Enhanced Therapeutic Effect against Liver W256 Carcinosarcoma with Temperature-sensitive Liposomal Adriamycin Administered into the Hepatic Artery

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ABSTRACT

The antitumor activity of Adriamycin encapsulated in temperature-sensitive liposomes combined with local hyperthermia (HT) was tested in rats bearing well-developed liver W256 carcinosarcoma tumors. Two h after rats received Adriamycin encapsulated in temperature-sensitive liposomes via either the hepatic artery (i.a.) or the femoral vein (i.v.) or free Adriamycin i.a., liver HT was applied at 42°C for 6 min. In animals treated with liposomal Adriamycin i.a., HT resulted in a 38% reduction in the tumor volume ratio and a 2.2-fold increase in the life span of the animals. In animals treated with liposomal Adriamycin i.v. or free Adriamycin i.a., HT did not alter the tumor volume ratio or life span of the animals. Administration i.a. of liposomal Adriamycin markedly increased the tumor drug levels (4-14-fold), reduced the systemic distribution of the drug, and slowed the drug decrease from both the tumor and liver compared with animals treated i.v. Liver HT in animals treated with liposomal Adriamycin i.a. further increased tumor drug levels by 1.5-2.6-fold, further slowed the drug decrease from the tumor, and resulted in a dissociation of the parallel decrease of drug and lipid from the tumor. This latter effect was not observed in the other groups. These pharmacological findings combined with the lack of beneficial effect from HT in animals treated with free Adriamycin i.a. or liposomal Adriamycin i.v. suggest that i.a. administration of Adriamycin encapsulated in temperature-sensitive liposomes results in a significant retention of intact liposomes in the tumor vasculature that are able to release the encapsulated drug into the tumor cell compartment upon raising the temperature to the phase transition level.

INTRODUCTION

Liposomes have been extensively explored in the past 20 years as carriers of antitumor drugs (1–6). However, because most tumors are not natural targets of liposomes, several methods have been developed to enhance their tumor-targeting efficiency. Some of these methods combine a targeting physical maneuver, such as the application of a magnetic field or HT,1 with a nontargeting maneuver, such as the administration of magnetic- or temperature-sensitive liposomes containing the antitumor agent. Encouraging results with the combination of HT and temperature-sensitive liposomes containing methotrexate have been reported previously (7, 8). With this method, tumor targeting is achieved by enhancing the release of the liposome content into the tumor cell compartment when HT is applied locally.

Liposomes injected i.v. distribute preferentially to the organs rich in reticuloendothelial system cells, such as the liver, spleen, and bone marrow (9, 10). Although it would seem that this phenomenon could be exploited for targeting liver tumors, it cannot. Unlike the liver parenchyma, which receives less than 30% of its blood supply from the hepatic artery and the rest from the portal vein, liver tumors receive almost 100% of their blood supply from the hepatic artery (11–15). For well-established liver tumors that have developed an independent blood supply, there is, therefore, no reason why i.v.-administered liposomes should be able to preferentially deliver the drug to the tumor cells.

Administration of antitumor agents into the hepatic artery with and without concomitant embolization for the treatment of liver primary and metastatic tumors has been extensively explored to achieve a higher tumor drug concentration and to increase the therapeutic effect (16). In experimental systems, i.a. administration of Adriamycin has been shown to increase the drug tumor levels by 3-fold without altering significantly the heart drug levels (17). In patients with malignant tumors, the i.a. administration of Adriamycin reduced the plasma AUC by about 30% (18).

In a previous study we showed that administration of LA via the hepatic artery resulted in a more selective distribution of the drug into the liver tumor than after i.v. administration (19). On the basis of these results, we hypothesized that temperature-sensitive liposomes administered into the hepatic artery may remain in the liver vasculature for some time. Therefore, local HT applied after drug administration might enhance the release of the encapsulated drug, increase the drug levels in liver tumor, and result in an increased therapeutic effect. To prove this hypothesis, we studied the tumor and normal tissue distribution and antitumor activity of Adriamycin encapsulated in temperature-sensitive liposomes administered i.v. and i.a. into the hepatic artery in combination with liver HT. Antitumor activity experiments were performed in rats bearing well-developed W256 carcinosarcoma liver tumors.

MATERIALS AND METHODS

Liposome Preparation and Characterization

The final liposome formulation was composed of 28.3 mg/ml of DPPC (M. W.) 735; purity of DPPC >98%; Nippon Oil and Fats Co., Ltd., Tokyo, Japan) and 7.05 mg/ml of Adriamycin (Adriamycin Injection; Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) in Tris-HCl buffer, pH 6.01. Liposomes were prepared by the reverse-phase evaporation method (20).

Liposomes were prepared by the reverse-phase evaporation method (20). The encapsulated drug was separated from the nonencapsulated drug by centrifugation at 75,000 × g for 20 min at 4°C, and the pelletted vesicles were resuspended in Tris-HCl buffer. This step was repeated 3 times. The final concentration of total Adriamycin was adjusted to 7.08 mg/ml. Concentration of nonencapsulated drug was <0.05% of the total concentration.

Radiolabeled liposomes containing Adriamycin were prepared using [14C]-DPPC (1.3-phosphatidylcholine, 1.2-di[14C]palmitoyl; 4.07 GBq/mmol; Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) mixed with the nonlabeled DPPC at a molar ratio of 1:1.76 × 108. The remaining steps of preparation were the same as those for nonradiolabeled liposomes.

The average diameter and size distribution of the liposomes were determined by a Laser Particle Analyzer (LPA-3000/3100: Otsuka Electronics Co., Ltd., Osaka, Japan) using the dynamic light scattering method.
The %EE and the leakage of drug from LA were determined by separately measuring both the free and total amount of Adriamycin in the liposomal preparation at 0 min, 6 min, 2 h, and 8 h; an aliquot of the initial LA preparation (200 μl) was transferred to Ultra-Free-C3GC centrifugal filtration tubes (MW, 10,000; Millipore Kogyo Co., Ltd., Tokyo, Japan). The tube was centrifuged at 2000 × g for 30 s at 4°C. The concentration of nonencapsulated drug in the filtered fraction at the bottom of the tube was determined by HPLC (21). Phosphorus determinations with the Bartlett assay (22) were used to assess phospholipid content. No phosphorus was detected in the filtered fraction thus confirming that the Adriamycin measured in that fraction was nonencapsulated drug. The standard Sephadex G-50 filtration method was also used to confirm the %EE in the same samples.

The phase-transition temperature (Tm) of LA was determined by differential scanning calorimetry (SSC/560; Seiko Denshi Instruments, Inc., Tokyo, Japan). A Tris-HCl solution containing Adriamycin at the same concentration (200 nM) was transferred to Ultra-Free-C3GC centrifugal filtration tubes (MW, 10,000; Millipore Kogyo Co., Ltd., Tokyo, Japan). The tube was centrifuged at 10,000 × g for 30 s at 4°C. The concentration of nonencapsulated drug in the filtered fraction at the bottom of the tube was determined by HPLC (21). Phosphorus determinations with the Bartlett assay (22) were used to assess phospholipid content. No phosphorus was detected in the filtered fraction thus confirming that the Adriamycin measured in that fraction was nonencapsulated drug. The standard Sephadex G-50 filtration method was also used to confirm the %EE in the same samples.

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Liver HT and Organ Distribution

Walker 256 (W256) carcinosarcoma cells (Shionogi & Co., Ltd., Osaka, Japan) were surgically implanted (5 × 10⁶ cells/rat) into the liver lobes of male SLC Wistar/ST rats (8 weeks old; Japan SLC Co., Ltd., Hamamatsu, Shizuoka, Japan). Nine days after the tumor inoculation, all animals had a laparotomy. Animals showing a visible liver tumor (90%) were divided into 4 groups of 30 rats each. Animals of groups 1 and 2 had the femoral vein cannulated; animals of groups 3 and 4 had the hepatic artery cannulated (Table 1). The positions of the cannulae were checked by a contrast X-ray system (Softex-CSI-2; Softex Co., Ltd., Tokyo, Japan). The cannulation methods have been described previously in detail by us (23). All animals received LA (2.35 mg/kg) through the cannulae. Injection time was 1 min in all cases.

The %EE and the leakage of drug from LA were determined by separately measuring both the free and total amount of Adriamycin in the liposomal preparation at 0 min, 6 min, 2 h, and 8 h; an aliquot of the initial LA preparation (200 μl) was transferred to Ultra-Free-C3GC centrifugal filtration tubes (MW, 10,000; Millipore Kogyo Co., Ltd., Tokyo, Japan). The tube was centrifuged at 2000 × g for 30 s at 4°C. The concentration of nonencapsulated drug in the filtered fraction at the bottom of the tube was determined by HPLC (21). Phosphorus determinations with the Bartlett assay (22) were used to assess phospholipid content. No phosphorus was detected in the filtered fraction thus confirming that the Adriamycin measured in that fraction was nonencapsulated drug. The standard Sephadex G-50 filtration method was also used to confirm the %EE in the same samples.

Table 1 Distribution of Adriamycin encapsulated in temperature-sensitive liposomes in liver tumor-bearing rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>5 min</th>
<th>2 h</th>
<th>2.5 h</th>
<th>8 h</th>
<th>24 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>i.v. LA</td>
<td>6.76 ± 1.19</td>
<td>3.87 ± 0.68</td>
<td>3.72 ± 0.30</td>
<td>2.55 ± 0.22</td>
<td>0.17 ± 0.02</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>i.v. LA + HT</td>
<td>3.98 ± 0.74</td>
<td>4.03 ± 0.30</td>
<td>2.75 ± 0.30</td>
<td>0.18 ± 0.03</td>
<td>0.13 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>i.a. LA</td>
<td>0.45 ± 0.09</td>
<td>0.11 ± 0.04</td>
<td>0.10 ± 0.04</td>
<td>0.05 ± 0.03</td>
<td>0.04 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>i.a. LA + HT</td>
<td>0.12 ± 0.04</td>
<td>0.42 ± 0.10</td>
<td>0.10 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>μg/ml ± SD</th>
<th>5 min</th>
<th>2 h</th>
<th>8 h</th>
<th>24 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>i.v. LA</td>
<td>2.56 ± 0.21</td>
<td>2.72 ± 0.21</td>
<td>2.79 ± 0.12</td>
<td>1.51 ± 0.11</td>
</tr>
<tr>
<td>2</td>
<td>i.v. LA + HT</td>
<td>2.86 ± 0.20</td>
<td>2.91 ± 0.13</td>
<td>1.55 ± 0.16</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>i.a. LA</td>
<td>0.45 ± 0.04</td>
<td>0.47 ± 0.04</td>
<td>0.52 ± 0.06</td>
<td>0.44 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>i.a. LA + HT</td>
<td>0.63 ± 0.07</td>
<td>0.69 ± 0.04</td>
<td>0.53 ± 0.03</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

| Liver | i.v. LA | 10.77 ± 1.24 | 12.74 ± 1.41 | 5.30 ± 0.87 | 1.37 ± 0.15 | 0.32 ± 0.04 |
| 2 | i.v. LA + HT | 12.90 ± 1.32 | 4.82 ± 0.81 | 1.25 ± 0.15 | 0.30 ± 0.04 |
| 3 | i.a. LA | 17.06 ± 1.40 | 16.49 ± 0.62 | 11.36 ± 0.94 | 5.21 ± 0.77 | 1.13 ± 0.09 |
| 4 | i.a. LA + HT | 14.83 ± 1.06 | 7.19 ± 0.77 | 1.41 ± 0.29 | 0.44 ± 0.08 |

| Tumor | i.v. LA | 7.03 ± 1.20 | 5.90 ± 0.94 | 2.29 ± 0.70 | 0.50 ± 0.11 | 0.10 ± 0.03 |
| 2 | i.v. LA + HT | 5.85 ± 0.88 | 2.24 ± 0.62 | 0.49 ± 0.09 | 0.10 ± 0.02 |
| 3 | i.a. LA | 27.78 ± 1.69 | 24.38 ± 1.51 | 16.99 ± 2.16 | 6.85 ± 1.75 | 0.63 ± 0.06 |
| 4 | i.a. LA + HT | 28.10 ± 2.02 | 28.95 ± 2.50 | 17.98 ± 2.25 | 0.94 ± 0.16 |

<table>
<thead>
<tr>
<th>% of Injected dose ± SD</th>
<th>5 min</th>
<th>2 h</th>
<th>8 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>i.v. LA</td>
<td>0.01 ± 0.00</td>
<td>3.42 ± 1.07</td>
<td>9.27 ± 1.73</td>
</tr>
<tr>
<td>2</td>
<td>i.v. LA + HT</td>
<td>3.55 ± 1.21</td>
<td>9.34 ± 1.97</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>i.a. LA</td>
<td>2 × 10⁻³ ± 0.00</td>
<td>0.18 ± 0.06</td>
<td>0.75 ± 0.13</td>
</tr>
<tr>
<td>4</td>
<td>i.a. LA + HT</td>
<td>0.48 ± 0.04</td>
<td>1.92 ± 0.20</td>
<td></td>
</tr>
</tbody>
</table>
resected and a plasma sample obtained. Urine was also collected at 5 min, 2 h, and 8 h through a catheter. A volume of Kuthoff buffer equal to 5 times the tissue sample weight was added, the tissues were homogenized, and the Adriamycin was extracted with butanol:toluene (1:1). The samples were centrifuged at 3000 \( \times g \) for 10 min. The supernatants were collected, dried, and dissolved in PBS:methanol (1:1). Adriamycin concentrations in these samples were determined by HPLC (21).

The tumor and liver parenchyma levels of radiolabeled LA (25 \( \mu Ci \) of \[^{3}H\]DDPC/ml of LA), which has the same physical and chemical properties as the unlabeled formulation, were studied in groups of 15 rats each following the experimental protocol described above. Tissue samples were treated with the combustion method (24), and the tissue radioactivity was measured with a scintillation counter (LSC-900; Aloka Co., Ltd., Tokyo, Japan). Adriamycin concentrations in the same samples were determined by HPLC.

### Antitumor Activity Studies

#### Tumor Growth Inhibition

Nine-week-old rats bearing single W256 liver tumors had a laparotomy on day 7. Animals with visible liver tumor were divided into 7 groups of 6-8 animals/group (Table 2). The animals were given injections of LA (2.35 mg/kg) into either the hepatic artery (groups 4 and 5) or the femoral vein (groups 6 and 7) or free Adriamycin (2.35 mg/kg) into the hepatic artery (groups 2 and 3). Animals in groups 3, 5, and 7 were treated with HT using the same techniques and procedures described above. Animals in the control group (group 1b) were treated with an equivalent volume of normal saline given via the hepatic artery. The longest and shortest diameters (\( a \) and \( b \)) of the tumors were measured before drug administration and 5 days later through a second laparotomy. The tumor volume was calculated as

\[
a \times b^2/2
\]

The tumor volume ratio (TVR) was calculated as

\[
TVR = \frac{\text{Tumor volume on day 12}}{\text{Tumor volume on day 7}}
\]

#### Increased Life Span

The design was identical to that of the tumor growth inhibition experiment, but a no-treatment group of 15 animals (group 1a) was added as control. Before drug administration, the existence of liver tumors was surgically confirmed. Animal survival was monitored until day 90 and the %ILS was calculated as

\[
\%\text{ILS} = \left( \frac{\text{Median survival of treated group}}{\text{Median survival of control group}} - 1 \right) \times 100
\]

Animals with a survival time >90 days were considered cured.

#### Statistical Analysis

Differences in biodistribution and tumor growth inhibition were analyzed for statistical significance using Student's \( t \) test, and differences in median survival between animal groups were analyzed using a log rank test.

### RESULTS

#### Characterization of LA

As assessed by the laser dynamic light scattering method, the LA preparation was a homogeneous liposome suspension. The mean particle size was 850 ± 50 (SD) nm in diameter, and the size of all particles (100%) ranged from 772 to 903 nm.

By two different methods, centrifugal filtration and gel filtration, the %EE of Adriamycin in the liposomes were 37.70 ± 1.72 and 38.14 ± 1.64, respectively, in at least five different samples. Physical stability (change of drug remaining in liposomes) of LA suspended in Tris-HCl buffer or human plasma at 2 h at 37°C was >95%.

To compare the \( T_m \) and temperature of maximum release of encapsulated drug from the liposomes, the variation in relative endothermic value and the percentage of release of encapsulated Adriamycin from LA suspended in Tris-HCl buffer is shown in the same figure (Fig. 1). The temperature of maximum release of encapsulated Adriamycin was 41°C, which corresponds to the endothermic peak (\( T_m \)). At this temperature, about 84% of the encapsulated drug was released from the liposomes. Similar results were obtained when LA was suspended in human plasma (data not shown) in accordance with previous results (25-27).

#### Biodistribution

Table 1 shows the organ distribution of liposomal Adriamycin in rats bearing liver W256 carcinosarcoma tumors. The route of administration, i.v. or i.a., was found to alter significantly the organ distribution, independently of the addition of HT. HT was found to alter the organ distribution only when applied after i.a. administration.

In liver and tumor, the drug levels in i.a. treated groups were 1.2-2.5 and 4-24.8 times higher than those in i.v.-treated groups, respectively. Application of liver HT 2 h after i.a. drug administration increased by 1.5-2.6-fold the tumor drug levels at all time points and resulted in sustained tumor drug levels for 6 h. In the liver parenchyma, HT had the opposite effect. The rate of drug disappearance was faster in animals treated with HT; between 2 and 120 h the liver drug levels were reduced by 14-fold in animals not receiving HT and by 33-fold in animals receiving HT.

Administration i.a. resulted in a decreased systemic distribution of liposomal Adriamycin. In heart, plasma, and urine, the drug levels in i.v.-treated groups were 2.2-5.7, 7.0-10.6, and 4.5-35.3 times higher than those in i.a.-treated groups. In animals treated i.v., the addition of liver HT did not alter the systemic organ distribution; in contrast, in animals treated i.a., it increased the plasma drug levels by 4-fold 30 min after completion of HT and the heart drug levels and urine excretion by 1.3- and 2.7-fold at 8 h, respectively.

#### Statistical Analysis

Differences in biodistribution and tumor growth inhibition were analyzed for statistical significance using Student's \( t \) test, and differences in median survival between animal groups were analyzed using a log rank test.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>TVR</th>
<th>% ILS</th>
<th>% Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>No treatment</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1b</td>
<td>Normal saline</td>
<td>4.81 ± 0.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>FA i.a.</td>
<td>1.53 ± 0.23</td>
<td>+27</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>FA i.a. + HT</td>
<td>1.55 ± 0.19</td>
<td>+26</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>LA i.a.</td>
<td>0.84 ± 0.29</td>
<td>+108</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>LA i.a. + HT</td>
<td>0.52 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+242&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>LA i.v.</td>
<td>1.89 ± 0.48</td>
<td>+26</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>LA i.v. + HT</td>
<td>1.87 ± 0.44</td>
<td>+25</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SD.

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**Fig. 1.** Phase-transition temperature and temperature-controlled release of Adriamycin encapsulated in temperature-sensitive liposomes. The relative endothermic value (\( T_m \)) was measured with differential scanning calorimetry. A mixture of hydrated DPPC and Adriamycin at the same concentrations as in the liposomal Adriamycin sample was used as control. The percentage of Adriamycin release (○ --- ○) was monitored at different temperatures (5 samples/point). Arrow, heating time.
The AUCs for each tissue and for plasma and urine were calculated and compared between animals treated with i.a. or i.v. LA, with or without HT (Table 3). In tumor and liver parenchyma, the AUCs after i.a. administration were 8.3–16.8 and 1.3–2.6 times greater than after i.v. administration, respectively. In heart, plasma, and urine, the AUCs after i.a. administration were 26–31. 6–9. and 7–18% of those observed after i.v. administration, respectively. In the i.a.-treated animals the addition of HT increased the tumor, plasma, and urine AUCs by 1.5–2.6-fold but had no effect on the heart AUC. No significant differences were observed between the animals treated i.v. with or without liver HT.

Fig. 2 shows the tumor and liver parenchyma percentage of injected dose decrease of [14C]DPPC and Adriamycin after i.a. administration of radiolabeled LA alone or in combination with HT. HT did not alter rate of [14C]DPPC disappearance in the tumor and liver parenchyma whereas it increased the tumor Adriamycin levels by 1.5–2.6-fold and decreased the liver parenchyma Adriamycin levels.

No significant differences in the clearances of [14C]-DPPC or Adriamycin were found between animals that received radiolabeled LA i.v., with or without liver HT.

**Tumor:Liver and Tumor:Heart Adriamycin Ratio.** Tumor:liver and tumor:heart drug level ratios were calculated for each animal and are shown in Fig. 3. The tumor:liver drug level ratios after i.a. administration without HT were about 1.50 until 24 h, and decreased to 0.61 at 120 h. With i.a. administration and HT the ratio greatly increased to 4.10 at 8 h and 12.5 at 24 h; at 120 h, it was 2.1. The tumor:liver drug ratios in i.v.-treated animals were much lower than those in i.a.-treated animals (0.30–0.66 versus 1.50) and were unchanged after the HT. Therefore, i.a. administration resulted in a preferential distribution of the drug into the tumor tissue compared with i.v. administration, and HT further enhanced the delivery of drug into the tumor, but only in animals treated i.a.

A similar phenomenon was found in the tumor:heart drug level ratio. The tumor:heart drug level ratios after i.a. administration (7.17–57.62) were at least 20 times higher than those after i.v. injection (0–2.57) at all time points. After liver HT, the tumor:heart drug level ratio in i.a.-treated animals was increased by 1.3–1.9-fold between 8 and 120 h after drug administration. No change was observed in the i.v.-treated animals.

**Antitumor Activity.** In the tumor growth inhibition experiment, the size of tumors in animals receiving i.a. LA with or without HT was decreased by 46.7 and 16.1%, respectively, 5 days after treatment (P < 0.05), whereas the tumors of the animals treated with free Adriamycin i.a. or LA i.v. with or without HT were increased by 55, 53, 87, and 89%, respectively (P < 0.01 compared with liposomal Adriamycin i.a.). The actual volumes of tumors of the normal saline control group were 0.08–0.10 cm³ on day 7 and 0.36–0.50 cm³ on day 12.

In the survival experiment, the %ILS was 4–9-fold higher in animals treated i.a. with LA than in animals treated i.a. with free Adriamycin or i.v. with LA (108% versus 27 and 26%, respectively, P < 0.05). When animals received both i.a. LA and HT, the %ILS was further increased by 2.2-fold to 242% (P < 0.01) and 50% of animals were alive on day 90 versus none in all other groups. On the other hand, HT did not alter the %ILS in animals treated with i.a. free Adriamycin or i.v. LA.

**DISCUSSION**

The results of our experiments show that subtherapeutic doses of liver HT applied 2 h after drug administration increase the tumor drug levels and enhance the antitumor activity of Adriamycin against liver tumors when the drug is encapsulated in temperature-sensitive liposomes and delivered via the hepatic artery. These effects were not observed when HT was combined with free Adriamycin administered into the hepatic artery or with Adriamycin encapsulated in the same liposomes administered i.v. Since about 85% of the Adriamycin encapsulated in this type of liposomes has been shown to leak out from the vesicles in vitro at 41°C, the most likely mechanism for the increased tumor drug levels and enhanced therapeutic activity appears to be drug leakage from liposomes retained in the tumor vasculature into the tumor cell compartment. Alternative mechanisms are possible but unlikely to account for most of the beneficial effect observed. Local HT may have profound effects on the tumor vasculature, ranging from dilation of small vessels and increased blood flow when heat at 42°C is applied for 30 min to severe vessel damage accompanied with stasis and extravasation of RBC and complete shutdown of the circulation when heat at >43°C is applied (28). A temperature-dependent chemoembolization or extravasation type of effect might, there-

**Table 3** Tissues, plasma, and urine AUC after i.a. and i.v. injection of liposomal Adriamycin alone or combined with hyperthermia

<table>
<thead>
<tr>
<th>Tissue and Fluid</th>
<th>AUC</th>
<th>i.a.</th>
<th>i.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>i.a.</td>
<td>i.a.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT</td>
<td>Non-HT</td>
</tr>
<tr>
<td>Tumor</td>
<td>87 ± 18b</td>
<td>88 ± 20</td>
<td>1469 ± 171b</td>
</tr>
<tr>
<td>Liver</td>
<td>199 ± 25</td>
<td>212 ± 26</td>
<td>255 ± 34b</td>
</tr>
<tr>
<td>Heart</td>
<td>141 ± 13</td>
<td>140 ± 10</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>Plasma</td>
<td>68 ± 10</td>
<td>65 ± 8</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Urine</td>
<td>42 ± 11</td>
<td>41 ± 9</td>
<td>8 ± 1b</td>
</tr>
</tbody>
</table>

b Mean ± SD.
drug decrease from both organs compared with the i.v. route. As a result, the tumor and liver AUC’s were about 8.2- and 2.6-fold higher, respectively, in animals treated i.a. than in animals treated i.v. The observed dissociation of the parallel decrease of \[^{14}C\]DPPC and Adriamycin after liver HT in animals treated i.a. with \[^{14}C\]DPPC LA is an additional indication that HT results in significant liposome disruption. Interestingly, application of HT had opposite effects in the drug level change in tumor and liver parenchyma. It accelerated drug decrease from the liver parenchyma, probably by enhancing drug uptake, metabolism, and biliary excretion from the vesicles retained in the liver capillaries into the liver parenchymal cells and slowed drug decrease from the tumor, resulting in sustained tumor drug levels for several h after application of HT, probably as a result of transfer of most drug bound to the liposomes in the tumor vascular bed into the tumor cellular compartment.

Large unilamellar vesicles were used because they have the best temperature-controlled drug release properties as a result of their homogeneous size and smaller curvature compared with small unilamellar vesicles (25-27). The %EE of the liposomes used in our study is significantly lower than that reported by others (30-33). The %EE can be easily increased to >70% by preparing the liposomes at a pH of >7.4 which enhances a tight binding between the lipids and the drug in non-ionic form. However, in these type of liposomes, the maximum percentage of drug release by HT is <60%. By preparing the liposomes at a lower pH (6.01), the Adriamycin in ionic form is encapsulated in the aqueous phase which increases the percentage of drug release by HT, although at the expense of a lower %EE.

The use of HT in combination with cytotoxic agents encapsulated in temperature-sensitive liposomes has been described previously in murine models of subcutaneous tumors using liposomal methotrexate and other cytotoxic agents administered i.v. and HT applied less than 30 min after drug administration rather than 2 h as in our study (7, 8, 34–36). In the initial reports, the authors stated that one of the limitations of this approach was its narrow spectrum of clinical use because the concept did not address the problem of disseminated malignancies. However, the development of improved local HT techniques has opened the possibility of applying HT to deep-seated tumors (e.g., liver tumors) (37). The studies presented here represent a step toward the development of this strategy for the treatment of liver metastatic disease, which is an extremely common occurrence in patients with epithelial malignancies. For example, intrahepatic artery administration of a cytotoxic agent encapsulated in temperature-sensitive liposomes followed by liver HT at the time of the surgical resection of a colon carcinoma would be a reasonable approach to investigate in view of the results obtained in our study and the high incidence and mortality from liver metastases in this disease. The dose and timing of HT deserve further investigation to optimize drug release into the tumor and to exploit and integrate the inherent antitumor effects of HT (28). By using the i.a. route of administration, our studies indicate that the application of HT may be delayed for at least 2 h, probably as a result of retention of intact liposomes in the tumor vasculature, and this should allow for a greater flexibility in designing the most appropriate treatment schedule.

Fig. 3. Tumor/liver (a) and tumor/heart (b) Adriamycin ratios obtained in the experiment described in Fig. 2. At each sampling time, 6 rats in each group were sacrificed and Adriamycin levels in liver tumor, liver parenchyma, and heart were determined.

Therefore, explain the results obtained. However, both types of effects appear very unlikely: the chemoembolization effect because HT was applied 2 h after drug administration; and the extravasation effect because of the mild characteristics of the HT treatment used (duration of 6 min and highest temperature reached of 42°C). Synergism between Adriamycin and HT has been described previously (29). Synergism between subtherapeutic doses of HT and Adriamycin or the lipid components of the liposomes can be ruled out with a high level of certainty since no enhancement of antitumor activity was observed when HT was applied to animals treated with free Adriamycin administered into the hepatic artery or LA administered i.v.

In animals treated with liposomal Adriamycin but no HT, the use of the intrahepatic route resulted in 4-fold and 50% increases in tumor and liver parenchyma peak drug levels, respectively, and a slower drug decrease from both organs compared with the i.v. route. As a result, the tumor and liver AUC’s were about 8.2- and 2.6-fold higher, respectively, in animals treated i.a. than in animals treated i.v. The slower drug decrease from the tumor and the liver parenchyma in animals treated i.a. is consistent with a significant retention of intra-vascular liposomes as a result of i.a. administration of liposomes. The observed dissociation of the parallel decrease of \[^{14}C\]DPPC (liposome marker) and Adriamycin after liver HT in animals treated i.a. with \[^{14}C\]DPPC LA is an additional indication that HT results

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