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The effects of liposome surface charge and size on the intracellular delivery of clodronate and gallium in vitro

Jukka Mönkkönen *, Riitta Valjakka, Maria Hakasalo, Arto Urtti

University of Kuopio, Department of Pharmaceutical Technology and A.I. Virtanen Institute, P.O. Box 1627, FIN-70211 Kuopio, Finland

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Abstract

Clodronate (dichloromethylene bisphosphonate) and gallium in liposomes are macrophage suppressive agents, and thus possibly useful in inflammatory diseases. The effects of liposome surface charge, liposome type, and size on the delivery of these compounds to RAW 264 macrophages and L929 fibroblasts were evaluated by growth inhibition assay in vitro. Unilamellar liposomes with neutral surface charge (100 mol% DSPC) were unable to deliver clodronate or gallium to macrophages, but inclusion of negatively charged DSPG in liposomes provided effective delivery of the compounds. Large multilamellar liposomes with neutral surface charge were also ineffective in delivering clodronate to macrophages. A decrease in the size of unilamellar DSPG liposomes by extrusion did not affect the delivery of the compounds to macrophages, but drastically increased their potency for fibroblasts. For macrophage delivery, unextruded DSPG liposomes prepared by the REV method were found to be the best formulation studied, while extrusion of liposomes broadened the effects to other cell types. Various lysosomotropic agents did not affect the potency of liposomal clodronate for macrophages, but decreased the potency of liposomal gallium, indicating that, despite their similarities in mechanism of action in macrophages, the intracellular processing of liposomal clodronate differs from that of gallium.

Key words: Clodronate; Gallium; Liposome; Surface charge; Size; Delivery; Macrophage; Fibroblast

1. Introduction

Liposomes have proved to be effective carriers of clodronate (dichloromethylene bisphosphonate) and gallium to macrophages, which otherwise do not readily internalize these compounds (Mönkkönen and Heath, 1993; Mönkkönen et al., 1993). As a free drug, very high concentrations (0.5–1 mM) of clodronate and gallium are required to inhibit the growth of RAW 264 macrophages in vitro, but the encapsulation of the drugs in unilamellar liposomes enhances their potency by a factor of about 50. The effects of liposomal clodronate and gallium are specific for highly endocytotic cells, which probably stems from the failure of liposomes to deliver sufficient intracellular drug concentration to affect the cells with lower endocytotic capacity (Mönkkönen and Heath, 1993; Mönkkönen et al., 1993). In vivo, clodronate encapsulated in multilamellar vesicles (MLV) eliminates macrophages from the spleen,

* Corresponding author.

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liver, lungs, and lymph nodes of mice, when administered via the appropriate routes (Van Rooijen, 1993).

The macrophage suppressive effects of liposomal clodronate and gallium suggest that they may be useful in the treatment of inflammatory diseases, like rheumatoid arthritis (Matkovic et al., 1991; Kinne et al., 1993; Mönkkönen and Heath, 1993; Mönkkönen et al., 1993; Van Lent et al., 1993) and autoimmune uveitis (Niesman et al., 1993), where macrophages have been suggested to be involved in pathological processes (Firestein and Zvaifler, 1990; Bresnihan, 1992). This approach is strongly supported by our findings that liposomal clodronate inhibits LPS-stimulated cytokine production by RAW 264 cells in vitro (Mönkkönen et al., 1994).

Macrophage depletion in vivo by liposomal clodronate was shown using MLVs prepared from neutral egg yolk phosphatidylcholine (EPC) and cholesterol (Claassen and Van Rooijen, 1986). In our in vitro studies we have used unilamellar negatively charged distearoylphosphatidylglycerol/cholesterol (DSPG/Chol) liposomes for the delivery of clodronate and gallium (Mönkkönen and Heath, 1993; Mönkkönen et al., 1993).

The encapsulation efficiency of the drugs in liposomes, liposome size, and surface charge all contribute to the liposome-mediated delivery of compounds in cells (Machy and Leserman, 1983; Heath et al., 1985). Thus, more information about these factors is required in order to establish the optimal liposome formulation for the delivery of clodronate and gallium in macrophages and possibly to other types of cells. In the present study, the effects of these factors on the delivery of clodronate and gallium in macrophages and fibroblasts have been evaluated, and also those of various lysosomotropic agents on the delivery of these compounds in macrophages have been studied in vitro.

2. Materials and methods

2.1. Materials

Clodronate (dichloromethylene bisphosphonate, disodium salt) was obtained from Leiras Pharmaceutical Co. (Tampere, Finland). Murexide (5,5'-nitrilodibarbituric acid), MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), chloroquine, primaquine, and hexamethylene tetramine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Gallium(III) nitrate, hematoxylin trihydrate, and nitrilotriacetic acid (NTA) were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). SDS was from Merck (Darmstadt, Germany) and *N,N*-dimethylformamide (DMF) from Fluka Chemie AG (Buchs, Switzerland). Distearoylphosphatidylglycerol (DSPG) and distearoylphosphatidylcholine (DSPC) were obtained from Orion Farmos Co. (Turku, Finland), and phosphatidylcholine (from egg yolk) and cholesterol from Sigma. Phospholipids and cholesterol were stored in chloroform at −20°C. Dulbecco's modified Eagle's medium (DMEM), 10000 units of penicillin and streptomycin, and fetal bovine serum were from Gibco (Grand Island, NY, U.S.A.), and Sephadex G-50 was purchased from Pharmacia LKB (Uppsala, Sweden). All other reagents were obtained from various suppliers and were reagent grade or better.

2.2. Preparation of liposomes

Stock solutions of clodronate (200 mM) and gallium (100 mM, complexed with NTA) for liposome encapsulation were prepared as described earlier (Mönkkönen and Heath, 1993; Mönkkönen et al., 1993). Unilamellar liposomes were prepared by reversed-phase evaporation (REV) (Szoka and Papahadjopoulos, 1978) from phospholipid/cholesterol (67:33) as described (Mönkkönen and Heath, 1993; Mönkkönen et al., 1993), and will be subsequently referred to by phospholipid content only. In some experiments, liposomes were extruded through 0.1 μm polycarbonate filters (MacDonald et al., 1991). Large multilamellar liposomes (MLV) containing clodronate were prepared according to the method of Claassen and Van Rooijen (1986) from 75 mg of egg yolk phosphatidylcholine (EPC) and 19 mg of cholesterol. The drug content of various liposomes was analyzed spectrophotometrically (Claassen and Van Rooijen, 1986; Zaki and El-

Didamony, 1988) after disrupting the liposomes with 0.1% Triton X-100. The lipid content of liposomes was measured by phosphorus analysis (Bartlett, 1959), and the size distribution by a Nicomp Submicron Particle Sizer utilizing laser light scattering (model 370, Nicomp, Santa Barbara, CA, U.S.A.).

2.3. Cells and cell growth studies

The growth-inhibitory properties of various liposome preparations were studied on a murine macrophage cell line (RAW 264), and on murine fibroblast cell line L929, which were obtained from T.D. Heath, University of Wisconsin (Madison, WI, U.S.A.). The cells were grown in DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C. Cells were plated at 4×10^3 per well in 96-well plates (Nunc, Roskilde, Denmark) and allowed to grow overnight. Quadruplicate wells were treated with 5 μl of drug solutions from half-logarithmic dilution series. On each plate, eight wells were left without cells for the blank column, and four control wells were treated with 5 μl of appropriate buffer. The plates were returned to incubator for 48 h, and at the end of the growth period the cell growth was evaluated using the MTT assay (Hansen et al., 1989; Nargi and Yang, 1993).

The effect of lysosomotropic agents on the growth inhibition properties of liposome-encapsulated clodronate and gallium was studied by performing the experiments in the presence or absence of 7.5 mM NH_4Cl, 10 μM chloroquine, or 10 μM primaquine, which were added 30 min before drug addition.

Experiments were repeated two to four times, and the maximum deviation from the average value was less than 15% in all experiments. The variance between experiments was larger (up to 50%), but in each experiment the difference between various treatments was consistently reproducible.

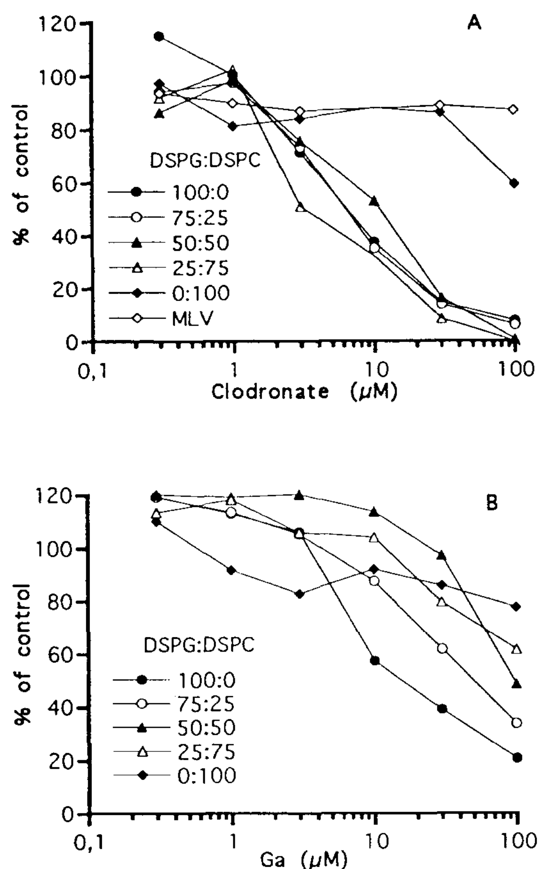

Fig. 1. Effect of various liposome formulations of clodronate (A) and gallium (B) on the growth of RAW 264 cells in vitro. Unilamellar liposomes were prepared from various mixtures (mol%) of negatively charged distearoylphosphatidylglycerol (DSPG) and neutral distearoylphosphatidylcholine (DSPC) by reversed phase evaporation. Large multilamellar liposomes (MLV) were prepared from neutral egg yolk phosphatidylcholine. Data represent mean of two to four independently conducted experiments.

3. Results and discussion

3.1. Effect of surface charge

Fig. 1 shows the growth-inhibitory effects of clodronate (A) and gallium (B) encapsulated in liposomes prepared from various mixtures of DSPG and DSPC on RAW 264 cells in vitro. The potency of clodronate was very similar in all preparations containing negatively charged DSPG, whereas liposomes containing only neutral DSPC were not effective for delivering clodronate in RAW 264 cells. This is clearly seen when the IC_{50} values (i.e., the concentration of

drug required to produce 50% inhibition of cell growth) of clodronate are plotted as a function of molar percentage of DSPG of total liposomal phospholipid (Fig. 2A). The results indicate that 25 mol% of DSPG in the liposomes is sufficient for optimal delivery of clodronate in RAW 264 cells.

In contrast, the potency of gallium gradually increased with increasing portion of DSPG (Fig. 1B), and optimal delivery was achieved with liposomes containing 100 mol% DSPG (Fig. 2B).

Non-loaded liposomes at the concentrations used for the delivery of clodronate and gallium did not significantly affect cell growth (data not shown), indicating that the observed effects were due to the drugs, and not to phospholipids.

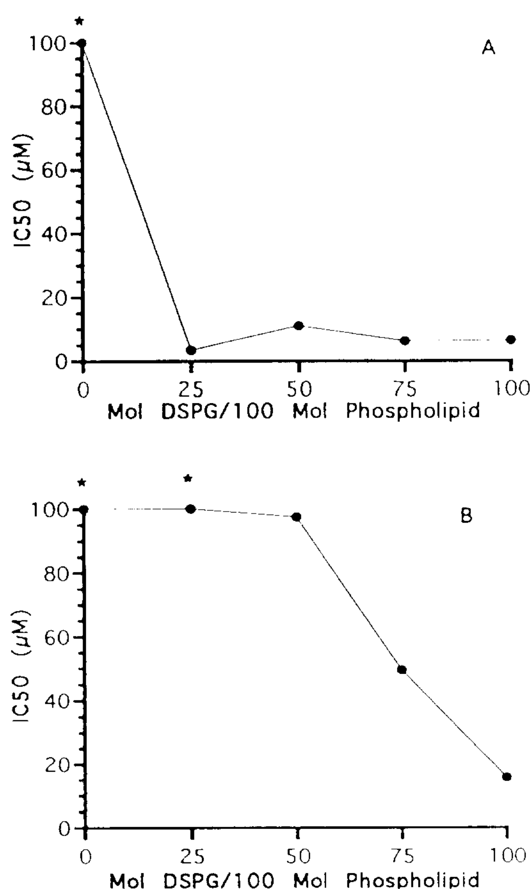

Fig. 2. Effect of surface charge density on the IC_{50} value of encapsulated clodronate (A) and gallium (B) for RAW 264 cells in vitro. *The IC_{50} is greater than the highest drug concentration used (100 μM).

Table 1
Drug/lipid ratio and size of various liposome formulations containing clodronate and gallium

Liposome	Drug/lipid ratio [a] (mol/mol)	Size [a] (nm)
Clodronate liposomes		
DSPG:DSPC [b] (mol%)		
100:0	1.27	168
75:25	1.38	210
50:50	1.46	177
25:75	0.84	190
0:100	1.01	182
Extruded [c]	0.40	128
MLV [d]	0.72	> 5000
Gallium liposomes		
DSPG:DSPC [b] (mol%)		
100:0	0.70	178
75:25	0.59	205
50:50	0.79	240
25:75	0.59	211
0:100	0.65	230
Extruded [c]	0.29	121

[a] Mean of two to three separately prepared batches.
[b] Prepared by REV method from distearoylphosphatidylglycerol (DSPG) and distearoylphosphatidylcholine (DSPC).
[c] Prepared from 100 mol% of DSPG.
[d] Multilamellar vesicles, prepared from egg yolk phosphatidylcholine (EPC) and cholesterol.

The amount of the drug in each liposome is an important determinant for optimal liposomal delivery of compounds (Schwendener et al., 1984; Heath et al., 1985). Thus, the differences in drug/lipid ratios in various liposomes may contribute to their drug delivery properties. However, the mixing of neutral DSPC phospholipid with negatively charged DSPG in unilamellar liposomes did not have any striking effect on the encapsulation of clodronate and gallium; the drug/lipid ratios varied between 0.8 and 1.5 mol of clodronate per mol of phospholipid, and between 0.6 and 0.8 mol of gallium per mol of lipid in various DSPG/DSPC liposomes, as shown in Table 1. We have previously reported similar drug/lipid ratios for clodronate (Mönkkönen and Heath, 1993) and gallium (Mönkkönen et al., 1993) encapsulated in DSPG liposomes. Hence, differences in encapsulation of clodronate and

gallium in different liposomes do not explain the variation in delivery of the drugs.

Liposome-encapsulated drugs rely on the adsorptive endocytosis of the liposome for their efficient delivery to cells (Heath and Brown, 1990). The liposome uptake in cells is a two-step process involving binding of the vesicles on the cell surface and subsequent endocytosis (Lee et al., 1992). In general, negatively charged liposomes are bound to and endocytosed by cells to a greater extent than neutral liposomes (Stevenson et al., 1984; Heath et al., 1985), although this difference is not as great for macrophages as seen for other cell lines (Heath et al., 1985; Daleke et al., 1990). Nevertheless, the present results show that the delivery of clodronate and gallium in macrophages is much more effective by negatively charged than neutral liposomes, as reported for some other liposome-dependent drugs, such as *N*-(phosphonacetyl)-L-aspartic acid (Heath and Brown, 1990). The reason why 25 mol% of DSPG is sufficient for optimal delivery of clodronate, while gallium appears to require 100 mol% of DSPG, is not clear, but may stem from the different intracellular growth inhibitory action vs intracellular concentration relationship of the drugs, making gallium more susceptible to a lower uptake rate.

3.2. Effect of liposome type and size

Clodronate encapsulated in MLVs prepared from EPC and cholesterol did not affect the growth of RAW 264 cells in vitro (Fig. 1A). The drug/lipid ratio was also much lower and the size much larger in MLVs than in unilamellar liposomes (Table 1). Van Rooijen (1989, 1993) has shown that clodronate encapsulated in this type of MLVs eliminates macrophages in spleen, liver, lungs, and lymph nodes in mice when administered via the appropriate routes, and this liposome-mediated macrophage elimination by clodronate has been used for the study of functional aspects of macrophages in vivo. In vitro data on the interaction of clodronate encapsulated in MLVs are, however, scarce. In one study, 0.7 mM clodronate in MLVs was required for the elimination of macrophages from spleen cell suspension (Claassen et al., 1990), while the IC_{50} of clodronate for RAW 264 cells in DSPG liposomes is 6–17 μM (present study; Mönkkönen and Heath, 1993). Macrophages are able to phagocytose particles of at least 2 μm in diameter (Darnell et al., 1990). Clodronate containing MLVs have a mean diameter of over 5 μm, but part of the liposomes are smaller than 2 μm, this proportion appearing to be sufficient for the elimination of macrophages in vivo. RAW 264 cells are probably incapable of ingesting enough large particles, especially when they possess neutral charge, and consequently, are not affected by clodronate MLVs. It seems obvious that unilamellar DSPG liposomes are much more effective for delivering clodronate in macrophages in vitro than multilamellar MLV liposomes, most probably because of their negative surface charge, smaller size, and greater encapsulation efficiency per mol of phospholipid. If this is also the case in vivo, much lower clodronate and phospholipid doses would be required to eliminate macrophages, when DSPG liposomes are used.

In order to define the effect of vesicle size of negatively charged unilamellar liposomes on the potency of liposomal clodronate and gallium, we extruded liposomes prepared from 100 mol% of DSPG through 0.1 μm polycarbonate filters. Extrusion reduced the size of the vesicles from 170–180 to 120–130 μm, and also decreased the drug/lipid ratio considerably (Table 1). The extrusion of DSPG liposomes did not affect the potency of clodronate on RAW 264 cells (Fig. 3A), whereas it somewhat decreased the potency of encapsulated gallium (Fig. 3B). It is unclear whether there is a different mechanism of macrophage uptake (e.g., phagocytosis vs less efficient endocytosis) of liposomes with different size (Schwendener et al., 1984). In the case of clodronate, our results suggest independence of size for the uptake of unilamellar liposomes, whereas the decreased potency of gallium in extruded liposomes indicates an even lower rate of uptake of smaller liposomes by RAW 264 cells. Nevertheless, the present results indicate that unextruded DSPG liposomes provide optimal delivery of both clodronate and gallium in macrophages.

The cells other than 'professional phagocytes' take up liposomes most probably through adsorptive endocytosis mediated by coated pits (Straubinger et al., 1983). This route for the uptake of liposomes is size dependent, as shown with lymphoma cells and L929 fibroblasts, which are sensitive to drug delivery by small liposomes, but not by larger vesicles (Machy and Leserman, 1983). We have previously demonstrated that L929 cells are insensitive to liposomal delivery of clodronate and gallium by unextruded DSPG liposomes, and suggested that this stems from the failure of the drugs to reach sufficient intracellular concentration to affect the cells with lower endocytotic capacity (Mönkkönen and Heath, 1993; Mönkkönen et al., 1993). Therefore, we determined whether L929 fibroblasts are susceptible to clodronate and gallium encapsulated in extruded DSPG liposomes. As shown in Fig. 4, the reduction of vesicle size by extrusion considerably increased the growth inhibitory potency of both drugs, whereas unextruded clodronate and gallium liposomes did not produce any growth inhibition of L929 cells. This indicates that extruded liposomes were small enough to be taken up through endocytosis by fibroblasts.

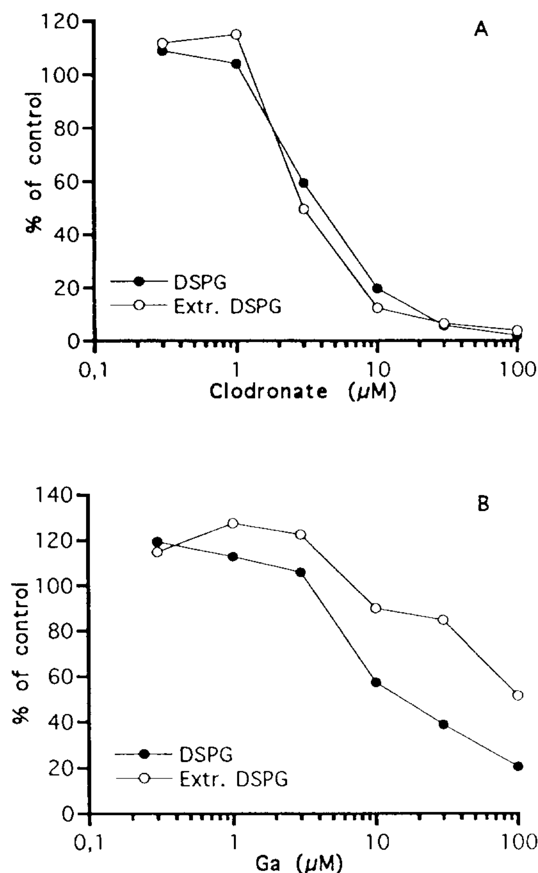

Fig. 3. Effect of extrusion of liposomes on the growth inhibition of RAW 264 cells by encapsulated clodronate (A) and gallium (B). Liposomes were prepared from 100 mol% of DSPG (DSPG), and extruded through 0.1 μm polycarbonate filters (extr. DSPG). Data are mean of two experiments.

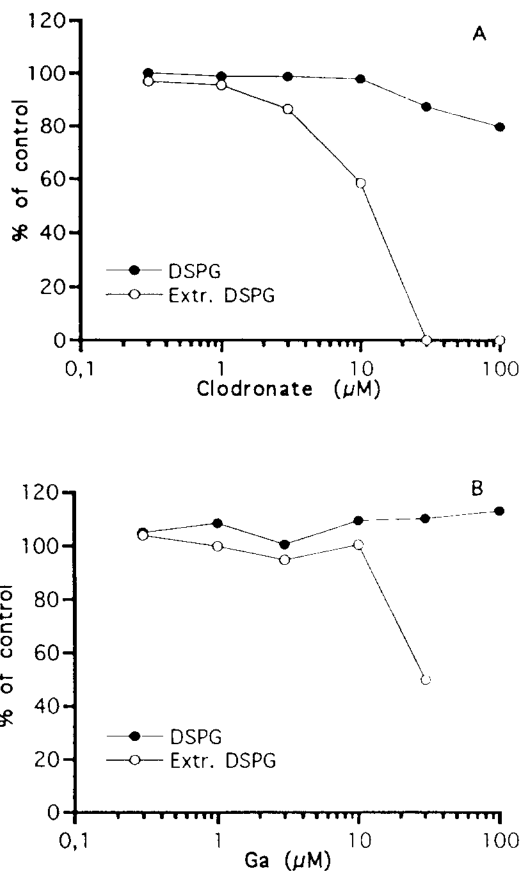

Fig. 4. Effect of extrusion of liposomes on the growth inhibition of L929 cells by encapsulated clodronate (A) and gallium (B). Liposomes were prepared from 100 mol% of DSPG (DSPG), and extruded through 0.1 μm polycarbonate filters (extr. DSPG). Data are mean of two experiments.

The major conclusion from these findings is that if it is desired to target the effects of clo-

dronate and gallium to macrophages, i.e., in inflamed joint cavity, and to avoid effects on other cells, then the optimal formulation would be unextruded DSPG liposomes. On the other hand, if both macrophages and fibroblasts play a major role in the pathogenesis of joint destruction in rheumatoid arthritis, as proposed by Firestein and Zvaifler (1990), extruded DSPG liposomes might be the best choice for the delivery of clodronate and gallium intraarticularly.

When liposomes are used 'locally', e.g., injected intra-articularly, it is unlikely that they will gain access to blood circulation and affect cells other than those in the joint cavity. However, liposome encapsulated radioactive gallium (^{67}Ga) complexed with NTA has been used for pharmacokinetic studies to monitor the in vivo distribution of liposomal content. In such studies the possible harmful effects of liposomal gallium on macrophages of reticuloendothelial system should be taken into account.

3.3. Effects of lysosomotropic agents

In earlier studies we found that the co-incubation of RAW 264 cells with liposomes and NH_4Cl did not significantly affect the growth inhibitory properties of liposomal clodronate (Mönkkönen and Heath, 1993), whilst it did inhibit the effects of liposome-encapsulated gallium (Mönkkönen et al., 1993). In the present study, we further examined the effects of lysosomotropic agents NH_4Cl (7.5 mM), chloroquine (10 μM), and primaquine (10 μM) on the growth inhibitory effects of encapsulated clodronate and gallium for RAW 264 cells. As shown in Fig. 5, NH_4Cl and chloroquine did not significantly affect the delivery of liposomal clodronate, while primaquine seemed to result in a slight increase in the potency of the drug (Fig. 5A). The effects of encapsulated gallium were inhibited strongly by NH_4Cl, and also slightly by chloroquine and primaquine.

NH_4Cl, chloroquine, and primaquine are known to elevate endosomal and lysosomal pH (Poole and Ohkuma, 1981; Reif et al., 1991), and the inhibition of drug delivery by NH_4Cl has been taken as evidence for the involvement of adsorptive endocytosis in the effective delivery of

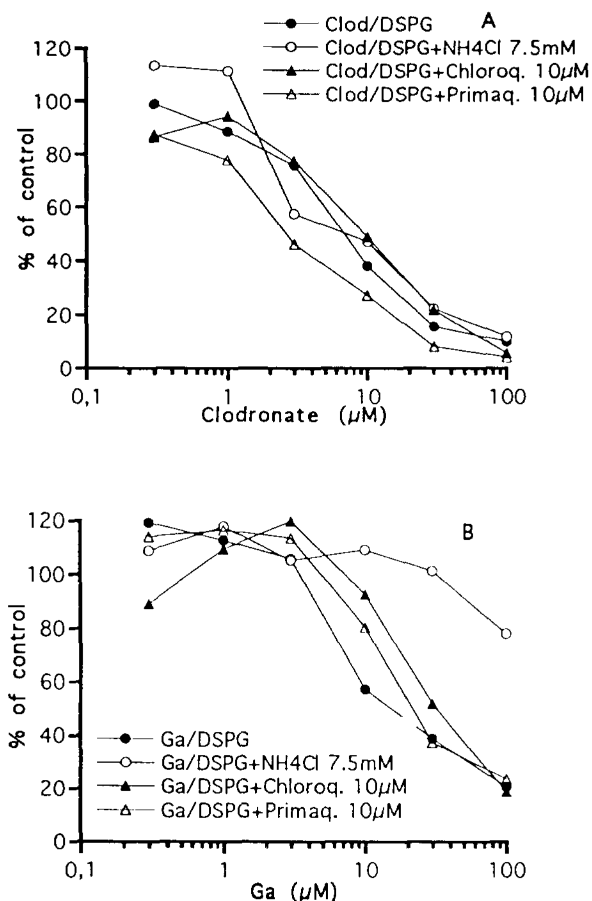

Fig. 5. Effects of NH_4Cl, chloroquine, and primaquine on the inhibition of RAW 264 cell growth by clodronate (A) and gallium (B) encapsulated in DSPG liposomes. Data are mean of two experiments.

the drug (Heath et al., 1985), although the effects of NH_4Cl on liposomal drug delivery have been variable depending on the cell line and drug studied (Heath et al., 1985; Heath and Brown, 1990). The finding that the delivery of liposomal clodronate is not affected by NH_4Cl and chloroquine, and even slightly enhanced by primaquine, is surprising, and suggests that the acidification of endosomes and lysosomes is not necessary for the intracellular uptake and effects of liposomal clodronate. The inhibition may have been only minimal because the concentrations of lysosomotropic agents used were rather low, since at higher concentrations they produce significant growth inhi-

bition of RAW 264 cells (data not shown), and the interpretation of resultant growth inhibition curves is difficult. However, 10 mM NH_4Cl and 1 μM of chloroquine led to a rise of more than 1 unit in intralysosomal pH in mouse peritoneal macrophages within 5 min after addition to the growth medium (Poole and Ohkuma, 1981), and the effects of liposomal gallium were inhibited at these concentrations (Fig. 5B), indicating that intracellular processing of liposomes was affected at the concentrations of lysosomotropic agents used.

It has been reported that, in macrophages, NH_4Cl blocks the fusion of endosomes to lysosomes, thus interrupting the endocytotic pathway (Gordon et al., 1980), while chloroquine enhances endosome-lysosome fusion (D'Arcy Hart and Young, 1978). Because NH_4Cl is unable to inhibit the effects of liposomal clodronate, we have previously speculated as to whether clodronate already acts at the endosomal compartment and does not have to reach lysosomes at all (Mönkkönen and Heath, 1993). The present results suggest that, in addition to independence of the acidification of intracellular vesicular compartments, the action of liposomal clodronate is also independent of the endosome-lysosome fusion rate.

All the lysosomotropic agents tested inhibited the action of liposomal gallium, suggesting that the action of gallium requires the acidification of endosomes and lysosomes, but is independent of the endosome-lysosome fusion rate.

Both liposomal clodronate and gallium seem to affect the growth of RAW 264 cells by depletion of intracellular iron from the cells (Mönkkönen and Heath, 1993; Mönkkönen et al., 1993). Although they probably share at least partially the same mechanism of action in the growth inhibition of RAW 264 cells, the corresponding forms of intracellular processing required for their action, however, differ from each other for as yet unclear reason(s). For example, whether the release of clodronate from liposomes in endosomes and/or lysosomes occurs at different pH than that of gallium, or whether clodronate can affect iron metabolism at higher pH than gallium, remains to be clarified in further studies.

4. Conclusions

The present study confirms the liposome-mediated delivery of clodronate and gallium in macrophage-like cells in vitro, and indicates that unilamellar liposomes prepared from negatively charged DSPG are by far more effective for the delivery of these compounds than liposomes containing neutral DSPC. Although only 25 mol% of DSPG is required for optimal delivery of clodronate in macrophages, the liposomes prepared from 100 mol% of DSPG have been chosen for further studies because of the simplicity of the liposome preparation procedure. The lack of potency of clodronate encapsulated in MLVs in vitro suggests that also in vivo unilamellar DSPG might provide more efficient delivery of the drug into macrophages. The delivery of clodronate and gallium in different cell types is dependent on liposome size; unextruded liposomes provide macrophage specific targeting of the compounds, while extrusion of liposomes makes these compounds also active against, e.g., fibroblasts. Thus, the choice of liposome size depends on the target for the delivery of clodronate and gallium.

Acknowledgements

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