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MULTIDRUG RESISTANCE BYPASS IN CELLS EXPOSED TO DOXORUBICIN-LOADED NANOSPHERES

ABSENCE OF ENDOCYTOSIS

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Abstract—Multidrug resistance bypass has been achieved *in vitro* with polyalkylcyanoacrylate nanospheres loaded with doxorubicin as previously shown by Cuvier *et al.* Fluorescence-imaging experiments were performed to determine if the endocytosis of the particles by the cells could be responsible for this activity. The results obtained with three lines of sensitive and resistant cells (K562, MCF7, and C6) demonstrate that the particles were not internalized by the cells, nor were they adsorbed onto the cell surface. In contrast, these nanospheres were very efficiently endocytosed by phagocytic cells such as macrophages. No correlation was observed between the fate of the particles, endocytosed or not, and their cytotoxic activity. It was concluded that no endocytosis step was involved in the mechanism of the multidrug resistance bypass obtained by associating a drug to polymeric particles.

Key words: nanoparticles; endocytosis; cell lines; macrophage resistance; fluorescence microscopy

Colloidal carriers such as liposomes [1–2] or polymeric nanospheres have been designed as drug carriers with the objective of selective delivery of the active molecule to the intended target and consequent improvement of the therapeutic index [3, 4]. Recently, the encapsulation of DOX into polymeric nanospheres has become the object of renewed interest. Indeed, the drug-loaded particles have been shown to overcome *in vitro* the multidrug resistance (MDR) of several cell lines, all characterized by the overexpression of the 170 kD membrane P-glycoprotein responsible for an accelerated drug efflux out of the cells [5]. Until now, the mechanism involved in this effect has not been elucidated. Earlier results from Couvreur *et al.* [6], obtained with polyacrylamide nanosphere-treated fibroblasts, suggested that these carriers could reach the cell lysosomes. This work has been taken as a basis to promote the hypothesis of nanosphere endocytosis as a determining step in the mechanism responsible for the observed MDR bypass.

We conjectured that the results of Couvreur *et al.* [6] could not necessarily be extrapolated to the cell and nanosphere system used to show the MDR overcome. To clear up this point, we undertook a fluorescence-imaging study of the cellular localization of NS – DOX with three cells lines relevant for the resistance bypass [5, 7]. To complete this study, we also examined what happens to the same polymeric nanospheres administered to peritoneal macrophages maintained in culture, as well as the cytotoxicity induced by the DOX-loaded nanospheres in this cell type well-known for its phagocytic activity.

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§ Abbreviations: DOX, doxorubicin; PIHCA, polyisohexylcyanoacrylate; NS, nanospheres; (NS – DOX), nanospheres loaded with doxorubicin during the polymerization process; (NS + DOX), preformed nanospheres added with doxorubicin.

MATERIAL AND METHODS

Drug and polymeric particles

Doxorubicin hydrochlorate was a gift from Carlo Erba (Milan, Italy). Solutions were prepared just before use and kept out of light. The polymeric NS were provided by Professor P. Couvreur (Centre d'études pharmaceutiques, Châtenay-Malabry, France), and were prepared as previously described [8]. Briefly, the isohexylcyanoacrylate monomer (IHCA) (Sopar, Brussels, Belgium) were added under gentle stirring at room temperature to a solution of 0.5% (w/v) citric acid, 1% dextran 70, and 5% glucose. After 6 hours' polymerization, a homogeneous suspension was obtained. Stirring was completed to 24 hours, and the suspension was then freeze-dried. The same protocol was followed to prepare NS – DOX, except that the drug was added in the polymerization medium. Before use, the NS were resuspended in pure water at a final IHCA concentration equal to 13.3 mg/mL and 1 mg/mL doxorubicin for loaded samples. DOX was also associated with NS by adding DOX to unloaded preformed NS in the same ratio (NS + DOX). The size of particles used was determined with a laser light-scattering apparatus (Nanosizer, Coultronics, France), and found equal to 386 ± 91 nm for unloaded NS and 332 ± 78 nm for loaded samples.

Cells and media

The three studied cell lines (K562, a human erythroleukemia; MCF7, a human breast adenocarcinoma; and C6, a rat glioblastoma) and their resistant variants (K562-R, MCF7-R, and C6 0,001) were provided by Dr J. Robert (Fondation Bergonié, Bordeaux, France). The MDR variants had been selected *in vitro* with increasing concentrations of DOX. K562 and MCF-7 were grown at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf

serum, 2 mM glutamine, and an antibiotic cocktail. The procedure was the same for C6 and C6 0,001, except that the culture medium was DMEM. All the culture products were supplied by Seromed.

Mouse resident peritoneal macrophages were prepared according to ref. [9] from Balb/c male mice (Centre d'élevage Janvier, France). They were collected in 5-mL cold phosphate buffer saline and allowed to adhere to 12-mm diameter coverslips (10^5 cells per coverslips) for 30 minutes. The macrophages were then rinsed with Hank's balanced saline solution, and cultured in wells containing 0.5-mL RPMI 1640 supplemented with 10% fetal calf serum, 25 mM HEPES, and 50 µg/mL gentamycin. Cultures were kept at 37°C in a 5% CO_2 humidified atmosphere.

Macrophage toxicity assay

The macrophages were rinsed with Hank's balanced saline solution and replaced in fresh culture medium. They were exposed to DOX concentrations ranging from 10^{-8} to 10^{-5} M under free, NS – DOX and NS + DOX forms. Controls were performed with unloaded NS at corresponding polymer concentrations. After 6 hours' incubation, the drug-containing medium was substituted for fresh medium and returned to the incubator for 21 additional hours. Cell survival was then scored using trypan blue exclusion test [10].

Fluorescence microscopy

The experiments were carried out using an Olympus Dapo 100 UV (×100), numerical aperture 1.3, oil immersion objective mounted on an Olympus IMT-2 (Tokyo, Japan) inverted microscope equipped for epifluorescence and using a 75W Xenon lamp. Excitation and emission wavelengths were selected using the Rhodamine filter set (Oriel, Les Ulis France). Exposure times were short enough to avoid photobleaching. Video images were obtained with a silicon-intensified converter. To increase the signal/noise ratio, 16 images were averaged after digitalization. Shading due to the camera target was corrected by dividing each image with a mask constituted by an image of a homogeneous doxorubicin solution. The dark current of the camera was substracted.

Prior to the fluorescence microscopy experiments, cells were cultured for one day on 3-cm diameter microscope slides mounted in centered hole petri dishes to allow further observations with the ×100 immersion objective and appropriate conditions for cell survival. This device permitted us to settle 3 ml of culture medium over the cells and to apply the treatments under the microscope, thereby allowing immediate observations of the fluorescence. For prolonged observations out of the controlled atmosphere of the incubator, the cells were added with 20 mM HEPES buffer to avoid significant pH shifts. We also used phenol red depleted medium to decrease the contribution of the medium to the fluorescent background. Cells were also treated in the incubator for delayed observations.

RESULTS

DOX-loaded nanosphere fluorescence

Doxorubicin-loaded NS were suspended in HEPES buffer to a total DOX concentration equal to 10^{-6} M when related to the total volume of the suspension. This corresponded to an NS concentration approximately equal to 10^9 NS/mL. When these NS – DOX suspended in HEPES buffer were allowed to settle over an untreated slide, a fraction of the particles stuck onto the glass surface, permitting the recording of fixed NS images (Fig. 1a). However, the majority of the NS of the sample remained suspended in the buffer unrestrainedly agitated by the Brownian motion, providing enlarged spots and shadowed rings depending on the course of the particles in and out of the focalization plane during the accumulation time of the image (Fig. 1b). Immobilized NS were thus easily distinguishable from free NS. It should be noted that when the NS were suspended in culture medium supplemented with 10% fetal calf serum, no significant adhesion on the slides was detected.

DOX-loaded nanosphere fluorescence in the presence of cells

K562, MCF7, and C6 cells were treated with 300 nM, 50 nM, and 50 nM DOX, respectively. The resistant variants were treated identically. These concentrations were chosen because they had been shown to induce 50% toxicity in sensitive lines when the drug was administered under its free form, and 50% toxicity in sensitive and resistant lines when DOX was administered under its NS – DOX form [5]. It has also been previously shown that the MDR bypass was totally acquired after 6 hours incubation of the cells with NS – DOX. We thus observed the cells treated with free DOX or NS–DOX during the first 6 hours of treatment under the microscope and then 15 hours later. The fluorescence level of the sensitive cells treated with free DOX increased after a 20-minute lag phase to reach a level that did not significantly evolve after two hours' incubation. The distribution of the fluorescence appeared to be mainly cytoplasmic, with a dark area corresponding to the nucleus position. Under these conditions, the fluorescence of the resistant lines remained at the level of the autofluorescence. This can be seen in Fig. 2, where the images recorded with C6 and C6 0,001 cells are represented. The images obtained with this cell line are representative of the situation found with the two other cell lines, a phenomenon observed throughout this study. We also tested ten-fold higher DOX concentrations, and observed that the fluorescence distribution did not differ greatly from sensitive to resistant cells. Only the total fluorescence level was clearly lower in resistant than in sensitive cells.

When the same DOX amount was administered to C6

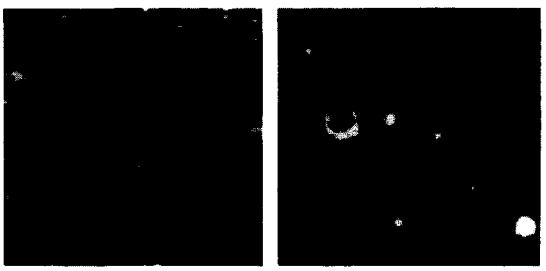

Fig. 1. Polymeric nanospheres loaded with doxorubicin (a) stuck onto the glass slide, and (b) mobile in the medium.

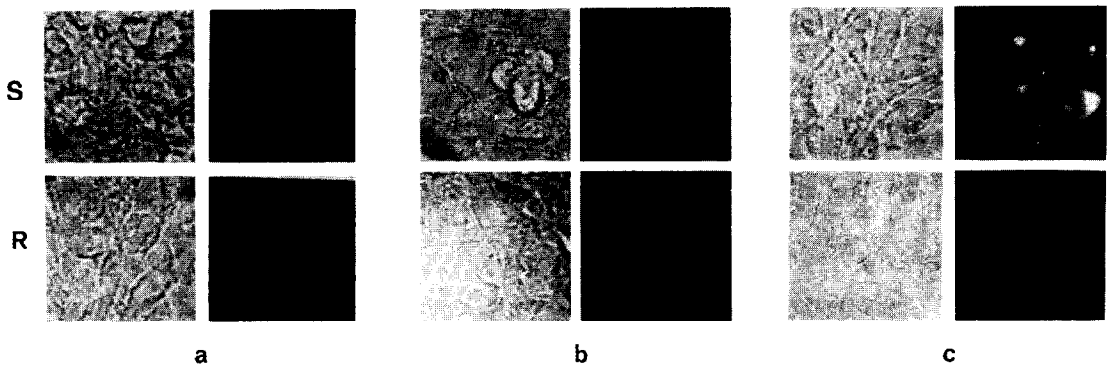

Fig. 2. C6 and C6 0,001 treated with 50 nM free DOX. Phase contrast (left panels) and fluorescence (right panels) images of sensitive cells C6 (top panels) and resistant cells C6 0,001 (bottom panels). Images were acquired just before treatment of the cells (a) (autofluorescence), (b) after 6 hours, and (c) after 15 hours of incubation.

and C6 0,001 under NS – DOX form, no significant cell fluorescence intensity changes were observed during the first six hours; both sensitive and resistant cell fluorescence patterns were found at the level of autofluorescence after 6 hours' incubation (Fig. 3). Most significant was the fact that no NS was detected adsorbed onto the cell surface. All the NS were mobile in the medium, and could be found over the focalization plane of the cells (see Fig. 3). Of all the samples examined (5.10^5 cells treated with 7.10^7 NS – DOX) only some cells with one adsorbed NS on their surface were detected. The frequency of this unusual event was estimated to be 10^{-4}, and considered without statistical meaning. Moreover, the scarce adsorption that we observed never resulted in a migration either onto the cell surface or inside the cell. In contrast, a kind of filament network, slightly fluorescently labelled in the presence of DOX-loaded NS, seemed to have trapped a fraction of the NS – DOX over the plane of the cells. These NS were still mobile, but had a restricted course around a mean position, unlike the other NS, which were entirely free in the medium. After 15 hours, almost all the NS had disappeared from the fluorescence images, and both sensitive and resistant cells were labelled in a quite similar manner to sensitive cells treated during the same time with the same concentration of free DOX (see Fig. 2). Similar experiments were also performed with a ten-fold higher DOX or NS – DOX concentration. The general pattern described above was not significantly modified, except that cell damage was clearly detected with phase contrast objective in sensitive cells, and that more intense fluorescent labelling appeared. No more NS adhesion occurred with the higher concentration, supporting the idea that the few bound NS observed were the result of accident. Neither NS adhesion nor endocytosis was detected for either sensitive or resistant cells with the three cell lines analyzed above.

Nanosphere capture by macrophages

To prove the capacity of our experimental procedure to allow these two events (adhesion and endocytosis) to occur, we applied the same treatment to peritoneal macrophages chosen for their professional phagocitic activity. NS – DOX were added to the peritoneal macrophage preparations under the microscope to reach total DOX concentrations equal to 50 or 500 nM. The treated samples were continuously examined for the first two hours following the addition. Bound or adsorbed NS were detected as early as the first five minutes following addition, with their number increasing during the next 20

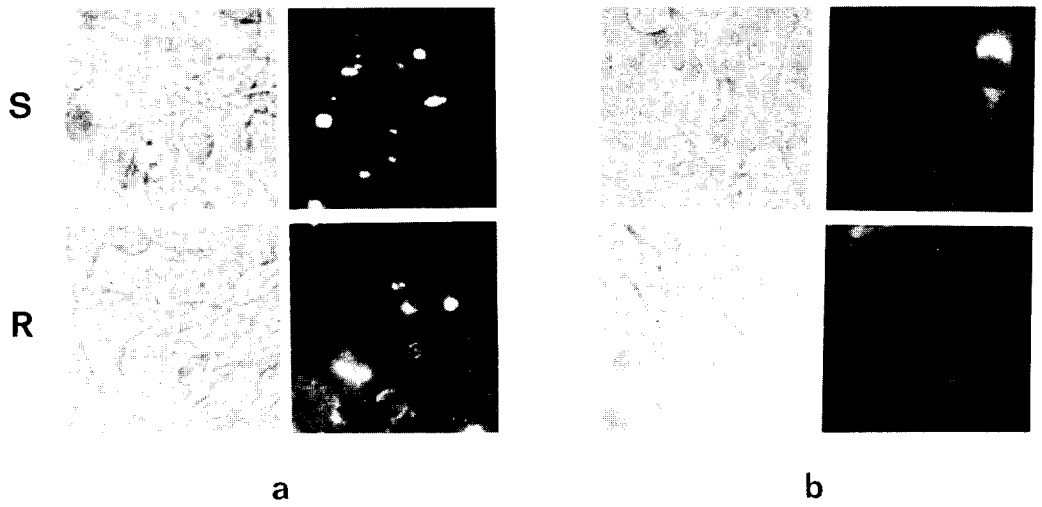

Fig. 3. C6 and C6 0,001 treated with 50 nM NS – DOX. Phase contrast (left panels) and fluorescence (right panels). The images presented are for (a) 6 hours' and (b) 15 hours' incubation for sensitive cells (top panels) and resistant cells (bottom panels).

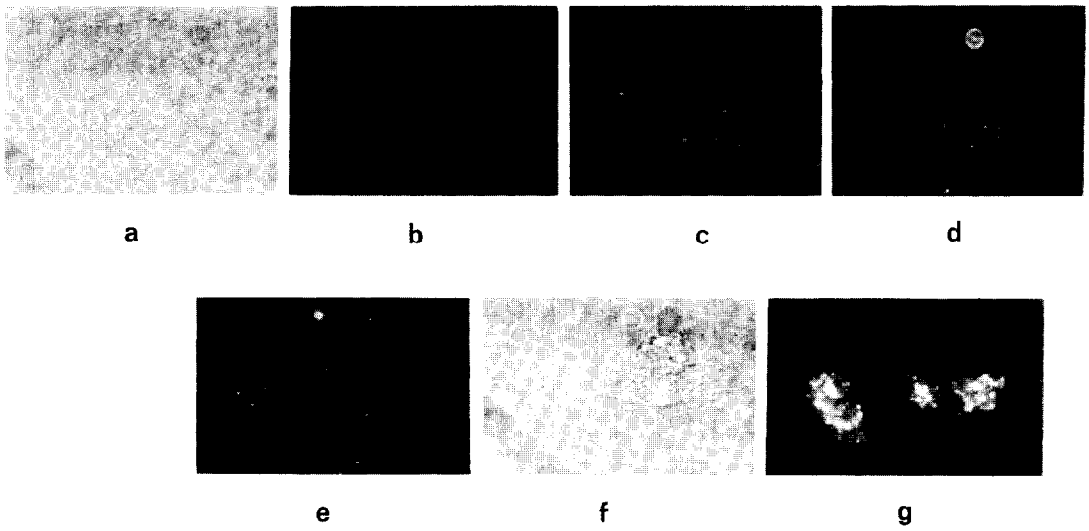

Fig. 4. Peritoneal macrophages treated with 50 nM NS – DOX. Phase contrast images (a) and (f) correspond to two different fields of the same sample. Images (b), (c), (d), and (e) are the fluorescence images of the field (b) just before treatment, (c) after 2 min, (d) after 10 min, and (e) after 10 min in the equatorial plane of the cells (g). Image (g) corresponds to field (f), and was acquired 2 hours later.

minutes to reach a plateau. At this point, only peripheral (bound) NS were sharply imaged when the focal plane of the objective corresponded with the equatorial plane of the cells as checked with the phase contrast, indicating that all the NS remained on the macrophage surface (Fig. 4d). Other sharply imaged NS – DOX appeared in the middle of the macrophage when the focal plane was moved to the equator of the cells (Fig. 4e). Over longer periods, macrophages internalized the NS, dozens of them being counted per cell. It should be noted that the internalization process was greatly favoured, and accelerated when cells were replaced in the CO_2 incubator rather than maintained under the microscope, which was not the case for the adhesion step. Also worthy of mention was the fact that only a fraction (30 ± 5%) of the macrophage preparation had trapped NS – DOX at the concentrations used, as can be seen by comparing transmission and fluorescence images. We also observed that depriving the macrophage medium of fetal calf serum before the addition of the NS led to the quasi total suppression of NS – DOX adhesion onto the cells. Finally, to bring out some relation between NS – DOX biological activity and the type of their interactions with the targeted cells, we examined the toxicity exerted by NS–DOX on macrophages as compared with that induced by free DOX.

Macrophage toxicity

The toxicity exerted towards macrophages was measured using a trypan blue exclusion test, as described in Methods. Results given in Fig. 5 show that at 10^{-6} M total DOX concentration, no toxicity was induced by free DOX or by unloaded NS of polymer concentration corresponding to 10^{-6} M NS – DOX (i.e. 4.3 10^{-5} M). In contrast, 95% of the cells were killed by 10^{-6} M DOX administered under NS – DOX form, and 50% by the mixture of DOX with unloaded NS (NS + DOX). From the toxicity curves, the DOX doses that killed 50% of the cells were 3.10^{-6} M, 10^{-6} M, and 0.8 10^{-6} M for free DOX, NS + DOX, and NS – DOX, respectively. The polymer concentration necessary to kill 50% of macrophages corresponded to a 15.10^{-6} M DOX dose in NS – DOX or NS + DOX.

When varying experimental conditions (incubation times increased and/or the cell-washing step omitted), the toxicity curves were shifted towards the lower concentrations, but the hierarchy of the different DOX forms was conserved.

DISCUSSION

The capacity of three lines of sensitive and resistant cells to adsorb and internalize DOX-loaded PIHCA NS under the concentration and incubation time conditions used to evidence MDR overcome was examined herein. The objective of this study was to determine whether this process was fundamental to understanding the mechanism of MDR overcome observed with these drug-loaded particles by Cuvier *et al.* [5]. This point had to be addressed before it would be possible to conceive of and optimize new routes in the overcome of the MDR pro-

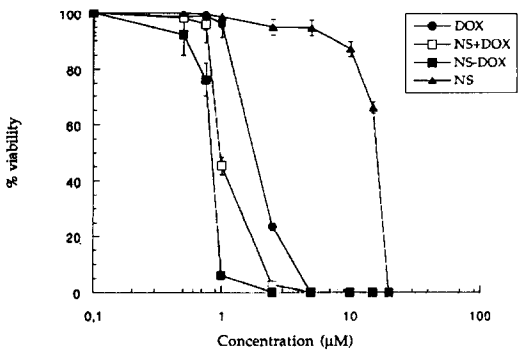

Fig. 5. Toxicity of DOX (●), NS – DOX (■), NS + DOX (□), NS (▲) on peritoneal macrophages. Macrophages were incubated as described in the Material and Methods section with drugs for 6 hours at the concentrations indicated. Macrophages were then washed and their viability assessed 24 hours later by trypan blue exclusion test. The results represent the mean of 4 assays.

cess and to make suppositions as to the nature of the mechanism of MDR itself. This was achieved by direct observation in fluorescence microscopy of the NS – DOX in the presence of various cell types. It appeared to us that the detailed analysis of fluorescence distribution into the cells may be difficult to interpret precisely in terms of DOX distribution. Indeed, problems arise from DOX fluorescence changes occurring when the molecule shifts from one environment to another. For instance, it has been extensively demonstrated that fluorescence is severely quenched by the intercalation of the drug molecule into the DNA helix [11]; this is very likely the process responsible for the lag phase observed in the fluorescence appearance after cell treatment with free DOX. The concentration of the drug in particular sites of the cells is also susceptible of inducing fluorescence quenching by self-association of the molecules. Another difficulty originates in the uncontrolled formation in culture media of DOX hydrophobic degradation products with higher affinity for cell membranes and modified fluorescence characteristics. For all these reasons, we deliberately decided to limit our investigations here to the observation of the whole fluorescence pattern. The results showed that under the conditions used to bypass MDR, no DOX-loaded NS were adsorbed onto the cell membrane or endocytosed by the three cell lines studied. The fluorescence distribution in NS – DOX treated cells was close to that of the free DOX-treated cells after 15 hours of treatment. This was observed both with the sensitive and resistant cells. On the other hand, phagocytic cells such as macrophages rapidly trap and internalize NS – DOX under the same conditions. This demonstrates that the experimental approach chosen here is well-suited to evidence adsorption and endocytosis when it occurs. The adsorption step, which is a prerequisite for internalization, requires the presence of fetal calf serum in the medium, in accordance with what is expected in a opsonization-mediated process involving the presence of specialized receptors on the cell surface. This suggests that the NS – DOX in complete culture medium are covered with opsonins contained in fetal calf serum, and that these opsonins are thereafter recognized and bound by specific membrane receptors present on the macrophage surface but very likely lacking on the surface of C6, K562, and MCF-7 sensitive and resistant cells. Moreover, it is interesting to note that the response of the macrophages to the different forms of DOX is significantly different from that described in previous works for the three cell lines used herein [5, 7]. First, although the NS – DOX form of the drug appeared to be more toxic towards macrophages than the free one, the toxicity gain was not as important as that obtained by Cuvier *et al.* on the resistant variants of the lines examined. Second, the NS + DOX form was as toxic for the macrophages as the NS – DOX form. This suggests that the mechanism of toxicity developed in macrophages where endocytosis of the DOX-loaded NS has been observed is basically different from that prevailing in MDR cell lines.

The absence of internalization evidenced with the three sensitive and resistant cell lines used in this work implies that the MDR bypass described by Cuvier *et al.* has an extracellular origin. Products arising from the polymeric matrix could be released in the extracellular medium, and act in synergism with the drug itself. The formation of modified chemical species when polymerisation is performed in the presence of DOX could explain the higher toxicity of (NS – DOX) compared to (NS + DOX) forms of the drug.

The existence of these species remains to be proven, and their nature has yet to be identified. However, this work strongly suggests that the mechanism of MDR bypass by the polymeric nanospheres is much more likely related to the chemical nature of the particles than to their colloidal character. The results obtained with DOX-loaded liposomes by different groups [12, 13] lend support to this idea. Indeed, the clearest examples of MDR bypass described with liposomal systems are those where the unloaded liposomes themselves affect cell survival.

This suggests that efficient MDR bypass is to be sought in an adequate synergism between the administered drug and the associated carrier, and indicates that endocytosis of the loaded carriers is not a determining step in the overcome process.

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