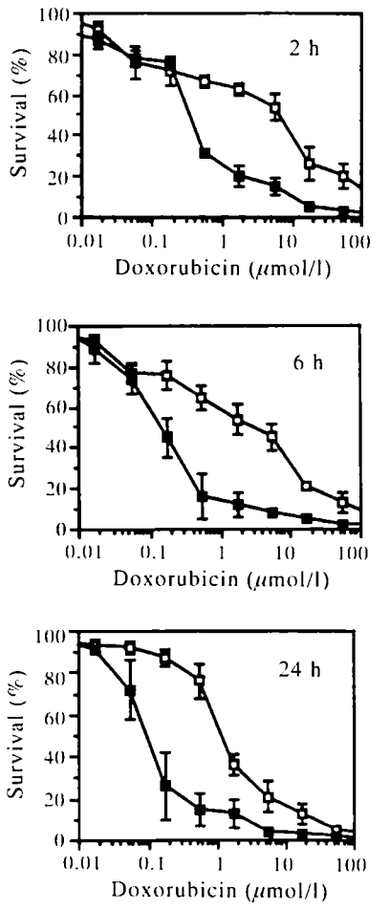

Fig. 2. Growth inhibition curves of the C6 0.1 line after 2-h, 6-h and 24-h incubations with free dox (–□– or dox-NS (–■–).

DISCUSSION

The data show that a circumvention of multidrug resistance may be obtained by the use of doxorubicin entrapped into PIHCA nanospheres. The overproduction of Pgp is the key mechanism of multidrug resistance, despite the fact that other mechanisms can be involved in the MDR phenotype, such as alterations of topoisomerase II or drug detoxification [4]. We have used several cell lines originating from the same strain and differing by their degree of resistance to doxorubicin or by the nature of the selecting agent. We had shown earlier that the C6 0.001 line displayed a pure MDR phenotype, whose characteristics could be explained by the sole overproduction of Pgp, whereas the other lines, especially the C6 0.5 line, presented additional mechanisms of resistance to doxorubicin [14]. Since the purely MDR cell line C6 0.001 was as sensitive to dox-NS as the wild-type C6 cell line, the circumvention of resistance by dox-NS must be attributed to a bypass of Pgp by the carrier, as was suggested by a previous study [11]. In the C6 0.5 line it was, however, not at all possible to restore doxorubicin sensitivity above the estimated participation of MDR to this resistance. It is worth comparing these results with those obtained with verapamil on the same lines [14]: this classical inhibitor of Pgp-mediated drug efflux was also able to completely restore doxorubicin sensitivity in the C6 0.001 line, but gave a residual resistance factor of 20 in the C6 0.5 line.

Table 2. Comparative intracellular accumulation of doxorubicin after incubation with dox or dox-NS

Cell line	Accumulation after a 2-h, 17.2 μmol/l exposure* Free dox	dox-NS	Accumulation after a 2-h, IC_{50} exposure* Free dox	dox-NS
C6	1550 ± 230	430 ± 80	27 ± 2	4.1 ± 0.7
C6 0.001	1000	250	40	2.7
C6 0.1	240	94	130	6.4
C6 0.5	220 ± 65	110 ± 30	500 ± 100	35.0 ± 3.0
C6 1V	200 ± 25	95 ± 15	92 ± 4	5.8 ± 0.6

* Accumulations are expressed in pmoles doxorubicin per million cells. Results are the means ± S.D. of one or two experiments performed in triplicate.

There has been recently some interest in the use of liposomes as carriers of doxorubicin for the circumvention of MDR. Liposomal doxorubicin has been shown to be as active on MDR cells as on sensitive cells [10]. This would suggest that the same general mechanism could explain the observations obtained with PIHCA nanospheres and with liposomes. However, it was shown that liposomes alone could block Pgp function, and were capable of exerting their effect whether the drug was encapsulated or not [23, 24]. This is quite different from what we have observed: doxorubicin nanospheres could only reverse MDR resistance if the drug was firmly associated with the polymer. This suggests that liposomes may act directly by inhibiting Pgp, allowing then a higher accumulation of doxorubicin, whereas nanospheres would simply prevent dox from being identified and effluxed by Pgp. The hypothesis recently developed by Gottesman [9], implying that Pgp removes drugs directly from the lipid bilayer, and not from the cytoplasm, may explain our observations on PIHCA-carried doxorubicin: the drug entering the cell under a particulate form would not be dissolved in the lipid bilayer, rendering then the multidrug transporter ineffective. Thus, escaping Pgp would allow the circumvention of MDR without any direct inhibition of the transporter by the nanospheres. This explanation was corroborated by the fact that doxorubicin intracellular accumulation was increased when the drug was associated with liposomes [10], whereas it was decreased when the drug was embedded into PIHCA nanospheres. This decrease of drug accumulation with nanospheres could be explained by a reduced uptake of the dox-NS. The mechanism by which dox-NS were more efficient than dox, even for lower intracellular concentrations, is still unclear. It is believed that the efficacy of the targeted formulation may result from a possible modification of the intracellular distribution of the drug. Whatever this mechanism is, the present data strongly suggest that under the form of nanospheres, doxorubicin is probably not recognised by Pgp as a normal drug. Finally, the key finding of this study is that the reversal of doxorubicin resistance by nanospheres was closely dependent on the nature of the resistance: nanospheres were only efficient on pure MDR phenotype cells and not on the additional mechanism of resistance to doxorubicin.

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Simple Cytokeratins in the Serum of Patients with Lung Cancer: Relationship to Cell Death

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An important role in differentiation and proliferation has been demonstrated for the 20 cytokeratin (CK) polypeptides. The serum of 24 patients with biopsy-proven non-small cell lung cancer (NSCLC) and a similar number of controls was examined for evidence of CK8 and CK18. Using enzyme-linked immunosorbent assay (ELISA), all the control sera were negative, but 9 of the 24 patients were positive (mean 2.62 ng/ml; range 1.4–5.8; $P = 0.0036$). Western blotting confirmed the results of the ELISA in all cases, and indicated full size CK polypeptides. Advanced stage disease patients were more likely to be seropositive ($P = 0.00024$). Biopsy specimens showed CK8 expression in all 24 cases by immunochemistry and CK18 in 22 cases. This is the first study to demonstrate that a subgroup of NSCLC patients have intact CK8 and CK18 peptides in their serum, and their detection may correlate with advanced disease.
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INTRODUCTION

CYTOKERATIN (CK) POLYPEPTIDE-CONTAINING intermediate filaments are the structural unit of epithelial cells, and are found in the malignant tumours derived from them [1]. There are a total of 20 CK polypeptides, sub-divided into two equal families based on a combination of biochemical and molecular biologicial properties—the type I (acidic) and type II (basic) CK—and CK intermediate filaments are made up of equimolar quantities of each family [2–4].

The simplest pattern of CK intermediate filament expression