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Effects of liposome-encapsulated drugs on macrophages: comparative activity of the diamidine 4′,6-diamidino-2-phenylindole and the phenanthridinium salts ethidium bromide and propidium iodide

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Abstract

Liposomes can be used for the intracellular delivery of drugs into macrophages. Previously, we developed a liposome-mediated macrophage 'suicide' technique based on the intraphagocytic accumulation of the liposomally delivered bisphosphonate clodronate. Later we found that the diamidine propamidine is even more effective in this approach. In the present study it is shown that liposome-encapsulated 4′,6-diamidino-2-phenylindole (L-DAPI), another well known DNA-binding diamidine, is the most effective drug in killing liver macrophages (Kupffer cells), when intravenously administered in rat. Compared to liposome-encapsulated propamidine (L-propamidine) it showed about 10-fold more activity on a molar basis. Furthermore, L-DAPI was found to induce cell death by inducing apoptosis. The structurally strongly related phenanthridinium salts ethidium bromide (EB) and propidium iodide (PI) exert marked differences in their efficacy. Whereas liposome-encapsulated PI (L-PI) was about 5 times more active in killing macrophages than L-propamidine, liposome-encapsulated EB (L-EB) showed a strongly reduced activity (10 times less than L-PI). As is shown here, PI remains mainly encapsulated in liposomes, while substantial amounts of EB leak out of liposomes. This may very well explain the differences in in vivo activity between L-EB and L-PI. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: 4′,6-Diamidino-2-phenylindole; Propamidine; Ethidium; Propidium; Liposome; Kupffer cell

1. Introduction

The liposome-encapsulated bisphosphonate clodronate can be used for in vivo depletion of macrophages from various organs and tissues. This so-called liposome-mediated macrophage suicide approach is widely applied in order to reveal macrophage functions and to obtain transient suppression of macrophage activity [1–4]. Whereas various other liposome-encapsulated bisphosphonates were inactive in this approach [5], we have recently shown that the liposome-encapsulated diamidine propamidine was about 5 times more efficacious than clodronate [6]. In general, working with L-drugs that are more effective could lead to a reduction of amounts of liposomal components used in experimental models. Furthermore, compounds like the diamidines show considerable toxic effects in vivo, despite their

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; EB, ethidium bromide; PI, propidium iodide; L-drug, liposome-encapsulated drug (L-DAPI, L-EB or L-PI)

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antimicrobial, antitumoral, and antiviral activities [10]. Concerning these effects, it may be obvious that a drug-delivery approach such as used in the liposome-mediated macrophage 'suicide' technique could be very suitable.

It was further shown that both intracellular clodronate and intracellular propamidine induce apoptotic cell death in macrophages [7,8]. Contrary to clodronate, propamidine is known to bind DNA in the minor groove and its cellular activity may well be related to this characteristic [9–12].

In this study we examined whether another well known minor groove binding diamidine, i.e. DAPI [13], is also efficacious in the liposome-mediated macrophage suicide approach. DAPI is frequently used as a dye for staining of DNA. Two other compounds that are fluorescent upon binding to DNA are ethidium bromide and propidium iodide. These phenanthridiniums are structurally well related and bind DNA in an intercalative fashion but not in the minor groove [14]. We determined their activities in the above mentioned approach. Also, we examined whether apoptosis is the main mechanism of cell death after internalization of L-DAPI, L-EB or L-PI in an in vitro assay, using the macrophage-like RAW264 cells.

2. Materials and methods

2.1. Preparation and administration of liposomes

Multilamellar liposomes were prepared as described previously [1]. Briefly, 86 mg phosphatidylcholine (Lipoid E PC, Lipoid, Ludwigshafen, Germany) and 8 mg cholesterol (Sigma, St. Louis, MO, USA) were dissolved (molar ratio 6:1 phosphatidylcholine/cholesterol) in 20 ml chloroform in a round-bottom flask. The lipid film formed on the interior of the flask after rotary evaporation under reduced pressure at 37°C was dispersed in 10 ml saline solutions of propamidine (Rhône-Poulenc Rorer, Dagenham, UK), DAPI, EB or PI (all three purchased from Sigma at various concentrations. Liposomes were not extruded. Finally, the liposomes were resuspended in 4 ml PBS. We estimated their size to be in the range of 0.2–6 μm.

In vivo each concentration of the L-drugs used was tested on a group of four male Wag/Rij rats (aged 9 weeks, approx. 220 g) by an intravenous injection in the tail vein of a 1.5 ml liposome suspension. An untreated group of four rats was taken as a control.

In culture each L-drug was tested in a ratio of 1:10 as volume of liposome suspension to total volume of culture medium.

2.2. Immunohistochemistry

Forty-eight hours after administration of the liposomes the rats were killed, livers removed, frozen in liquid nitrogen, and stored at −80°C. Frozen sections were stained for the presence or absence of Kupffer cells. Staining of the Kupffer cells was performed as described earlier [6,15]. In short, frozen tissues were cut on a cryostat and sections of 8 μm thickness were picked up on glass slides. After air-drying sections were fixed in anhydrous acetone and a two-step immunoperoxidase method using the monoclonal antibody ED2 [16] for detection of Kupffer cells in the rat liver was carried out. ED2 was diluted 1:250 with a 0.1% BSA/PBS solution. A 1:300 dilution of rabbit anti-mouse Ig-peroxidase in 0.1% BSA/PBS solution was used, which could be visualized with 3,3′-diaminobenzidinetetrahydrochloride (DAB; Sigma).

Since ethidium, propidium and DAPI are all fluorescent compounds, uptake of the liposome-encapsulated form of these drugs by liver cells could be visualized on 8 μm sections with a Zeiss fluorescence microscope.

2.3. Determination of Kupffer cells

Numbers of Kupffer cells were determined using a normal light microscope (200× magnification) as described previously [6]. Per liver a total area of 3 mm^2 (in two different sections) was screened for ED2-positive Kupffer cells. The mean number of Kupffer cells in the group of four untreated rats (control group) was taken as 100%.

2.4. Cell cultures

The macrophage-like cell line RAW264 [17] was cultured in 75 cm^2 tissue culture flasks with RPMI

1640 medium (Gibco, New York, USA) containing penicillin-streptomycin (50–50 U/ml) (Gibco) and 10% newborn calf serum. At 20 h, 48 h or 72 h after addition of liposome-encapsulated drugs cells were harvested in 10 mM EDTA/RPMI and washed with fresh medium. In case of addition of free drugs, cells were harvested in 5 mM EDTA/PBS and washed with PBS. Cell viability was determined by trypan blue staining. For leakage studies, after 1 h (L-EB) or 24 h (L-PI, L-DAPI) incubation of L-drug in RPMI 1640 medium, liposomes were removed by centrifugation ($22\,000 \times g$) twice and free drug was measured spectroscopically ($\lambda_{EB} = 280$ nm; $\lambda_{PI} = 302$ nm; $\lambda_{DAPI} = 346$ nm).

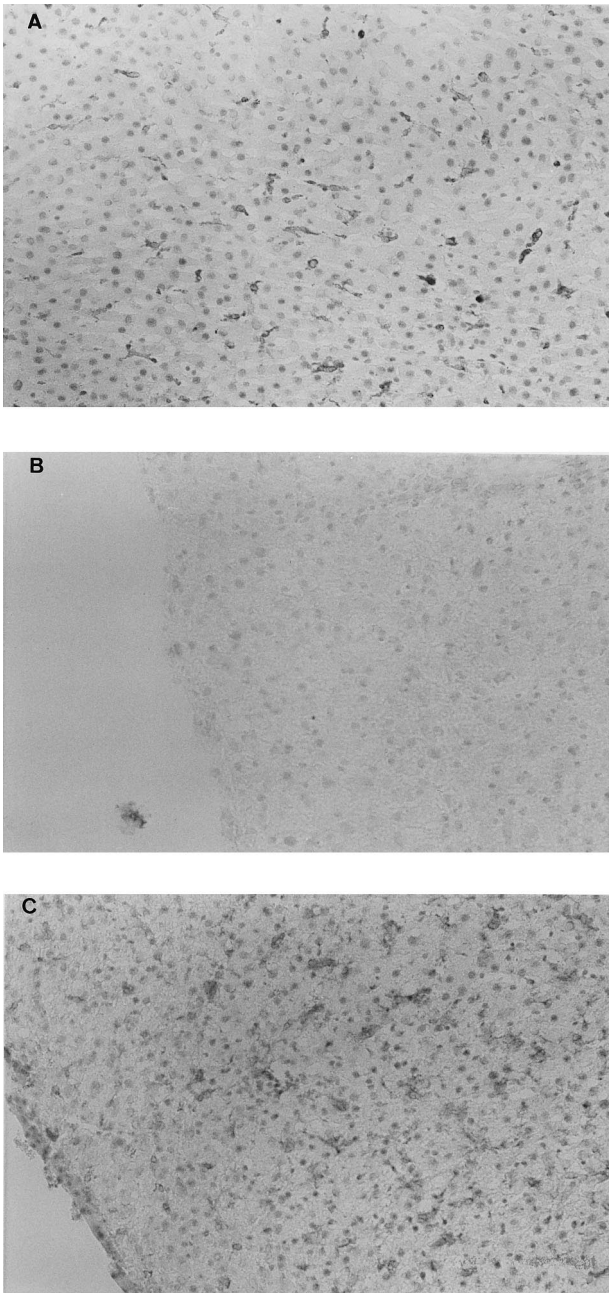

Fig. 1. Kupffer cells on rat liver sections stained with the monoclonal antibody ED2. (A) Normal rat liver, animals at 48 h after intravenous injection of 1.5 ml, (B) 500 μM L-DAPI or (C) 2.5 mM L-EB. Note the absence of ED2-positive cells in B and the swelling of these cells in C.

2.5. Determination of apoptotic cell death

To determine whether apoptosis occurred in RAW264 cells we made use of two methods. Firstly, we measured the appearance of a sub-G1 peak [18] by flow cytometry, using a Becton Dickinson FACScan. This method has been used before to determine apoptosis in RAW264 cells and peritoneal macrophages after treatment with liposome-encapsulated clodronate [7]. In short, 2×10^6 cells were fixed in 70% ethanol at 4°C for at least 24 h, permeabilized in 0.1% NP40/PBS at 0°C for 10 min and incubated for 30 min at 0°C in 50 μg/ml propidium iodide (Calbiochem) in PBS for DNA staining with the addition of 25 μg/ml RNase A (Sigma). Secondly, we analyzed the oligonucleosomal DNA fragmentation by gel electrophoresis [7]. Briefly, cells and debris were taken up in 5 ml lysine buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% SDS, 1 mg proteinase K (Sigma)). After extraction with 1:1 (v/v) phenol/chloroform and pure chloroform, the aqueous phase was collected and incubated with 100 μg RNase A for 1 h at 20°C. Then 450 μl of a 2 M NaCl solution and 2 vols. of 96% ethanol were added for overnight precipitation. After centrifugation pellets were dried and dissolved in 50 μl TE buffer (50 mM Tris-HCl, 1 mM EDTA (pH 8.0)). DNA fragmentation was analyzed on a 1.5% agarose gel with 5 μM ethidium bromide.

3. Results

3.1. In vivo effects of L-EB, L-PI, and L-DAPI on Kupffer cells

Propamidine is effective in the liposome-mediated macrophage suicide technique in lower doses than

the widely used clodronate [6]. In order to find other effective drugs, we focused on the intracellular activity of compounds structurally related to propamidine, i.e. DAPI, PI and EB. In our first experiment we injected intravenously 1.5 ml of the liposome suspensions containing various concentrations of DAPI, PI or EB. While 500 μM L-DAPI (Fig. 1) and 2.5 mM L-PI (not shown) were effective in reducing numbers of ED2-positive Kupffer cells, 2.5 mM L-EB was not (Fig. 1). However, L-EB did affect the macrophages, which resulted in a swollen shape of the cells.

As EB, PI and DAPI are all fluorescent dyes, phagocytosis of the liposome-encapsulated form of these compounds could be visualized on non-fixed liver sections using a fluorescence microscope. Fig. 2 shows a fluorescence image of a liver section of a rat treated with 500 μM L-PI. The picture is illustrative for treatment with L-PI. Similar pictures (not shown) were obtained after treatment with 2.5 mM L-EB and 100 μM L-DAPI. These doses were not effective in killing Kupffer cells. The picture clearly shows the uptake of L-PI.

We studied the effects of administration of these liposome-encapsulated drugs on the survival of Kupffer cells in livers of treated rats and compared it to the survival after treatment with L-propamidine. Previously [6], we found that an injection of 2.0 ml 1.0 mM L-propamidine resulted in a more than 50% reduction of numbers of ED2-positive macrophages. In Fig. 3 it is shown that 1.5 ml 2.5 mM L-propamidine resulted in a 60% decrease in numbers of ED2-positive cells compared to untreated controls. Furthermore, of all compounds tested L-DAPI exhibited the highest intracellular toxicity. Even at 500 μM it induced a nearly complete depletion of ED2-positive Kupffer cells. On the other hand, L-EB showed only limited intracellular activity. Interestingly, liposome-encapsulated PI, which is structurally more related to EB than any of the other compounds described here, was roughly 10 times more effective in eliminating Kupffer cells than L-EB.

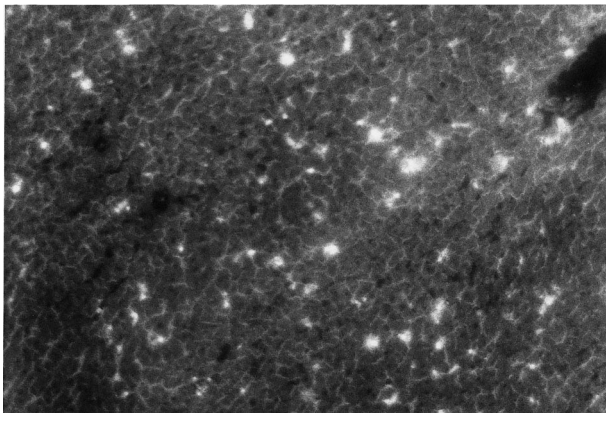

Fig. 2. View on a non-fixed rat liver section using fluorescence microscopy. Liver of an animal at 48 h after intravenous administration of 500 μM L-PI. This picture clearly shows the uptake of L-PI.

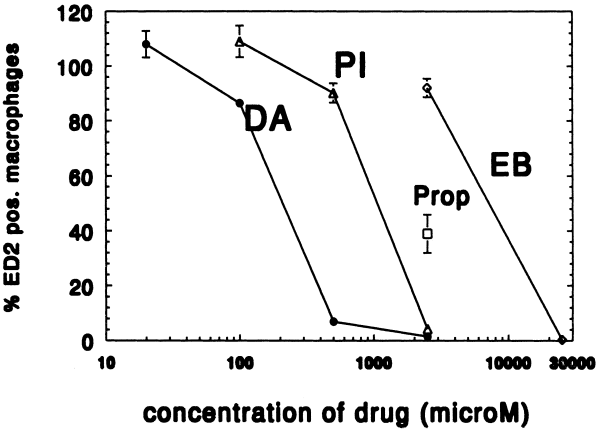

Fig. 3. Effects of various concentrations of 1.5 ml L-drugs on number of ED2-positive Kupffer cells. Types of L-drug are indicated in the figure and their effects were determined on rat liver sections 48 h after intravenous injection. Results of each concentration of L-drug ($n=4$) were correlated with the number of ED2-positive Kupffer cells in untreated controls ($n=4$), which was taken as 100% with S.E. = 17.3. Error bars within each group represent the S.E. as a percentage of the controls (if S.E. <10 ($\approx 2.5\%$), error bars are not drawn).

In order to determine the specificity of the effects of the liposome-encapsulated drugs, we also injected free PI, DAPI and propamidine in a concentration of 50 nmol. On liver sections of these animals we did not find differences in ED2 positivity compared to untreated animals. Also, regarding PI and DAPI, we were not able to detect fluorescence on non-fixed liver sections (data not shown).

3.2. *In vitro determination of apoptosis*

The flow cytometrical detection of the appearance of a sub-G1 peak is a reliable method to determine programmed cell death [18]. We incubated RAW264 cells with in vivo effective doses of 500 μM L-DAPI

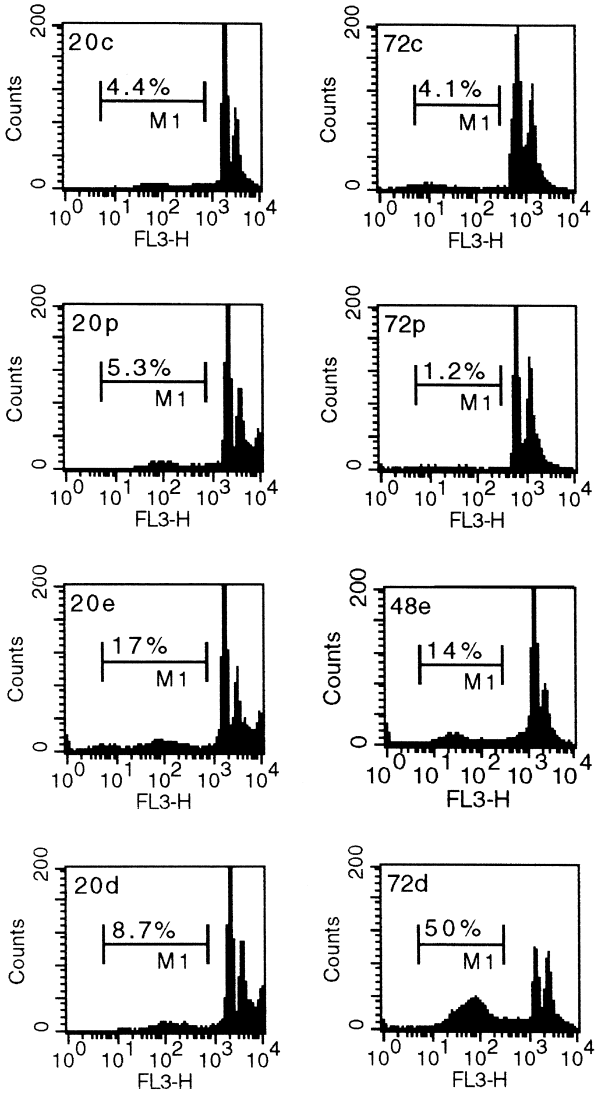

Fig. 4. DNA histograms of control (c) RAW264 cells cultured for 20 h (20c) and 72 h (72c) without liposomes, and RAW264 cells cultured with 2.5 mM L-PI (p) or 500 μM L-DAPI (d) for 20 h and 72 h (20p, 20d and 72p, 72d respectively). In case of 25 mM L-EB (e) the results of 20 h and 48 h are shown (20e and 48e). Percentages of apoptotic cells are included in each histogram.

and 2.5 mM L-PI for 20 h and 72 h. In the case of 25 mM L-EB there are only the results of the 20 h and 48 h incubation, which had already a dramatic effect on cell viability as determined with trypan blue staining (results not shown). After even longer incubation (72 h), only cell debris remained, resulting in noise in the DNA histograms (not shown). In Fig. 4 the DNA histograms of the L-DAPI, L-PI and L-EB treated cells are shown. Untreated cells were taken as control and exhibited a low percentage of apoptosis (about 4%) at both 20 h and 72 h. Similar data were obtained with PBS liposomes (between 5 and 7%, data not shown). Obviously, L-PI did not induce apoptosis, neither at 20 h nor at 72 h (Fig. 4). In addition, relative to the controls, the cultures incubated with L-PI seemed to be unaffected when cell numbers and growth were compared. Even after 72 h no differences could be observed (data not shown here). In contrary, L-EB had a major effect on the viability of the cultures. Already after 20 h there was 17% of apoptosis (Fig. 4) and after 48 h the percentage of apoptotic cells was still 14%. Trypan blue staining showed no living cells after 72 h (results not shown). L-DAPI induced the highest percentage of apoptosis in this experiment, ranging from 8.7% at 20 h to 50% at 72 h.

To be sure that the appearance of the sub-G1 peak in the DNA histograms correlates with the oligonucleosomal DNA fragmentation seen during apoptosis we analyzed the DNA of RAW264 cells incubated with liposomes by gel electrophoresis. Fig. 5 shows the agarose gels with DNA of cells treated 20 h with 25 mM L-EB, 72 h with 500 μM L-DAPI or 72 h with 2.5 mM L-PI. Only L-DAPI induced the DNA pattern typical for an apoptotic process, confirming the presence of the corresponding (L-DAPI) sub-G1 peak from Fig. 4.

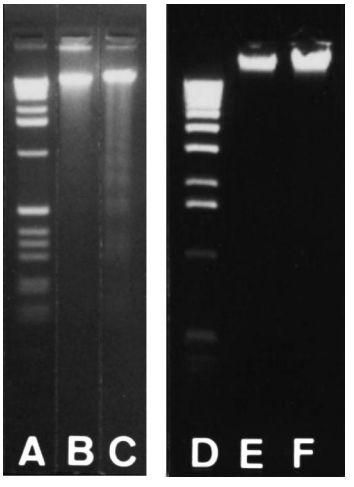

Fig. 5. Agarose gel electrophoresis of DNA from RAW264 cells treated with liposome-encapsulated drugs. Lanes A and D show 1 kb DNA ladders, the other lanes show DNA of cells incubated 20 h with 25 mM L-EB (B), 72 h with 500 μM L-DAPI (C) or 2.5 mM L-PI (E) and DNA of untreated RAW264 cells (F). Only L-DAPI incubation induced DNA fragmentation.

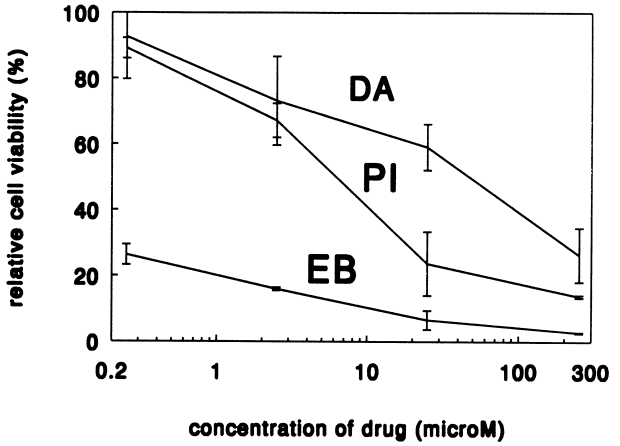

Fig. 6. Cytotoxic effect of free drugs on RAW264 cells. Cell viability was measured using trypan blue staining and related to the viability of control RAW264 cells (100%). DAPI, EB, and PI were tested in four concentrations, as indicated in the figure. We estimated that in culture the amount of 1 ml 2.5 mM liposome-encapsulated drug corresponds roughly to 1 μM free drug. Note the differences in effects on cell viability between DAPI and PI on the one hand and EB on the other at lower concentrations (0.25–2.5 μM).

3.3. Cytotoxicity of free EB, PI and DAPI

Except for L-EB, all L-drugs so far tested exhibited more intracellular activity in vivo than in vitro. Contrary, L-EB was more efficacious in vitro than in vivo. For example, whereas 2.5 mM L-EB was not capable of depleting Kupffer cells in vivo (Fig. 3), it had a major reducing effect on cell numbers in cultures, already after 20 h (results not shown). To investigate this phenomenon we tested the effects of free EB, PI and DAPI on cell viability. Fig. 6 shows these effects for various concentrations. Concentrations of the free drugs in the range of 0.25–2.5 μM were comparable to the amounts administered in the corresponding liposome-encapsulated form. (We estimated that 1 ml 2.5 mM L-drug corresponds to about 1 μmol of free drug.) It appeared that the viability as determined by trypan blue staining after incubation of RAW264 cells with free drug varied with concentration and type of drug. Free EB led to the highest reduction in cell numbers. Its effect was comparable to that of similar liposome-encapsulated amounts (see Section 3). On the other hand, free DAPI and PI in concentrations comparable to their liposomal forms had minor effects on cell numbers, even after 72 h (data not shown).

In order to find out whether the cytotoxic effects of L-EB in vitro are a result of leakage of EB from liposomes, we determined spectroscopically ($\lambda = 280$ nm) the amount of free EB after incubation of 100 μl liposome suspension in 1 ml culture medium. Already after 1 h we detected an amount of 0.04 μmol EB. For L-PI and L-DAPI we could not detect leakage of drug, even after 24 h incubation.

4. Discussion

The so-called liposome-mediated macrophage suicide approach is based on the selective intracellular delivery of liposome-encapsulated drugs and its subsequent accumulation in phagocytes [1]. One of the most frequently used drugs in this technique is clodronate. Especially because of its high hydrophilicity versus low lipophilicity and its rapid secretion from the body by the renal system, this bisphosphonate has the characteristics for proper liposome encapsulation and in vivo use. It will not easily cross liposomal or cellular membranes, so leakage rates will be low and side effects after administration will be minimal. In a recent study [19] it has been demonstrated that the formation of a non-hydrolyzable ATP metabolite may play an important role in induced cell death by clodronate in macrophage-like cells. However, until now it is unknown how clodronate exerts its intracellular effects finally leading to apoptotic cell death in vivo.

Propamidine is an aromatic diamidine with antimicrobial effects. Like clodronate, it is highly soluble in water and together with its low lipophilicity it is well suited for encapsulation in liposomal form. Recently it was found that L-propamidine is even more active in Kupffer cell depletion than L-clodronate and that propamidine, as clodronate, induces apoptotic cell death [6,7]. As with clodronate, the mode of intracellular action of propamidine is not known although its high DNA-binding capacity may play a major role. This interaction with DNA takes place mainly at the minor groove and has been investigated in detail [10]. Since L-propamidine exerts its major intracellular effects probably as a result of its DNA-binding ability, we decided to investigate the intracellular activity of the structurally related compound DAPI and the well known DNA-binding

compounds ethidium and propidium, all in liposomal form.

Like propamidine, DAPI is an aromatic diamidine. At low compound to base pair ratios DAPI binds exclusively to AT base pair sequences in the minor groove [13,20]. Examining the nature of the interaction between DAPI and DNA is particularly relevant considering the development of other sequence dependent DNA-binding drugs. Moreover, unlike propamidine, DAPI is a strong fluorescent agent, which enables us to determine its localization after in vivo administration of the liposome-encapsulated form. Two other strong fluorescent dyes are ethidium bromide and propidium iodide which are frequently used compounds in staining of DNA. These two structurally related compounds bind to DNA in an intercalative fashion and not in the minor groove [14].

We found L-DAPI to be the most potent drug in vivo in reducing the number of ED2-positive cells in the liver. Compared to L-propamidine it is even about 10 times more effective. Apparently the binding of a drug to AT sequences in the minor groove of DNA can be sufficient to disturb cell metabolism eventually leading to cell death. Interestingly, L-PI reduced numbers of ED2-positive macrophages more than L-propamidine. It is likely that propidium will mainly bind DNA after its intracellular release from the liposome. This intercalative interaction with DNA, which does not take place in the minor groove as with DAPI and propamidine, might also be enough to kill the cell. Since these compounds (PI, propamidine and DAPI) show only minimal leakage from liposomes ([21]; this study), variation in the leakage rates of these three molecules does probably not explain differences in efficacy. More probably, differences in DNA binding kinetics or sequence dependence might explain the variation in efficacy. Of all compounds tested here, L-EB had a markedly low effect on the presence of ED2-positive cells in the liver. We have shown here that EB leaks out of liposomes. This probably caused a less effective targeting to macrophages. This is supported by our observations that L-EB showed much more cytotoxic effects on RAW264 cells in culture than any other L-drug tested and, moreover, that these effects are comparable to the ones seen when treating RAW264 cells with similar amounts of free EB. Recently, a method has been developed to detect leakage of ethidium and propidium from PC/cholesterol liposomes [21]. As in this study, it was found that propidium has much lower leaking rates than ethidium. This is probably due to the lower lipophilicity of propidium compared to its hydrophilicity as determined from the octanol/medium partition coefficients.

Furthermore, by making use of the fluorescent properties of EB, PI, and DAPI it is possible to demonstrate, on liver sections, the uptake of the liposome-encapsulated form of these drugs. In addition, to determine the specificity of the effects of liposome-encapsulated drugs in vivo, we analyzed the effects of injection of free PI, DAPI and propamidine on ED2 positivity. On liver sections, we could not find differences in ED2 positivity when compared to untreated animals. Also, regarding PI and DAPI, we could not detect fluorescence on non-fixed sections. Altogether, these results demonstrate the specificity of the in vivo effects of liposome-encapsulated DAPI and propamidine on Kupffer cells. So, for diamidines like DAPI and propamidine but also for propidium iodide, liposomes are suitable carriers to target these drugs to macrophages and may very well be used to suppress toxic side effects. Moreover, encapsulation allows us to compare the intracellular activity of related compounds. For instance, the results from our experiments indicate that DAPI has about 10-fold higher intracellular activity than propamidine.

We clearly demonstrated that L-DAPI, like L-propamidine [7], induced apoptotic cell death in macrophage-like RAW264 cells. Although L-EB also induced a sub-G1 peak on the DNA histogram, we were unable to show DNA fragmentation on an agarose gel. However, the high reduction of cell viability, as determined with trypan blue staining, after incubation of cells with L-EB clearly demonstrated cell death. Earlier it was stated that a rapid process of cell death will favor necrosis [22]. We argue that cell death induced by L-EB in vitro is a fast process and that L-EB induces at least for a part necrotic cell death. Because EB can traverse membrane structures easily, uptake of EB in culture by the target cell can be regarded as liposome independent. In other words, endocytosis of liposomes is not a key step in the intracellular accumulation of EB. Therefore, in vitro EB will probably accumulate in the target

cell more rapidly than drugs that are dependent on liposomes for their uptake (e.g. PI and DAPI). This makes sense when taking into account relatively slow processes as receptor mediated phagocytosis and lysosomal degradation of liposomal components preceding the intracellular release of free drug. It explains the relatively delayed effects of L-DAPI on RAW cells in culture compared to L-EB.

In general, we conclude that in view of the leakage from liposomes and capacity to traverse biomembranes, DAPI and PI, but not EB are suitable for labeling of liposomes or use in the liposome-mediated macrophage 'suicide' technique.

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