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# Hepatic tissue distribution of doxorubicin-loaded nanoparticles after i.v. administration in reticulosarcoma M 5076 metastasis-bearing mice

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**Summary.** In our previous studies, doxorubicin-loaded polyisohexylcyanoacrylate nanoparticles have been proven to increase dramatically the antitumoral activity of the cytotoxic agent in metastasis-bearing mice. The experimental model consisted of metastases induced by i.v. inoculation of reticulosarcoma M 5076 cell suspension to $C_{57}BL/6$ mice. The improved efficacy of the drug was noted in terms of either metastasis count or survival. Therefore, tissue-distribution studies of this drug delivery system within the metastatic liver after i.v. administration were undertaken to gain more insight into the mechanism of action. Doxorubicin measurements in healthy hepatic or neoplastic tissue were carried out together with histological examinations using transmission electron microscopy. These results demonstrated the hepatic tissue to be an efficient reservoir of the drug when it was injected associated with nanoparticles. Accumulation of biodegradable nanoparticles with associated doxorubicin in Kupffer cells created a gradient of drug concentration for a massive and prolonged diffusion of the free drug towards the neoplastic tissue.

## Introduction

The ability to target cytotoxic drugs selectively into tumor cells in vivo has been a long-cherished goal in cancer chemotherapy. In the last few years, many efforts have been undertaken in the field of anticancer drug targeting, especially by means of liposomes [10, 17, 23]. Numerous publications have indicated improved anticancer activity for liposome-associated doxorubicin [7, 21], including its metastatic inhibition activity [8, 13, 14, 16]. However, the two main factors that have limited the development of liposomes are the instability of the drug in solution within the vesicles and the rapid leakage of hydrophilic compounds across the phospholipidic bilayer. Therefore, Couvreur et al. [3, 5] more recently proposed biodegradable polyalkylcyanoacrylate nanoparticles as a potential cancer-drug delivery system. Experiments previously carried out on liver metastatic model M 5076 have shown tripled efficacy for doxorubicin-loaded nanoparticles as compared with the free drug [2]; an 8-fold increase of drug level in the liver was also demonstrated. The in vivo fate of these nanoparticles in terms of their organic and cellular localization in healthy animals has been studied [9, 12].

This paper is an attempt to elucidate the mechanism of the enhanced efficacy of doxorubicin-loaded nanoparticles given i.v. to metastasis-bearing mice. Doxorubicin measurements in both metastasis cores and neighboring, healthy hepatic tissue provided quantitative information concerning the respective proportions of the drug that distributed within these tissues. Histological examinations carried out using transmission electron microscopy also showed the hepatic localization of these nanoparticles at the cellular level.

## Materials and methods

*Chemicals.* Lyophilized doxorubicin-loaded polyisohexylcyanoacrylate nanoparticles with a diameter of 300 nm were furnished by Sopar (Sart-Dames-Avelynes, Belgium). Doxorubicin nanoparticles were then dispersed by mechanical stirring in an isotonic solution of 5% glucose. The preparation was found to be sterile and non-pyrogenic at a final doxorubicin concentration of 1 mg/ml, the concentration of polymer in suspension being 13.3 mg/ml [22]. Free doxorubicin (Adriblastina 10) was obtained from Farmitalia (Brussels, Belgium). All other chemicals were of analytical grade and were used as purchased.

*Hepatic metastatic model.* Adult $C_{57}BL/6$ mice weighing 18–20 g were obtained from Iffa Credo (Arbresles, France). Reticulum cell sarcoma M 5076 was obtained through the courtesy of Dr. Lavelle (Rhône-Poulenc, Vitry-sur-Seine, France); it has been shown to have a unique capacity for metastasizing to the liver [1]. Histological and immunological studies showed the tumor to be histiocytic in origin [20], although originally classified as a carcinoma [19].

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**Table 1.** Tissue-distribution studies in tumor-bearing mice, showing the doxorubicin (DXR) equivalent (μg/g wet tissue) in healthy (HT) and tumoral (TT) tissue at given times after free (f-) and nanoparticulate (npc-) drug administration

| | 15 min | 30 min | 1 h | 3 h | 6 h | 24 h | 48 h |
|---|---|---|---|---|---|---|---|
| f-DXR/TT | 6.8 ± 1.6 | 8.3 ± 3.3 | 12.5 ± 4.0 | 8.8 ± 3.7 | 6.5 ± 3.2 | 2.2 ± 0.7 | 0.7 ± 0.7 |
| f-DXR/HT | 3.0 ± 0.2 | 2.9 ± 1.0 | 3.0 ± 1.0 | 2.0 ± 0.3 | 3.9 ± 1.3 | 1.9 ± 0.8 | 0.5 ± 0.4 |
| npc-DXR/TT | 8.4 ± 2.7 | 5.1 ± 3.2 | 15.8 ± 5.6 | 16.0 ± 2.3 | 7.6 ± 2.3 | 11.1 ± 6.3 | 3.1 ± 1.8 |
| npc-DXR/HT | 60.3 ± 7.0 | 36.6 ± 1.5 | 73.6 ± 14.4 | 51.1 ± 12.6 | 32.2 ± 3.5 | 13.4 ± 4.3 | 7.1 ± 2.8 |

The tumor was maintained by subcutaneous passage with a trocar every 2 weeks and was used after its 11th passage. Tumors obtained 2 weeks after implantation were minced with scissors and disaggregated by exposure to trypsin (0.3%) in Versene and phosphate-buffered saline (PBS) medium for 30 min at room temperature. The cells were washed twice with 50 ml buffered solution (0.85% NaCl, 36.5% $Na_2HPO_4$, 3.5% $Na_2HPO_4$) and finally resuspended in the same medium. A total of $7 \times 10^3$ tumor cells in 0.2 ml were then injected i. v. Animals were used for the pharmacokinetic studies 16 days after tumor-cell inoculation. At this time, liver metastases appeared as distinct, whitish colonies whose size ranged from 1.5 to 2 mm. Histological sections were also prepared from the same group of animals.

*Liver pharmacokinetic studies.* Doxorubicin was injected i.v. into 16-day-old metastasis-bearing mice in its free or nanoparticulate form at a dose of 10 mg/kg, corresponding to 133 mg/kg polymer. Animals were sacrificed at 15 and 30 min and at 1, 3, 6, 24 and 48 h after drug administration. The liver was removed and fixed in Fekete solution. Metastasis cores were excised with a scalpel, dried on a piece of absorbant paper and weighed (about 50 mg). The sample was then added to 20 times its weight of saline solution. The mixture was homogenized by Potter homogenizer (Bioblock, Paris, France) and kept at –20° C. An equal amount of healthy tissue surrounding metastasis cores was excised from the same animal and submitted to the same treatment. Groups of four animals were used for doxorubicin determination at each time point.

*Determination of doxorubicin.* Doxorubicin was extracted from tissue homogenate after thawing and defecation by acetonitrile. Analytical measurements were carried out by HPLC [18]. A column packed with nucleosil C18 (Novapak, Waters, St Quentin-en-Yvelines, France) was used. The mobile phase consisted of a methanol-acetate buffer (0.01 M) and concentrated acetic acid (60:39:1). Spectrofluorimetric detection (247–550 nm) was used (FS 970 LC Fluorimeter, Schoeffel Instrument, West Germany). Daunorubicin was chosen as internal standard.

*Electron microscopic studies.* Samples (0.5 ml) of doxorubicin-loaded nanoparticles (corresponding to a doxorubicin dose of 10 mg/kg) were injected i. v. into metastasis-bearing mice. Animals were sacrificed and the liver was excised at 15 min and at 4, 18 and 36 h after drug administration. Small pieces of liver (about 1 $mm^3$) were fixed as soon as possible in 1.6% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.3) for 24 h. After two rinses in cacodylate buffer, the tissues were post-fixed for 1 h in 2% osmium buffered in 0.1 *M* cacodylate (pH 7.3). The tissue pieces were dehydrated in increasing concentrations of ethanol, cleared in propylene oxide and embedded in Epon. The sections obtained from selected blocks were maintained on grids, stained with uranyl acetate and lead citrate and examined with an EM 400 Philips electron microscope.

## Results

### *Liver pharmacokinetic studies*

Mean concentration-time profiles for doxorubicin in hepatic and tumoral tissues after i. v. administration of the cytotoxic drug in both forms are presented in Fig. 1. The terminal slope (24–48 h) of concentration decay was nearly identical in all cases. It can be concluded that the rate of doxorubicin elimination was in the same range for both types of tissue and was not influenced by the nanoparticulate form. Pharmacokinetic profile differences should then be attributable to the distribution process within the metastasis-bearing liver.

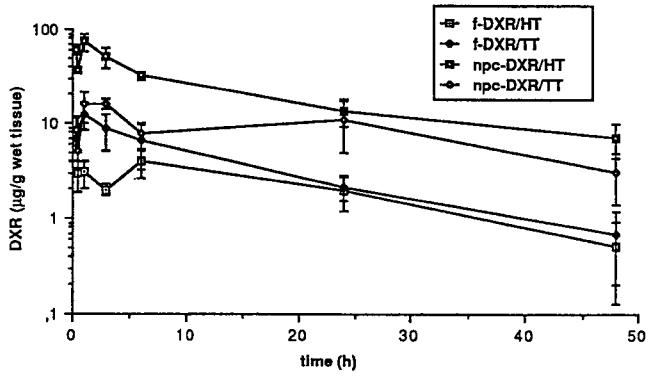


**Fig. 1.** Doxorubicin (*DXR*) concentration vs time curves in healthy hepatic (*HT*) and tumoral (*TT*) tissues after i. v. administration of doxorubicin (10 mg/kg, corresponding to 133 mg/kg PIHCA) in its free (*f-*) or nanoparticle-bound (*npc-*) forms in mice

The drug concentration peaks were obtained 1 h after administration except for free doxorubicin in healthy hepatic tissue, where no peak could be observed. At this time, the concentration of doxorubicin injected in nanoparticulate form was 24-fold higher (73.6 ± 14.4 μg/g wet tissue) than that of free doxorubicin in healthy tissue (3.0 ± 1.0 μg/g wet tissue). Nevertheless, the concentrations of doxorubicin in neoplastic tissue for both forms of drug delivery were practically identical (12.5 ± 4.0 vs 15.8 ± 5.6 μg/g wet tissue; Table 1). it should be noted, however that 24 h after drug administration, the concentration of nanoparticulate drug was 5-fold higher than that of free doxorubicin in neoplastic tissue.

**Table 2.** AUCs corresponding to the pharmacokinetic profiles of doxorubicin (DXR) injected in its free (f-) and nanoparticulate (npc-) forms in healthy hepatic (HT) and neoplastic (TT) tissues at different intervals

| AUC | 0–6 h | 0–48 h |
|---|---|---|
| f-DXR/HT | 16.1 | 98.3 |
| f-DXR/TT | 51.5 | 163.8 |
| npc-DXR/HT | 289.3 | 946.1 |
| npc-DXR/TT | 74.2 | 413.7 |

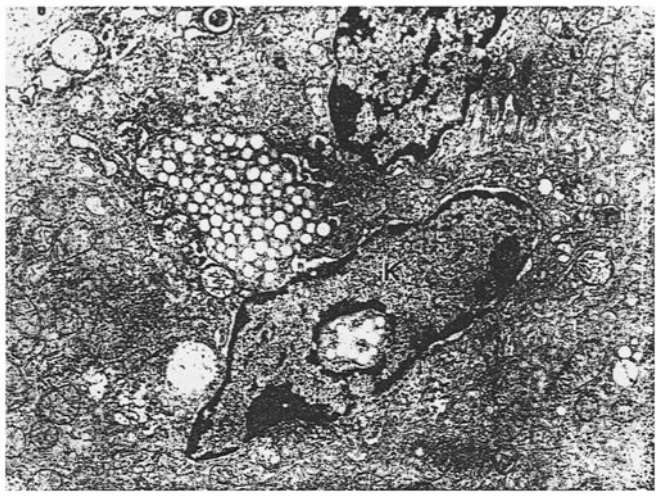

Fig. 2. Numerous spherical, electron-lucent nanoparticles agglomerated within a Kupffer cell's lysosome (*K*) 15 min after i. v. administration of nanoparticle-bound doxorubicin in mice

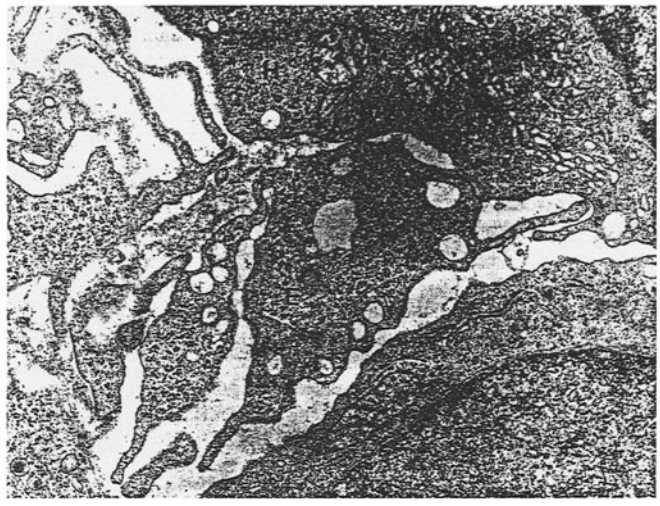

Fig. 4. Partly degraded polymeric nanoparticles remained in the lysosome of a Kupffer cell (*K*) 36 h after i. v. administration

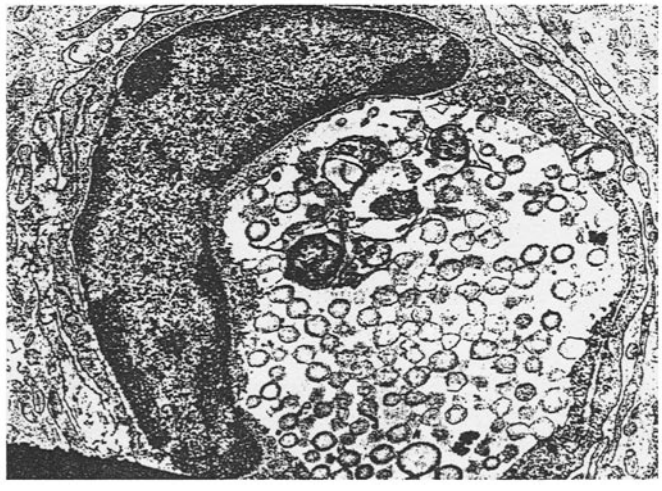

Fig. 3. Rare nanoparticles could be also observed in hepatocytes (*H*) and endocytes (*E*) 18 h after i. v. administration

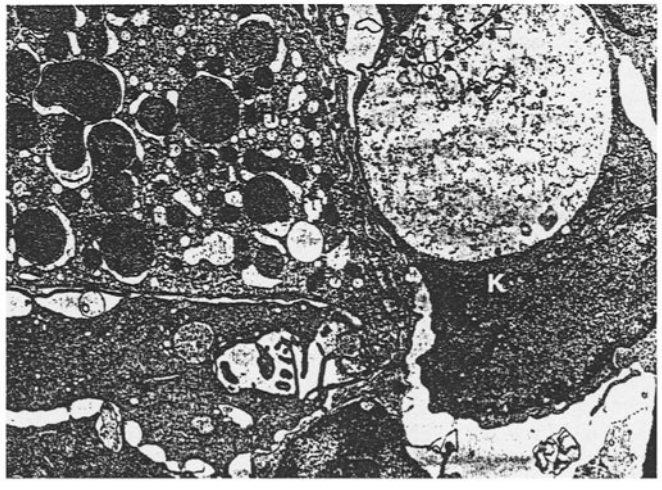


Fig. 5. Nanoparticles were almost entirely degraded within the Kupffer cells (*K*). Hepatocytes (*H*) seemed to be activated, with an enlarged endoplasmic reticulum

The AUC was calculated over different intervals (0–6 and 0–48 h) by the trapezoidal rule (Table 2). For each delivery form (either free or nanoparticle-bound doxorubicin), the tumor/liver AUC ratio over a given interval was used as an indicator of the relative drug exposure in these tissues. After administration of the free drug, the tumor/liver ratio was 3.2 for the 0- to 6-h interval and 1.7 for the 0- to 48-h interval. The latter ratio was in agreement with the high affinity of doxorubicin for cellular DNA, presumably abundant in rapidly dividing tissue, whereas the 0- to 6-h ratio indicated more rapid diffusion of the drug from the blood to the neoplastic tissue. After administration of the nanoparticle-bound drug, the tumor/liver ratio increased from 0.26 for the 0- to 6-h interval to 0.43 for the 0- to 48-h interval. Of particular interest was the observed increase in doxorubicin concentration in neoplastic tissue over the 6- to 24-h interval, which reached the levels recorded in healthy hepatic tissue (Fig. 1). As the drug carrier could not move from one tissue to another, it was clear from this result that free doxorubicin became available in hepatic tissue for diffusion in tumor tissue. As the rate of drug elimination from both tissues was low, this diffusion led to an increase in doxorubicin concentration in neoplastic cells.

Doxorubicin concentrations were 18-fold higher in the liver over the 0- to 6-h interval after its administration in nanoparticulate form than after free-drug administration (Table 2). This result was related to the rapid uptake of the colloidal carrier by Kupffer cells. In neoplastic tissue, nanoparticulate-associated doxorubicin administration resulted in only a 1.4-fold increase in drug exposure over the 0- to 6-h interval. This indicated clearly that nanoparticles had no special affinity for tumor cells. However, during the 0- to 48-h interval, drug exposure in metastatic tissue was found to be 2.5-times more important for the nanoparticle-bound doxorubicin. Moreover, the drug concentration in tumor tissue was clearly higher for the nanoparticulate formulation after 24 and 48 h (11.1 vs 2.2 μg/g wet

tissue at 24 h and 3.1 vs 0.7 μg/g wet tissue at 48 h; see Table 1).

*Tissue localization*

Transmission electron microscopy was used to determine both the appearance and the cellular localization of doxorubicin-loaded nanoparticles after i.v. administration to liver metastasis-bearing mice.

As shown in Fig. 2, Kupffer cells were observed to be filled with numerous spherical, electron-lucent nanoparticles as soon as 15 min after i.v. administration. Nanoparticles were also taken up, albeit to lesser extent, by hepatocytes and endocytes (Fig. 3). Phagocytosis of nanoparticles by circulating polynuclear cells in the sinuses was also observed. Furthermore, although some rare spherical vesicles could be observed in tumor cells, they could not be clearly identified as nanoparticles. Liver examination at later times (18 and 36 h after administration) showed that partly degraded nanoparticles remained in lysosomal vesicles of Kupffer cells (Fig. 4). Hepatocytes particularly close to Kupffer cells had a differnt appearance from those observed at previous time (Fig. 5); they appeared to be activated, with an enlarged endoplasmic reticulum and numerous transparent vesicles.

## Discussion

Previously published pharmacokinetic studies [2] carried out on total liver homogenate from both healthy and metastasis-bearing mice have shown considerable capture of nanoparticulate doxorubicin by the liver, whereas no difference in total hepatic concentrations was noted between healthy and cancer-bearing animals.

Our results clearly demonstrated that when doxorubicin was bound to nanoparticles and injected i.v. into M 5076 metastasis-bearing mice, the drug concentrated dramatically in the healthy hepatic tissue, which in turn could act as a sort of reservoir for the cytotoxic drug. This probably enabled a higher exposure of neoplastic cells to the drug. These pharmacokinetic data were consistent with our histological observations showing a considerable accumulation of nanoparticles (30–50 particles/cell) in lysosomal vesicles of Kupffer cells, whereas nanoparticles could not be clearly identified in neoplastic cells. This confirmed the observations of Lenaerts et al. [12] in healthy rats; after quantitative cell separation, these authors showed that Kupffer cells were the major site for nanoparticle cell internalization. Free doxorubicin is possibly released from Kupffer cells as a consequence of the bioerosion of cyanoacrylic polymers. The increase in the exposure of tumor cells to doxorubicin that was observed with nanoparticles could be due to the diffusion of drug from the healthy tissue, which was dramatically impregnated with doxorubicin.

In vivo uptake and release of drug in hepatocytes and Kupffer cells have been studied using liposomes by Lautersztein et al. [11]. These authors showed a non-constant uptake of drug-loaded liposomes by Kupffer cells; rapid uptake was immediately followed by a subsequent decrease in drug-loaded liposome capture. Our pharmacokinetic data concerning doxorubicin-loaded nanoparticles in hepatic tissue were consistent with this study; however, in contrast, we observed that the release of targeted drug from Kupffer cells involved a slow process, which could last some 10 h after the phagocytosis of nanoparticles.

Using the same metastatic model used in the present study, Perez-Soler et al. [15] confirmed the presence of a cisplatin analogue in the cytoplasm of tumor cells 5 min and 2 h after i.v. administration of liposome-encapsulated drug. In that case, liposomes were found to cross the liver sinusoidal capillaries and gain access to both healthy hepatocytes and M 5076 reticulosarcoma cells. This passage involved transport within endocytic vesicles rather than movement through endothelial fenestrae. In our studies, only rare spherical vesicles were noted in certain tumor cells. Therefore, we assumed that the high levels of doxorubicin found in neoplastic tissue after nanoparticle-mediated drug delivery could to a minor extent be due to the direct interaction of the carrier with the tumor cells and to a major extent be attributable to an intense capture by the Kupffer cells, followed by release of the drug into the microenvironment and its uptake by neighboring tumor cells. This was previously suggested by Gabizon et al. [8] in a study using doxorubicin-loaded liposomes.

## Conclusion

The mechanism by which nanoparticle-associated doxorubicin was much more effective is probably rather complex. However, apart from the unlikely direct uptake of nanoparticles by neoplastic tissue, the 2.5-fold increase in the exposure of tumor tissue to doxorubicin (as demonstrated by our data) probably resulted from its dramatic permeation into healthy tissue, mediated by its considerable capture by the Kupffer cells. The hepatic tissue acted as a reservoir for the drug, capable of inducing a gradient of drug concentration favorable for a massive and prolonged diffusion of the free drug (from nanoparticles entrapped in Kupffer cells' lysosomes) towards the neighboring malignant cells.

This leads to the question concerning the long-term effect of an 18-fold increase in doxorubicin levels in the liver. Although preliminary results have shown that doxorubicin-loaded nanoparticles did not exert any major or unexpected toxicity in the liver [6], this question should be more thoroughly explored in the near future. In other studies [4], nanoparticles have been found to reduce the acute toxicity of the drug and to diminish cardiac levels of doxorubicin.

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