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# Increase of doxorubicin penetration in cultured rat hepatocytes by its binding to polymethacrylic nanoparticles *

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## Summary

The anticancer agent, doxorubicin (DXR), was bound to a particulate carrier consisting of polymethacrylic nanospheres (0.3 μm diameter). The uptake of free and nanoparticle-bound doxorubicin in adult rat hepatocytes maintained in primary culture was compared using high-performance liquid chromatography. At concentrations ranging from 50 to 500 ng DXR/ml of nutrient medium, neither free nor bound DXR induced cytotoxicity, as evidenced by light microscopy and leakage of lactate dehydrogenase. In the case of doxorubicin-loaded nanoparticles compared to free DXR, after a 24 h incubation, DXR concentrations in the hepatocytes were increased by 64% and 25% for respectively initial concentrations of 50 and 500 ng DXR/ml of medium Moreover, the endocytosis of the nanospheres by the hepatocytes was observed by electron microscopic examination. These results emphasize the usefulness of polymethacrylic nanoparticles as a new intracellular carrier for the enhancement of the penetration of drugs, such as anthracyclines, into cells.

## Introduction

The antitumor antibiotic, doxorubicin (DXR) (adriamycin), is one of the most used drugs in the field of cancer chemotherapy (Bender et al., 1978; Young et al., 1981; Speth et al., 1988). Three mechanisms of action are ascribed to the anthracyclines: DNA intercalation (Di Marco, 1975; Schwartz, 1975; Daskal et al., 1978; Sinha et al., 1984); membrane binding (Murphree et al., 1976; Tritton et al., 1978; Tritton and Yee, 1982); and lipid peroxidation (Myers et al., 1977).

Although doxorubicin is widely used in the treatment of neoplastic diseases, such as leukemias and various solid tumors, it unfortunately produces both minor side-effects such as alopecia and nausea as well as severe effects such as myelosuppression and congestive heart failure which are dose-dependent (Lenaz and Page, 1976; Billingham et al., 1978; Van Vleet et al., 1980; Henderson and Frei, 1980; Ferrans, 1983; Haq et al., 1985).

One way to reduce DXR toxicity and to increase its therapeutic activity could be to target

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that drug to specific sites by using colloidal drug-carriers such as the polymethacrylic nanoparticles previously described (Rolland et al., 1986a and b). Since this anthracycline is actively metabolised by liver parenchymal cells (Riggs et al., 1977; Reich, 1978; Loveless et al., 1978; Le Bot et al., 1988) and is considered as the reference antitumoral drug in the treatment of hepatocarcinoma (Olweny et al., 1975; Vogel et al., 1977; Ihde et al., 1977; Bern et al., 1978; Chan et al., 1980; Ballet et al., 1984; Chlebowski et al., 1984), the penetration and accumulation of free and bound DXR in cultured rat hepatocytes have been compared in the present paper.

## Materials and Methods

*Hepatocyte isolation and culture*

The liver of adult male Sprague–Dawley rats (180–200 g) (Iffra-Credo, France) was excised and perfused with a 0.025% collagenase solution buffered with Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) pH 7.4 at 37 °C (Guguen et al., 1975). Cell suspensions were filtered on gauze and washed 3 times by centrifugation.

Isolated hepatocytes were seeded at a density of $5 \times 10^5$ cells per 10 $cm^2$ Petri dish containing 2 ml of medium which was a mixture of 75% minimum essential medium and 25% medium 199 supplemented with 200 $\mu$g/ml bovine serum albumin, 10 $\mu$g/ml bovine insulin and 10% fetal calf serum. The cultures were maintained under 5% $CO_2$–95% air humidified atmosphere. The medium, to which $7 \times 10^{-5}$ M hydrocortisone hemisuccinate had been added, was removed 4–8 h after cell seeding and just before the experiment. Rat hepatocytes were used after 24 h of culture.

*Manufacture of polymethacrylic nanoparticles*

Polymethacrylic nanoparticles were prepared by aqueous emulsion copolymerization of methacrylic monomers (previously described by Rolland et al., 1986a, b and c). The monomers, methyl methacrylate (MMA), 2-hydroxypropyl methacrylate (HPM), methacrylic acid (MA) and ethylene glycol dimethacrylate (EGD) (Merck, France) were purified by distillation as previously described (Rolland et al., 1986a; Le Verge and Rolland, 1987).

Briefly, 2.5 ml (2.5% suspension) or 5 ml (5% suspension) of mixed monomers (56% MMA; 30% HPM; 10% MA; 4% EGD) were added with stirring to 97.5 ml or to 95 ml of distilled water, respectively; 10 mg of potassium persulphate (Merck, France), a free radical initiator, was added and the copolymerization lasted for 1 h at 90 °C. After filtration (glass filter 10–20 $\mu$m), the suspension of nanospheres was purified by dialysis (Rolland et al., 1986b). The nanoparticle mean sizes, measured using a Coulter Nano-Sizer (Coultronics, France), were 250 nm and 311 nm for the 2.5% and 5% suspensions, respectively.

*Preparation of free and nanoparticle-bound DXR*

A 2.5% suspension of nanoparticles ($2.4 \times 10^{12}$ particles/ml) was adjusted to pH 8.4 with 0.01 M sodium acetate and 1 N sodium hydroxide, and an aqueous solution of DXR (1 mg/ml) (Adriblastine, Roger Bellon Lab., France) was added dropwise with stirring to give a final concentration of either 0.1 mg DXR/ml (A) or 1 mg DXR/ml (B), with more than 99% of binding in both cases. The mechanisms involved in the DXR binding and its stability at physiological pH have already been published elsewhere (Astier et al., 1988).

The suspensions were diluted in culture medium in order to obtain equivalent concentrations of DXR in each case (i.e. the number of nanoparticles was 10-fold higher in suspension A than in suspension B).

*Incubation conditions*

Twenty-four hours after cell seeding, the cultures were incubated for 24 h in total darkness with either free or nanoparticle-bound DXR at concentrations ranging from 50 to 500 ng of DXR/ml of culture medium.

*Evaluation of DXR toxicity*

Cytotoxicity of free DXR and of both free and DXR-loaded polymethacrylic nanoparticles was assessed by examination of the cultures with phase-contrast light microscopy and by measurement of lactate dehydrogenase activity in the culture medium.

*Electron microscopic analysis*

To visualize the penetration and accumulation of nanoparticles into the hepatocytes, a part of Petri culture dishes was set up for transmission electron microscopic examination. Hepatocyte cultures were first washed with phosphate-buffered saline (PBS, pH 7.4) then fixed in situ with a 2.5% glutaraldehyde solution buffered to pH 7.4 with 0.1 M sodium cacodylate for 30 min. Thereafter the monolayer was postfixed for 30 min in a 1% osmium tetroxide solution in cacodylate buffer (pH 7.4), dehydrated in graded ethanols and embedded in Epon.

*Quantitative determination of DXR in hepatocytes*

DXR concentrations were measured in both medium and cell extracts by reversed-phase high-performance liquid chromatography (HPLC) as described elsewhere (Rolland, 1988). To 0.5 ml of sample, 0.1 ml of internal standard (daunorubicin aqueous solution a 1 μg/ml) and 0.25 ml of 0.5 M Tris buffer pH 8.6 were added. The sample was then extracted with 4 ml of ethylacetate (Merck, France) by mixing for 4 min on a horizontal shaker. After centrifugation for 5 min at 4000 rpm, the upper organic phase was collected and mixed with 100 μl of 0.1 N sulfuric acid for 2 min. After centrifugation for 5 min at 3000 rpm, the upper phase was removed and the aqueous lower phase (100 μl) was quickly transferred into a tube in which 100 μl of a 0.2 M methanolic solution of sodium acetate was previously dried.

An aliquot of the extract was chromatographed by reversed-phase HPLC (μBondapak $C_{18}$ column at 30 °C; automatic injector Wisp 710B; constant flow pump 6000A; a 254 nm fixed wavelength detector model 240 Millipore-Waters, France). The mobile phase consisted of methanol/0.01 M aqueous solution of sodium acetate/acetic acid (70 : 30 : 1 v/v/v) at a flow rate of 1 ml/min.

## Results

*Toxicity studies*

After a 24 h incubation, no hepatocyte toxicity was found with both nanoparticle suspensions (2.5% and 5%) for concentrations ranging from 1.8

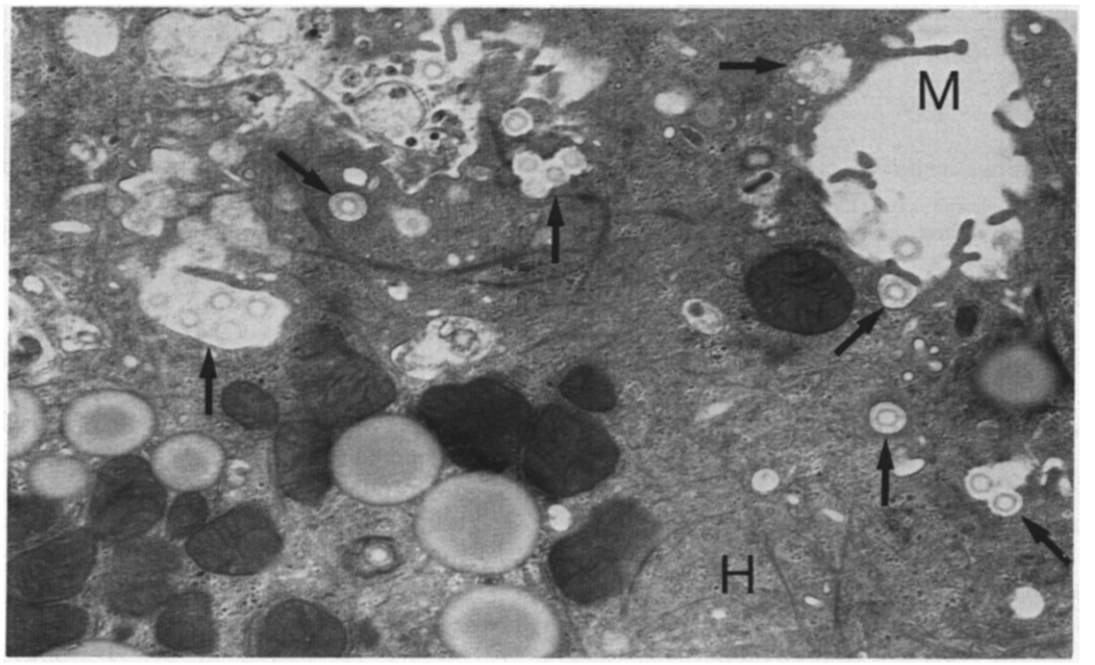


Fig. 1. Endocytosis of polymethacrylic nanospheres (2.5% suspension) by cultured rat hepatocytes (transmission electron micrograph, ×11,000). H, hepatocyte; M, extracellular medium. Intracytoplasmic vacuoles containing the nanospheres can be seen (arrows).

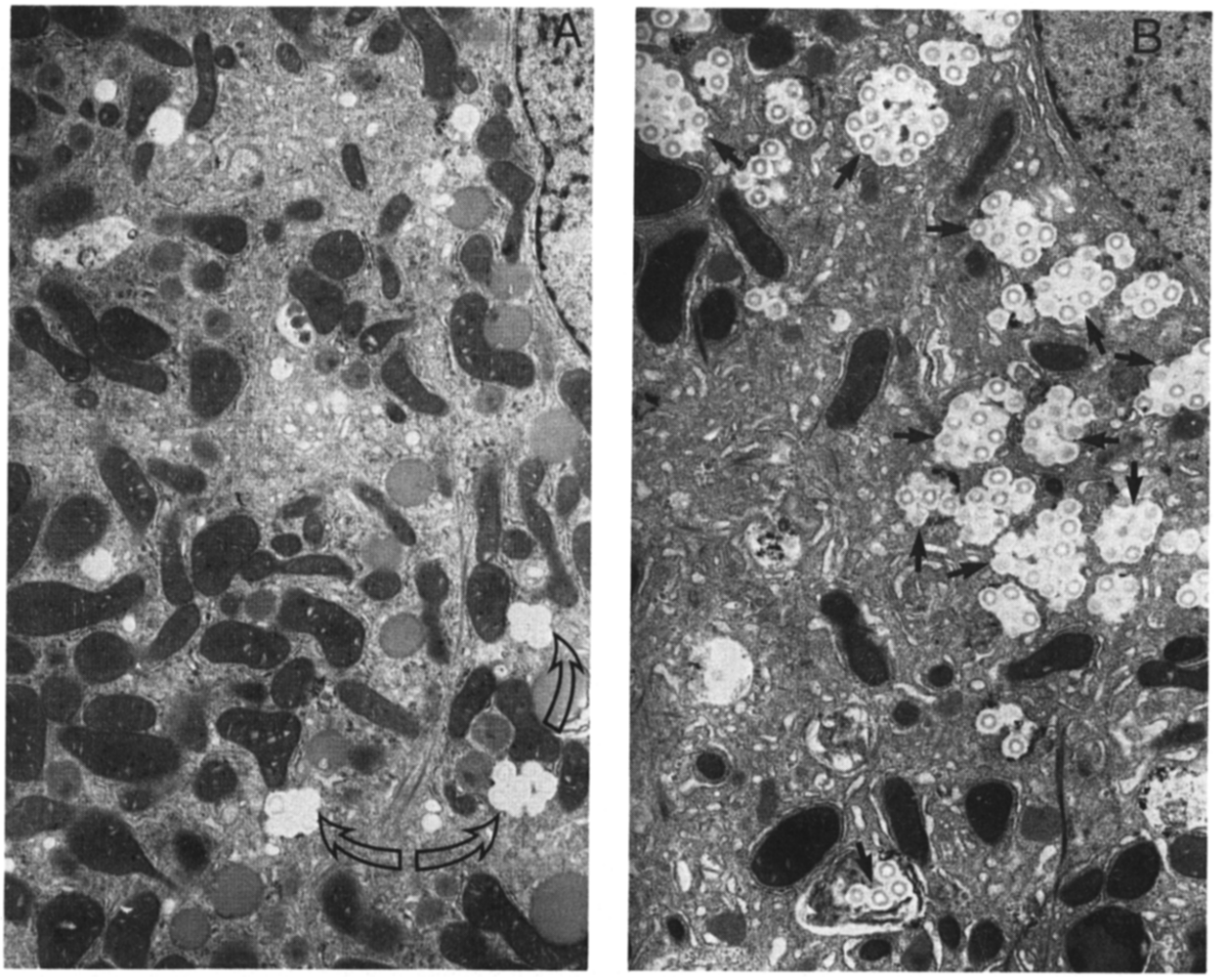


Fig. 2. Intracytoplasmic localization of polymethacrylic nanoparticles (arrows) in cultured rat hepatocytes after a 24 h incubation (transmission electron micrograph, ×7280). Incubation ratio: (A) 4800 nanospheres per hepatocyte; (B) 24,000 particles/cell.

to 90 μg of particles/ml of medium (data not shown). No cytotoxicity was observed up to an incubation ratio of about 24,000 and 34,000 nanospheres per cell for the 2.5% and 5% suspensions, respectively. Electron microscopic examination of hepatocyte cultures showed no evident subcellular alteration after a 24 h incubation in the presence of nanoparticles.

Whatever the concentrations of free or bound DXR, neither morphological alteration nor LDH leakage were evidenced in rat hepatocyte cultures after a 24 h incubation (data not shown).

*Nanoparticle uptake by cultured rat hepatocytes*

As observed by transmission electron microscopy (Fig. 1), nanospheres were observed both inside and outside the cells. They were localised within intracytoplasmic vacuoles suggesting that their uptake occurred via an endocytic process. The number of intracellular nanoparticles was observed to be dose-dependent; the higher the particle-to-hepatocyte incubation ratio was the higher the number of particles that were endocytosed. For instance, for an incubation ratio of about 4800 particles/hepatocyte only few particles were

TABLE 1

*Intracellular concentrations of doxorubicin (DXR) in rat hepatocytes incubated for 24 h at 37°C with various concentrations of free or bound DXR (n = 2)*

| Initial DXR concentrations (ng/ml) | Intracellular DXR concentrations (ng/ml) | | |
|---|---|---|---|
| | Free DXR | Bound DXR (A) (0.1 mg/ml)* | Bound DXR (B) (1 mg/ml)* |
| 50 | 16.34 | 26 82 (+64%) | 26 37 (+61%) |
| 100 | 42.76 | 54.41 (+27%) | 56.67 (+33%) |
| 250 | 121.13 | 144.43 (+19%) | 134.42 (+11%) |
| 500 | 210 89 | 260.59 (+24%) | 264 15 (+25%) |

* For equivalent initial concentrations of DXR, the number of nanospheres is 10-fold higher in suspension A than in suspension B.

taken up by the cells (Fig. 2A), whereas for a higher ratio of 24,000 particles/cell several intracytoplasmic vacuoles containing a large number of nanospheres were visualized (Fig. 2B).

*Determination of free and bound DXR in cultured rat hepatocytes*

Free or nanoparticle-bound DXR was used at concentrations ranging from 50 to 500 ng/ml. The highest concentration of DXR corresponded to a concentration of 90 μg of particles/ml of seeded cells and to 9 μg/ml for the 2.5% A and B suspensions, respectively (Table 1). Table 1 displays the intracellular DXR concentrations found after 24 h incubation of rat hepatocytes with free or bound DXR. When the anthracycline was bound to the nanospheres, its concentration into hepatocytes was enhanced, the increase varying from 11% to 64%: the lower DXR concentration resulted in the higher intracytoplasmic accumulation.

## Discussion

Since after a 24 h incubation, 2.5% and 5% polymethacrylic nanoparticle suspensions did not induce any morphological changes or LDH leakage at an incubation ratio as high as 34,000 particles per cell (90 μg/ml), they appear far less cytotoxic than other particulate carriers, e.g. polyalkylcyanoacrylate nanoparticles. Polybutylcyanoacrylate particles were indeed reported to provoke cellular damage after only a 4 h incubation with hepatocytes at a concentration of 150 μg of particles/ml of seeded cells, corresponding to about 20,000 particles per cell (Kante et al., 1982). The $LD_{50}$ was found to be about 0.4 mg/$2 \times 10^6$ and 1 mg/$2 \times 10^6$ cells for polybutylcyanoacrylate and polyhexylcyanoacrylate nanoparticles, respectively (Kreuter et al., 1984).

Similarly, no morphological alteration or LDH leakage were observed with free and polymethacrylic nanosphere-bound DXR for concentrations ranging from 50 to 500 ng/ml.

In addition to a previous publication (Rolland et al., 1987) where in vitro polymethacrylic nanoparticle phagocytosis by human monocytes and polymorphonuclear cells was shown, the present study demonstrates their dose-dependent endocytosis by cultured rat hepatocytes after a 24 h incubation (Figs. 1, 2). These observations differ from those made by Kreuter et al. (1984) showing no uptake of polybutylcyanoacrylate particles by hepatocytes after 1 h incubation, perhaps due to the too short incubation period.

The results reported here also show that DXR concentrations in cultured rat hepatocytes were enhanced when the drug was first bound to polymethacrylic nanospheres. At the lowest analysed DXR concentration, there was a significant increase (64%) in the accumulation of the nanoparticle-bound DXR as compared to the free drug. This enhancement of DXR uptake was not so important for higher DXR concentrations, probably due to a saturation of the transport process. This increased DXR penetration can be related to the ability of the nanoparticles to be endocytosed by the hepatocytes.

These nanoparticles could present a great interest to target tumoricidal drugs within intracellular compartments that would not normally be readily accessible to the free drugs. The present observations finally raise at least two questions: firstly, is the nanoparticle uptake higher in hepatoma cells than in normal hepatocytes; and secondly, what are the cytotoxic effects of these nanospheres after long-term intracellular accumulation? These considerations are presently under investigation.

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