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Liposome and Multiple Emulsion Formulations Augment the Anticalcifying Efficacy of Phosphocitrate in a Cutaneous Calcergy Model

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Abstract

The anticalcifying agent phosphocitrate was incorporated into phosphatidylcholine/cholesterol liposomes by reverse-phase evaporation. The compound was entrapped to the extent of 11·6% (mol mol^{-1} of lipid) and the liposomes exhibited prolonged retention of the compound when incubated with rat plasma.

Phosphocitrate's ionic contribution in solution adversely influenced the encapsulation efficiency but improvements were made through ion-pairing with the quaternary ammonium detergent cetrimide, or with the inclusion of stearylamine in the lipid phase. The liposomal dose that could be practically administered in-vivo was restricted to 2·5 mg phosphocitrate kg^{-1} day^{-1}. The formulation of a multiple emulsion preparation of phosphocitrate, however, offered an alternative delivery mode permitting infrequent dosing to be successfully investigated. In a rat calcergy model, both vehicles effectively reduced the formation of induced subcutaneous calcified plaques at doses for which the phosphocitrate salt alone was inactive.

The current formulations demonstrate that the therapeutic efficacy of phosphocitrate can be markedly improved through an appropriately designed drug delivery system, signalling a new approach for the future therapeutic application of this compound.

Pathological mineralization is evident in urolithiasis, crystal induced arthropathies, atherosclerosis and miscellaneous disorders in which calcification is causal to or perpetuates the morbidity. Prosthetic implants, too, are often impaired by insoluble salt deposits and some medical procedures (e.g. balloon angioplasty) can unavoidably produce damaged tissue that may calcify. Surgery offers a short-term solution but many calcific diseases (Anderson 1983) are by nature chronic or recurrent and often require prolonged prophylactic therapy.

Phosphocitrate is a compound with a demonstrated ability to interfere with the formation and crystallization of some pathologically significant calcium and magnesium salts (Tew et al 1980; Williams & Sallis 1981; Sallis et al 1988). It therefore offers the potential to not only help in the control of calcium related diseases (Tew et al 1981; Shankar et al 1986; Krug et al 1993) but also in such diverse situations as the mineralization of implanted prosthetic materials (Tsao et al 1988) and the protection of cell membranes against crystal-induced damage (Sallis et al 1989). Its action is believed to stem from a favourable stereochemistry and strong negative charge:size ratio (Ward et al 1987). Phosphocitrate has been identified biologically (Lehninger 1977; Williams & Sallis 1981), evidence for an enzymatic biosynthesis of the compound having recently been obtained in liver and kidney homogenates (Moro et al 1990; Romanello et al 1993). So far, its exogenous use in rodents has not revealed any toxic effects in respect to growth or bone mineralization (Tsao et al 1988; Krug et al 1993) and serum and haematological parameters appear completely unchanged after 90-day high-dose experiments (Shankar 1986).

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A suitable method of administering the compound to animals to allow maximum efficiency of its therapeutic potential now offers a challenge. There is evidence of limited intestinal absorption of phosphocitrate, reducing bioavailability by the oral route (Cooper & Sallis 1993). Injecting phosphocitrate intraperitoneally or intramuscularly circumvents the intestinal problem but maintaining a constant drug concentration at all times within the therapeutic range also becomes an important consideration. To date, studies with common laboratory animals have required daily parenteral phosphocitrate doses of 50–100 mg kg^{-1} to effectively combat induced calcific states. Bolus injections provide a rapid availability to tissues (Williams & Sallis 1981) but, for remedial drug action, a site of ongoing pathological calcification would clearly benefit from a continuous sustained availability of phosphocitrate in the circulation over a 24-h period. In this respect, a liposome or multiple emulsion preparation could enhance the potential of the compound and perhaps offer other useful benefits. The therapeutic efficacy of drugs is known in many instances to be improved through altered biodistribution patterns and a local depot effect. With liposomes and multiple emulsion formulations, there is a choice of biocompatible materials and techniques that might be used, and both systems are reported to have high encapsulation efficiencies for water-soluble compounds (Szoka & Papahadjopoulos 1978; Davis & Walker 1983). Further, their use is gaining clinical acceptance (Davis & Walker 1987; Ostro & Cullis 1989). Presentation of anticalcifying agents via such systems has not been widely pursued although liposomal entrapment of dichloromethylene diphosphonate to eliminate macrophage-like tumour cells has been reported (Van Rooijen et al 1988).

In the current study, we have investigated the feasibility and benefits of entrapping phosphocitrate into liposomes

and multiple emulsions. To determine whether a reduction in dose level or frequency was achievable with the new systems, their inhibitory activity against subcutaneous calcification was tested in a rat model and compared with that of the phosphocitrate salt alone.

Materials and Methods

Chemicals

Unlabelled and [1,5-^{14}C]phosphocitrate (sp. act. 50·7 MBq mmol^{-1}) was synthesized in our laboratory and converted to the sodium salt at pH 7·4 as previously described (Tew et al 1980; Williams & Sallis 1980). L-α-phosphatidylcholine (PtdCho) type V-E from egg yolk, cholesterol (Chol), stearylamine (SA) and distearoylphosphatidyl-choline (DSPtdCho) were purchased from the Sigma Chemical Co., St Louis. Polyoxyethylene sorbitan monooleate (Tween 80), and sorbitan monooleate (Span 80) were products of Ajax Chemicals, Sydney, while BP grade cetrimide and light liquid paraffin BP (medicinal quality) were obtained from ICI, Manchester and F. H. Faulding Co. Ltd, Thebarton, South Australia respectively. The polyacrylamide gel type used was Biogel P-6 (Bio-Rad, Richmond, CA). All other chemicals and solvents were of reagent grade with the exception of di-iso-propyl ether which was purified prior to use by passage through a 1 × 15 cm aluminium oxide column (basic activity II) to remove peroxides. Phosphate-buffered saline (PBS) was prepared as described by Szoka & Papahadjopoulos (1978).

Preparation and in-vitro testing of liposomes

Liposomes were produced by adapting the technique for reverse-phase evaporation vesicles (REV; Szoka & Papahadjopoulos 1978). Accordingly, for a routine preparation, phosphocitrate (112 mM) dissolved in 1·5 mL 10% PBS (pH 7·4) was used as the aqueous phase and subsequently emulsified by sonication into 9·0 mL chloroform/di-iso-propyl ether (1 : 1, v/v) containing PtdCho (49·5 μmol) and cholesterol (49·5 μmol). Organic solvent was then removed under vacuum. The resulting liposomes were centrifuged (25 000 *g*, 15 min), resuspended in PBS and extruded under nitrogen pressure through a 0·2 μm polycarbonate filter. Unentrapped phosphocitrate was removed by gentle centrifugation (800 *g*, 60 s) of the liposome suspension through 5·0 mL Biogel P-6 polyacrylamide gel minicolumns. The Biogel was initially equilibrated in PBS and before use, excess buffer was removed by centrifugation (800 *g*, 30 s).

The final concentration of phosphocitrate liposomes in suspension was established by extracting the lipids with a 9 : 1 (v/v) chloroform/methanol solution and assaying for lipid phosphorus (Chalvardjian & Rudnicki 1970) and cholesterol (Heider & Boyett 1978). Phosphocitrate is not extracted under the organic solvent conditions. The liposomal size distribution was confirmed by transmission electron microscopy. Sample (10-μL drop) was applied to copper Celloidin-coated grids preconditioned with a drop of wetting agent (bacitracin, 0·1 mg mL^{-1}), and negatively stained with 2% (w/v) uranyl acetate prior to viewing. The phosphocitrate liposomes prepared under these conditions were used for all in-vitro and in-vivo studies. Alterations to this standard procedure were made in respect to varying the composition of the lipids or of the aqueous phase for the purpose of investigating alternative entrapment strategies. For DSPtdCho liposomes a sonication temperature of 60–62°C was necessary and the organic phase consisted exclusively of di-iso-propyl ether. Solvent evaporation was carried out at 30–35°C to prevent excessive boiling of solvent and loss of material.

To measure the degree of phosphocitrate entrapment, vesicles were first lysed with 1% (v/v) Triton X-100 in 0·5 mL 10 mM glycine buffer. The liberated phosphocitrate was then hydrolysed to citrate and phosphate by adding 5 μL alkaline phosphatase (7500 units mL^{-1}) to the lysing medium and incubating for 1 h at 37°C. A total estimate of phosphocitrate was obtained by assaying for citrate before and after hydrolysis using a previously described coupled enzyme assay (Williams & Sallis 1980). The lipid and detergent components did not interfere with the assay conditions.

Liposomal stability was assessed in-vitro in the presence of rat plasma by monitoring the release of [^{14}C]phosphocitrate. Freshly prepared liposome suspensions (0·5 mL) containing 42 μmol lipid and [^{14}C]phosphocitrate at a total count of 170 000 d min^{-1} were incubated with rat plasma (2·5 mL) at 37°C in an orbital shaker (125 rev min^{-1}). Aliquots (125 μL) were removed in duplicate from the incubation mixture at specified time intervals. One aliquot was placed directly in scintillation fluid to obtain total radioactivity (C_{total}) for the sampled volume. The second aliquot was purified from released phosphocitrate by Biogel P-6 minicolumn centrifugation and the eluent counted for retained activity ($C_{retained}$). The percentage of liposomally-retained [^{14}C]phosphocitrate (% R_t) was calculated as

$$\% R_t = 100 \cdot C_{retained}/C_{total} \quad (1)$$

Control experiments with free [^{14}C]phosphocitrate showed no evidence of the compound binding to plasma proteins since the polyacrylamide columns displayed a better than 96% efficiency in removing radioactivity from plasma at the maximum experimental concentrations.

Preparation of w/o/w emulsions

Parameters favourable for the formation of a stable w/o/w emulsion with maximal entrapment of water-soluble compounds were selected on the basis of work by Davis & Walker (1983). Phosphocitrate (112 mM) in 10% PBS was emulsified (5 min, vortex mixer) with light liquid paraffin oil at a primary phase volume ratio ($\phi w_1/o$) of 0·2. Span 80 was included in the oil phase at 10% (w/w). The primary phase was further emulsified for 30 s into PBS containing 10% Tween 80 (v/v) at a secondary phase volume ratio ($\phi w_1/o/w_2$) of 0·2. The dispersed primary emulsion droplets were examined by photomicroscopy.

Treatment of subcutaneous calcergy in rats with liposomal and emulsion phosphocitrate

The relative efficacy afforded by the liposome and emulsion vehicles, compared with that of phosphocitrate alone, was tested in a rat model of subcutaneous calcergy (Doyle et al 1979). Male hooded Wistar rats, 190–220 g, were injected at two dorsal subcutaneous sites with 0·2 mL 0·1% (w/v) $KMnO_4$ solution. Treatment groups were established so

Table 1. Entrapment of phosphocitrate by phosphatidylcholine/cholesterol (1 : 1) liposomes.

Aqueous phosphocitrate (mM)	% Entrapment[a]		Captured volume μL (mg lipid)$^{-1}$
	w/w	mol/mol	
10	0·61	0·77	1·35
50	3·82	4·85	1·69
112	9·14 ± 0·67[b]	11·6	1·81 ± 0·13[b]

[a] Calculated as mg phosphocitrate/100 mg lipid and mol phosphocitrate/100 mol lipid (mean of 2 measurements). [b] Mean of six independent preparations ± s.d.

that for the next 10 days, rats (5–10 per group) were given intraperitoneal injections of either freshly-prepared phosphocitrate liposomes, phosphocitrate emulsions or for comparison, equivalent doses of the compound in solution. Liposomes were administered daily whereas a separate study with the emulsion formulation examined the effectiveness of a dose injected at 3-day intervals. Control groups for both experiments received either empty vehicles or PBS as a placebo. All animals were fed standard laboratory chow and had free access to water for the duration of the experiment. After ten days the rats were killed, and the calcified plaques removed and weighed. Internal organs were routinely examined, with special reference to the liver and spleen, and were found to be unaffected by the treatment. Plaque samples (50–100 mg) were digested at 75°C for 5 h with a mixture comprising perchloric acid (70% w/v, 200 μL) and hydrogen peroxide (30% w/v, 400 μL) and the extent of plaque mineralization was subsequently assessed by assaying for calcium and phosphate (Cooper & Sallis 1993). The data obtained was subjected to statistical analysis using a single-tailed Student's *t*-test.

Results and Discussion

Liposomes prepared by the REV method using the basic routine conditions described produced a mean entrapment of 9·14 ± 0·67 mg phosphocitrate per 100 mg lipid (equivalent to 11·6% mol mol^{-1}, see Table 1). The total captured volume of the formulation (1·81 L mg^{-1} lipid) represents an efficiency of approximately 7%. The REV technique should ideally lead to an efficiency value between 40 and 60% as it was conceived originally so as to obtain high aqueous space-to-lipid ratios. Optimum values are, however, known to be adversely affected by concentrated ionic solutions and it has been suggested that the overall ionic strength should not exceed 0·15 (Szoka & Papahadjopoulos 1978). Phosphocitrate is a highly polar compound owing to its ester phosphate hydroxyls and the three carboxylic acid functional groups. Together, they can contribute a net negative charge of 3·5 per molecule at physiological pH (Ward et al 1987) which also might have led to a poor entrapment. In the present experiments however, a reduction in the concentration of phosphocitrate from 112 to 10 mM with a corresponding adjustment to the buffer medium, failed to

Table 2. Ionic strength and osmolality values of solutions entrapped in liposomes.

Solution	I[a]	Osmolality (mOsm)
Phosphocitrate		
112 mM in 10% PBS	0·898	301
50 mM in 50% PBS	0·474	276
10 mM in 90% PBS	0·223	276
Phosphate-buffered saline	0·160	276
Phosphocitrate		
50 mM	0·393	n.d.
10 mM	0·079	25

[a] Calculated for Na_xphosphocitrate where x = 3·5. The ionic strength $I = 0{\cdot}5\ \Sigma\ mz^2$, where m is the molality and z is the valence of each ionic species.

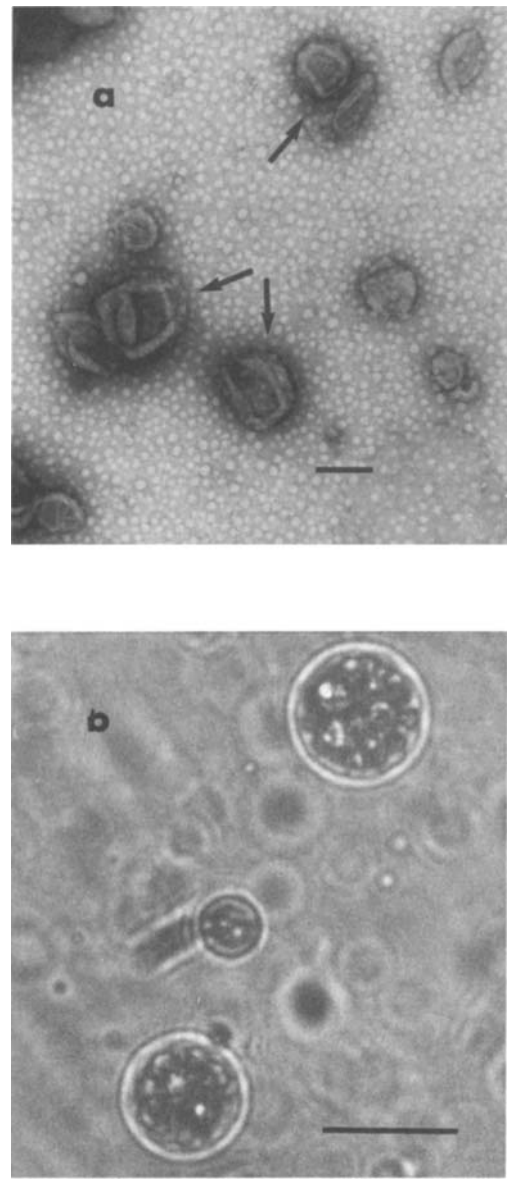

FIG. 1. Phosphocitrate drug delivery systems. a. Liposomes viewed by transmission electron microscopy. Vesicles prepared using the conditions described as routine in the text and filtered through a. 0·2-μm polycarbonate filter (bar = 150 nm). b. Multiple w/o/w emulsions viewed by light microscopy (bar = 20 μm).

Table 3. Influence of lipid variations and cetrimide on liposome entrapment of phosphocitrate.

Lipid composition (mole ratio)	Cetrimide (mM)	% Entrapment[a]		Captured Volume μL (mg lipid)$^{-1}$
		w/w	mol/mol	
Phosphatidylcholine/cholesterol (1 : 1)	3·7	12·1	14·9	2·38
Phosphatidylcholine/cholesterol/ stearylamine (2 : 1 : 1)	—	23–27[b]	(24–29)	3·9–4·7
Distearoylphosphatidylcholine/ cholesterol (2 : 1)	—	10·2	14·8	2·08
Distearoylphosphatidylcholine/ cholesterol (2 : 1)	6·31	7·83	10·9	1·60

[a] Calculated as mg phosphocitrate/100 mg lipid and mol phosphocitrate/100 mol lipid" (mean of two measurements). Aqueous phosphocitrate concentration was 112 mM for each formulation. [b] Value estimated from total [^{14}C]phosphocitrate and lipid recovered following Biogel P-6 polyacrylamide minicolumn purification.

lower the ionic strength sufficiently (see Table 2) to facilitate an increase in the captured volume. Improvements could be expected at least for a 10 mM phosphocitrate solution, if the PBS were to be replaced by an ionically inert osmotic buffer such as sucrose. Unfortunately, this apparent advantage would be largely offset by the much lower initial aqueous drug concentration. Calculations indicate that even at a maximum attainable entrapment efficiency for REVs, this would result in a final molar drug-to-lipid ratio that is less than for the current basic preparation. Liposome extrusion through a 0·2 μm polycarbonate membrane could also have compromised the total entrapment. Although the procedure produces a narrow vesicle size distribution when viewed by transmission electron microscopy (Fig. 1) which ensures a more defined pharmacokinetic profile, 20–30% decreases in captured volume are an inherent disadvantage (Szoka & Papahadjopoulos 1978).

Considerable stability was evident when liposomes containing 112 mM phosphocitrate were incubated with rat plasma at 37°C. Release was constant and slow to the extent that greater than 90% of radiolabelled phosphocitrate was retained after a 5-h incubation. Phosphocitrate possesses some structural features in common with α-hydroxy carboxylic acids which in these latter compounds have been shown to contribute to a low permeability of lipid bilayers (Akeson & Munns 1989). With a higher concentration of phosphocitrate (224 mM), an osmolality effect seems to take over with an initial rapid loss of about 33% in 30 min followed by a period of slow release. The osmotic activity of large unilamellar vesicles has been attributed to a less stable packing of the phospholipid alkyl chains which is related to a lower degree of bilayer curvature compared with small vesicles (Lichtenberg et al 1981). It is postulated then, that osmotic swelling through water influx would disrupt the forces that are preserving the liposomal stability thus permitting temporary rapid phosphocitrate release until the restoration of osmotic balance. This biphasic response could in fact be a useful characteristic as it might ensure that phosphocitrate's minimum effective concentration is rapidly attained whilst progressively providing a sustained supply.

In respect to an in-vivo response, it was observed that standard liposomes administered intraperitoneally at 2·5 mg phosphocitrate kg^{-1} day^{-1} could inhibit the formation of induced subcutaneous plaques by 21% ($P < 0·0005$) whereas an equivalent dose of the phosphocitrate salt alone was ineffective. This result is encouraging as it has been demonstrated previously that a 90% reduction in plaque formation requires daily intraperitoneal treatment with phosphocitrate at a level of 100 mg kg^{-1} (Cooper & Sallis 1993).

An attempt was made to improve the liposomal entrapment of phosphocitrate by including a positively charged species during liposomal preparation (Table 3). To explore the potential, the quaternary ammonium detergent cetrimide was added to the aqueous phase to ion-pair with phosphocitrate. This enhanced the total capture by 36% compared with the standard preparation probably as a result of a preferable partitioning by the more lipophilic complex into the lipid membrane. Other investigators have usefully applied ion-pairing for the liposomal encapsulation of basic drugs (Lee et al 1988) but invariably at excess counter-ion concentrations. In this respect the current approach was limited due to cetrimide's low critical micellar concentration (CMC) of 3·79 mM at 25°C (Evans et al 1984), above which complex formation and incorporation is hindered. Liposomes were therefore prepared using DSPtdCho at 60–62°C with a view to utilizing a higher CMC for cetrimide. However, as shown in Table 3, the anticipated benefit over the control batch was not apparent presumably because of the impracticality of maintaining the higher temperature during solvent evaporation. The most efficient procedure for entrapping phosphocitrate invoked the inclusion of stearylamine at 25 mol % into the liposome bilayer. It is suggested that the more than twofold increase compared with vesicles composed of the neutral lipids is due to direct binding of phosphocitrate to internal cationic sites, as gel filtration is an effective means of removing surface-absorbed material. For any future clinical application, however, an alternative to the use of either stearylamine or alkyltrimethylammonium halides would be advisable as toxicity concerns remain with these compounds even though liposomes can mask the effects to some degree (Pinnaduwage et al 1989). Despite the fact that entrapment of phospho-

citrate into liposomes appears currently to be restrained, the values obtained compare more than favourably with those reported for other multi-negatively charged compounds including sodium cromoglycate (Taylor et al 1990), ATP (Xu et al 1990), phosphonoformate and phosphonoacetate (Szoka & Chu 1988).

The limited phosphocitrate payload in liposomes made a more detailed dose-response study impractical so that an investigation into reducing the dosing frequency also became unfeasible. For this reason attention was focussed on the possibility of using a multiple emulsion. The delivery system is well-suited to phosphocitrate since it does not suffer the encapsulation restrictions characteristic of liposomes provided that the internal and external phases are osmotically balanced (Davis & Walker 1983; Davis & Walker 1987). Isotonic conditions were carefully maintained and photomicroscopy established that with phosphocitrate the selected formulation can produce true w/o/w droplets of average size 20–40 μm. Furthermore, after seven days of refrigerated storage not only did a cursory examination reveal no bulk creaming of the emulsion but microscopic observation confirmed that individual droplet size remained stable and the enveloped multiple aqueous droplets had not coalesced (Fig. 1b). In-vivo, a total of three emulsion doses each containing 50 mg phosphocitrate kg^{-1} applied on days 1, 4 and 7 of the 10-day experiment produced a significant decrease ($P < 0{\cdot}0005$) in plaque weight of 34%, a result validated by matching reductions of 39 and 38% respectively in plaque calcium and phosphate levels. An identical dosage regime with phosphocitrate alone diminished plaque weight by just 7% ($0{\cdot}25 < P < 0{\cdot}20$).

Whilst presently a comparison of responses between liposomal and multiple emulsions is not justified, it is a fact that both types of delivery systems have the ability to divert low molecular weight compounds from a systemic to a lymphatic transport (Sasaki et al 1985). Consequently, a considerable increase in circulation half-life can be gained over that of free drug (Parker et al 1981; Rosa & Clementi 1983). This would benefit phosphocitrate which displays a rapid clearance rate, in excess of 90% at 1 min post-injection (Cooper 1992). We recognise that even in a liposomal form an inevitable clearance by the reticuloendothelial system reduces the potential efficiency, whilst the vehicles remain unmodified (Allen et al 1989). However, the favourable anticalcifying response achieved currently with the comparatively low-dose preparations does justifiably pose a challenge for further dosage optimisation.

In summary, the present studies demonstrate that a highly charged small water-soluble molecule such as phosphocitrate can be presented in the form of a liposome or multiple emulsion preparation and be capable of exerting a positive action against dystrophic calcification. Possibilities now exist to build on these observations. The recent success reported with liposomal loading of basic drugs in response to a pH gradient (Mayer et al 1990) may be adapted as a useful strategy to help optimize phosphocitrate loading. Consideration can also be given to improving entrapment of phosphocitrate by introducing more lipophilicity into the molecule. Thus, the delivery of phosphocitrate through liposomes or multiple emulsion systems certainly would enhance the therapeutic capabilities of this compound.

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