Damage of Egg Phosphatidylcholine Liposomes by DNA-Binding Cytotoxic Agents

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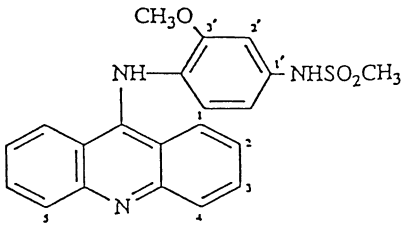
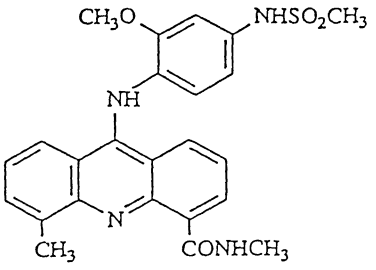
The stability of small unilamellar vesicles (SUV) formed by egg phosphatidylcholine has been examined in the presence of four DNA-intercalating drugs—carboxamides of acridine, anthracene, phenylquinoline, and phenylbenzimidazole. The physicochemical stability of the vesicle-membrane changed as the total drug concentration increased and, of the four drugs investigated, the phenylquinolinecarboxamide was the most destructive. The presence of cholesterol in the liposomal bilayer increased resistance to physicochemically induced damage by the drugs except the anthracenecarboxamide. In all cases, coating the liposomes with a polysaccharide made the liposomal surface more hydrophobic and led to the vesicle rupture by the cytotoxic agents. The cytotoxic 9-anilinoacridine derivatives, amsacrine and 9-[2-methoxy-4-(methylsulfonylamino)-anilino]-5-methylacridine-4-*N*-methylcarboxamide (CI-921), were too insoluble to be encapsulated within the aqueous core of liposomes, but the neutral form of CI-921 was intercalated into the liposomal membrane of pullulan-derivative-coated liposomes. In this formulation, CI-921 was more effective against BM 314 carcinoma-cells than the free drug, but an anticarcinoembrionic antigen (anti-CEA) antibody binding liposome provided no additional advantage.

N-[4-(9-Acridinylamino)-3-methoxyphenyl]methanesulfonamide, amsacrine (**1**) was the first synthetic DNA-intercalating agent successfully to enter clinical use,[1] and has been shown to be beneficial in the treatment of leukemia but not for the more common solid tumors.[2] Multi-parameter structure-activity relationships for amsacrine analogues suggested that alterations in the DNA-binding acridine moiety would be beneficial.[3] While high activity was associated with strong binding to DNA, it was also important to develop analogues with better distributive properties which could reach remote tumor sites. The requirement was, therefore, for weakly basic compounds which would have a higher proportion of the diffusable free base, and focussed attention on electron-withdrawing substituents with hydrogen bonding donor/acceptor properties.[4]

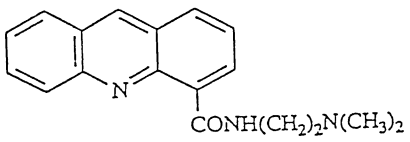
In 1982 the analogue 9-[2-methoxy-4-(methylsulfonylamino)anilino]-5-methylacridine-4-*N*-methylcarboxamide (CI-921) (**2**) was selected for clinical evaluation on the basis of its experimental solid tumor activity and its different physicochemical prop-



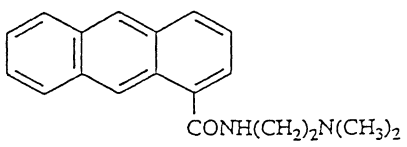
Amsacrine
1



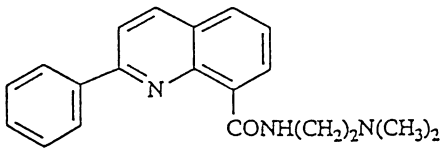
CI-921
2



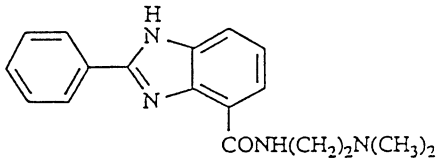
Acridine-4-*N*-
(2-dimethylaminoethyl)carboxamide
3



Anthracene-1-*N*-
(2-dimethylaminoethyl)carboxamide
4



2-Phenylquinoline-8-*N*-(2-dimethylaminoethyl)carboxamide
5



2-Phenylbenzimidazole-4-*N*-(2-dimethylaminoethyl)carboxamide
6

erties compared with amsacrine (stronger DNA-binding, higher water-solubility, and lower pK_a).[5]

Later studies showed that, although high DNA-binding affinity correlates with high in vitro and in vivo drug potency for several series of DNA-intercalating agents, it also limits the distributive properties of the compounds. Emphasis was therefore placed on the development of weakly-binding cationic DNA-intercalating agents.[1,6] One of the first of these was the acridinecarboxamide (**3**), which showed extraordinary activity against the remotely-sited Lewis lung carcinoma in mice.[6] A considerable number of tricyclic carboxamides has now been prepared and evaluated, and these are represented in this study by the linear tricyclic anthracene (**4**)[7] and two '2-1' tricyclic systems, the phenylquinoline (**5**)[8] and the pheylbenzimidazole (**6**).[9] This last cornpound, which possesses the chromophore of lowest aromaticity, displays the lowest DNA-binding constant of all the tricyclic carboxamides which have been investigated.[9]

Liposomes have been of particular interest in cancer chemotherapy, where treatment is required to be systemic to deal with metastatic disease, and is nearly always limited by the toxic side-effects of the drugs. Drug encapsulation within tumour-cell specific liposomes has been chosen to permit smaller quantities to be administered, reducing general side effects. Active targeting of the liposomes to specific tissues such as the lung, or to specific cell types such as alveolar macrophages, may be enhanced by coating them with naturally occurring polysaccharide-derivatives such as mannan and amylopectin[10] anchored by a cholesterol moiety. However, the most deliberate approach to the specific targeting of liposomes has been to attach them to monoclonal antibody fragments capable of recognizing tumor associated antigens. Recent work has shown that such "immunoliposomes" bind selectively to target tumor cells in vitro[11] and in vivo,[12] and that doxorubicin encapsulated into such liposomes is more effective against human lung tumor xenografts in mice than either free drug or drug encapsulated in conventional liposomes.[13]

In this paper we examine first the extent of damage of a variety of egg phosphatidylcholine liposomes caused by the antitumor agents (**3—6**) and how this varies with drug hydrophobicity. Through this experiment, we are able to obtain two different sorts of information. The first is information about the membrane toxicity of these drugs since liposomes are considered to be a simple model of intact cells. The more destructive drug for liposomes should, in general, be more membrane toxic to any kind of cells. The second is information of more technical concern; namely, if one wishes to encapsulate an anti tumor drug into the liposomal drug carrier, the carrier liposome should not be destructed by drugs during the encapsulation procedure. We, therefore, report on the effectiveness of **2**, encapsulated in a carcinoembrionic antigen (CEA)-specific immunoliposome, against a CEA-producing cell line, BM 314, and a CEA-nonproducing cell line, RPMI#4788.

The drugs investigated herein belong to the DNA-intercalating class of antitumor agents. They possess planar aromatic ring systems capable of intercalating reversibly between the basepairs of the double helix and distorting the DNA geometry.

Materials and Methods

Materials. Egg yolk phosphatidylcholine was isolated and purified from egg yolk as described in the literature[14,15] and found to be pure by thin layer chromatography (using a precoated silica gel plate and development with chloroform/methanol/water (65:25:4, v/v/v). Carboxyfluorescein (CF), the marker entrapped in the liposomes, was from Eastman Kodak. The polysaccharide [CHP-50-1.9 (AECM 2.1)] (i.e. pullulan of average molecular weight 50000, and the primary alcohol moieties are substituted by 2.1 free *N*-(2-aminoethyl)carbamoylmethyl (AECM) residues per 100 glucose units, of which 1.9 residues are bound to cholesterol), was similar to that used in previous work.[11]

Cell Lines. Human colon carcinoma cells (BM 314 and RPMI#4788) were maintained in RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 10% heat inactivated Fetal Calf Serum (FCS, Protein Institute, Osaka University).

Cytotoxic Assay. 3×10^4 BM 314- or RPMI#4788-cells in 0.5 ml of RPMI 1640 (10% FCS) were added into each of a 24 well culture plate (Corning). Subsequently, an aliquot of CI-921-encapsulated liposome or immunoliposome was added to the cell suspension. The mixture was maintained at 37 °C under 5% CO_2-95% air condition for 3 days. After the cells were stripped from the culture wells by treatment with 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA), the cell number was counted by trypan blue dye

exclusion.

Preparation of Liposomes. Small unilamellar vesicles, SUV, encapsulating 6-carboxyfluorescein in the interior water phase, were prepared by the method described elsewhere.[16] Polysaccharide-coated liposomes were prepared as follows. After sonication of a suspension of lecithin (30 mg) in a 4 ml solution of carboxyfluorescein (200 mM, 1 M=1 mol dm^{-3}) in tris(hydroxymethyl)methanamine (Tris) buffer (20 mM, pH 8.6) containing 200 mM NaCl, the polysaccharide, CHP-50-1.9 (AECM 2.1) (3 mg), in 0.5 ml of Tris buffer containing 200 mM NaCl, was added. The 1 : 10 (by wt) mixture of polysaccharide: liposome was stirred gently for 30 min and then separated from the multilamellar vesicles (MLV) by gel chromatography. Cholesterol containing liposomes (1 : 10 mol percent) were prepared by adding cholesterol (1.5 mg) to the solution of lecithin (30 mg) in an appropriate amount of chloroform at the beginning of the preparation. For all preparations, the concentration of phosphatidylcholine in the liposomal suspension was determined as inorganic phosphate according to the procedure of Eibl and Lands.[17]

Large unilamellar vesicles (LUV) were prepared by the method of reversed-phase evaporation. Lecithin (60 mg) was dissolved in diethyl ether (8 ml) and an aqueous solution (4 ml) of the material to be encapsulated was added. The mixture was sonicated in an ultrasonicator bath until formation of a homogeneous emulsion was obtained. This step is important, as difficulties in achieving high encapsulation or a primarily unilamellar population of vesicles may arise from failure to obtain a good emulsion. The organic solvent was then removed by rotary evaporation for 60 min at 350 mmHg[a] followed by a further 30 min at 650 mmHg.[a] The jelly-like residue was dissolved in Tris-HCl buffer, 20 mM, in 200 mM NaCl and centrifuged for 30 min at 35,000 G. After each centrifugation the supernatant was decanted and replaced by fresh Tris buffer and the process was repeated three times. The addition of excess buffer to the gel state has been found to decrease the time required to go from the gel state to the final liposomal dispersion, without reducing significantly the percent encapsulation of the initial aqueous material.[18] Sodium chloride is required in the buffer solution to balance the osmolarity between the interior and exterior of the liposome.

Preparation of Immunoliposomes. Dry pyridine (5 ml) was added to 3 ml dry dimethyl sulfoxide containing the CHP-50-1.9 (AECM 2.1) (300 mg) and *N*-[4-maleimidobutyryloxy]succinimide (50 mg) (Dojindo Laboratories, Kumamoto) at room temperature. Substitution of the 4-maleimidobutyryl (GNB) group into the free AECM group in the polysaccharide[11] was completed in 48 h, as confirmed by the fluorescamine test.[19] The reaction mixture was poured into 300 ml ethanol, stored overnight, and then the water soluble portion of the precipitate was lyophilized to yield the polysaccharide derivative coded as GMB-CHP.

Egg phosphatidylcholine liposomes containing CI-921 were prepared in Tris-buffer (200 mM NaCl), pH 8.6. Immediately after sonication, the liposome suspension was mixed with 1 ml Tris buffer (pH 8.6) containing 200 mM NaCl, and containing 3 mg of CMG-CHP. After stirring for 1 h at room temperature, 2 ml of the mixture were adjusted to pH 6.2 with 0.1 M HCl. Phosphate buffer (1.5 ml, pH 6.2) containing 72.5 μg of the anti-carcinoembrionic antigen (CEA) antibody fragment Fab′ (personal donation from S. Nishi, the First Department of Biochemistry, School of Medicine, Hokkaido University) was added and the reaction mixture was incubated with gentle stirring for 20 h at 4 °C. After gel filtration of the immunoliposomal suspension (using phosphate buffer (pH 6.2) as eluant), the MLV fraction (3.6 ml) contained 30.5 μg CI-921 and 4.6 Fab′ as determined by the fluorescamine test[20] (22% of the antibody employed) per ml. The corresponding GMB-CHP-coated multilamellar liposomes, kept at pH 8.6, contained 42.4 μg CI-921 per ml after gel filtration. Thus the loss of CI-921 during the change of pH from 8.6 to 6.2 and the conjugation of the antibody fragment was ca. 30%.

Monitoring the Release of 6-Carboxyfluorescein. A 3 ml sample of 0.1 mM 6-carboxyfluorescein-loaded SUV suspen-

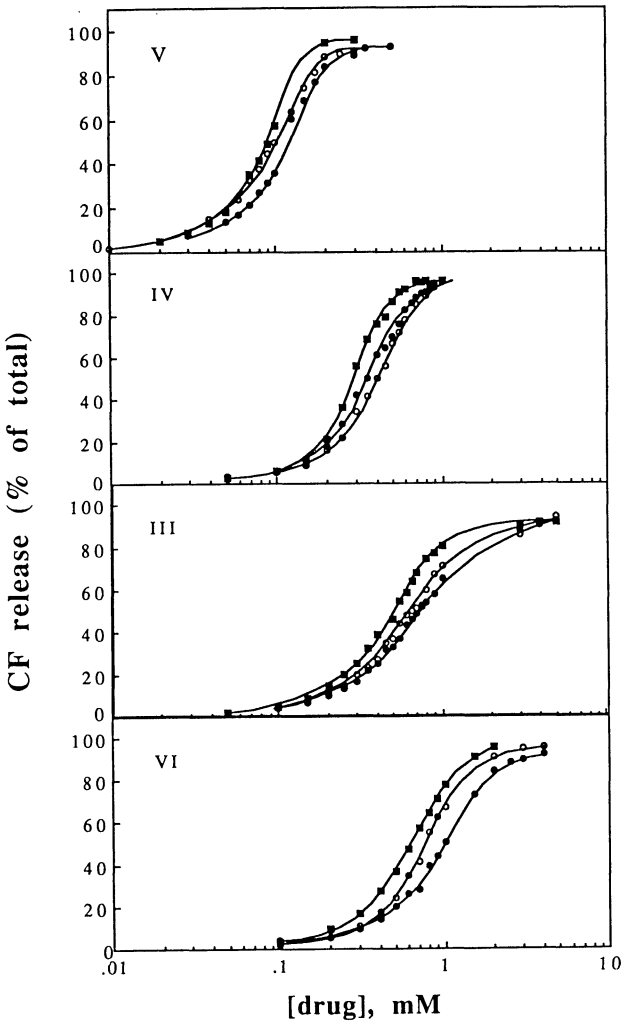


Fig. 1. Cytotoxic agent-induced leakage of 6-carboxyfluorescein (CF) from a 0.1 mM egg phosphatidylcholine small unilamellar vesicle suspension in 200 mM NaCl at 36.5 °C: ○, uncoated liposomes; ●, liposomes containing 10 mol % cholesterol: ■, liposomes coated with pullulan anchored by a cholesterol moiety. The drugs used were the carboxamide derivative of acridine (**3**), anthracene (**4**), the phenylquinoline (**5**), and phenylbenzimidazole (**6**).

[a] 1 mmHg=133.322 Pa.

sion in 20 mM Tris-HCl buffer (pH 8.6) containing 200 mM NaCl was placed in a thermoregulated cuvette cell and preincubated for 10 min at 36.5 °C. During the preincubation, we found no spontaneous release of the marker. The carboxyfluorescein-release (induced by adding 200 μl of an aqueous solution of the drug under investigation) was followed by monitoring an increase in the fluorescence intensity, I, at 520 nm, by excitation at 470 nm on a Shimadzu RF540 fluorescence spectrometer. The intensity of the total amount of the liposome-encapsulated marker, I_∞, was determined after complete destruction of the liposomes with 100 μl of Triton X-100 (10% v/v). The measured percentage of 6-carboxyfluorescein released was obtained by the following equation: % 6-carboxyfluorescein released=$((I_t-I_0)/(I_\infty-I_0))\times 100$, where t and 0 refer to times of sampling and to the instant when the solution of the drug was applied to the liposomal suspension. A standard time of 5 min was used to determine the relative ability of the drugs to effect lysis. Duplicate experiments were performed and the data shown in Fig. 1 represent the mean of two measurements. The experimental error was within ±2%.

Due to interference between the fluorescent properties of the probe and the compounds under investigation (e.g. the acridinecarboxamide, **3**, and the anthracene carboxamide, **4**, possessed fluorescence maxima at excitation wavelengths of 380 and 402 nm, and emission wavelengths of 450 and 472 nm, respectively), it was necessary to apply a correction factor equal to (I_∞(in the absence of drug))/(I_∞(in the presence of drug)) to the measured percent probe released.

Results

Stability of Liposome-Encapsulated Drug Formulations. The amount of amsacrine in the liposomal fraction was below the spectroscopic (λ_{max} 435 nm, ε=12300) detection limit (<80 ng ml^{-1}), but the concentration of neutral CI-921 (λ_{max} 445 nm, ε=10300) dissolved in the lipid membrane increased to 3—8 μg ml^{-1} for the cationic form. Coating the liposomes with cholesterol-substituted pullulan increased the concentration of neutral drug embedded in liposomal bilayer to 70—80 μg ml^{-1} CI-921 and 1.2 μg ml^{-1} amsacrine. The neutral form of CI-921 precipitates rapidly from the uncoated liposomes after sonication and gel filtration, but the polysaccharide-coated liposomes are stabilized and release the drug slowly: at 0, 8, and 11 days the relative concentrations were 90, 66, and 55 μg ml^{-1}, respectively. At the same time the lipid concentration decreased from 2.5 to 2.3 mM.

However, the tricyclic carboxamides (**3**—**6**) were not able to be encapsulated, and in fact degraded liposomal membranes. The rate of this destruction was measured by carboxyfluorescein release. Figure 1 shows comparative data for the percent release of liposome-encapsulated carboxyfluorescein by increasing concentrations of the acridine-(**3**), anthracene-(**4**),

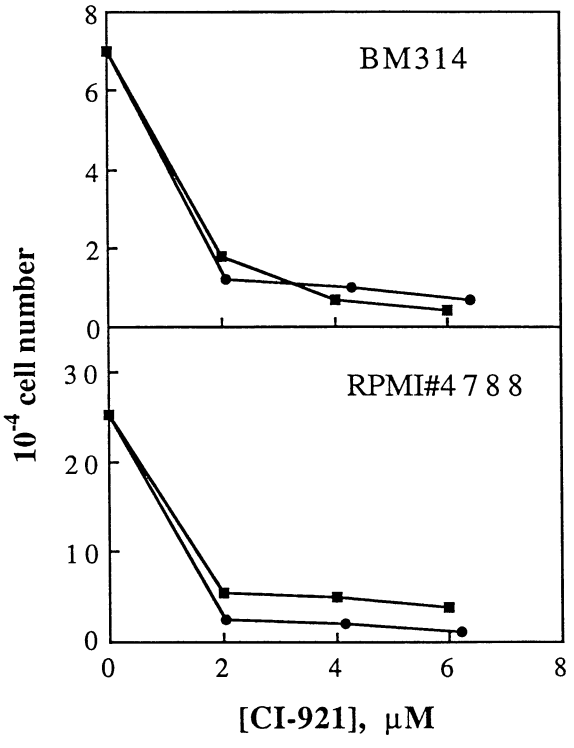


Fig. 2. Antitumor activity of CI-921. The protonated free drug, ●, and the deprotonated form of the drug encapsulated in 60 μM egg phosphatidylcholine MLV, ■, were incubated with CEA-producing BM-314 cells (upper plot) and RPMI#4788 (lower plot) for 3 days at 37 °C. The initial cell number was 3×10^4.

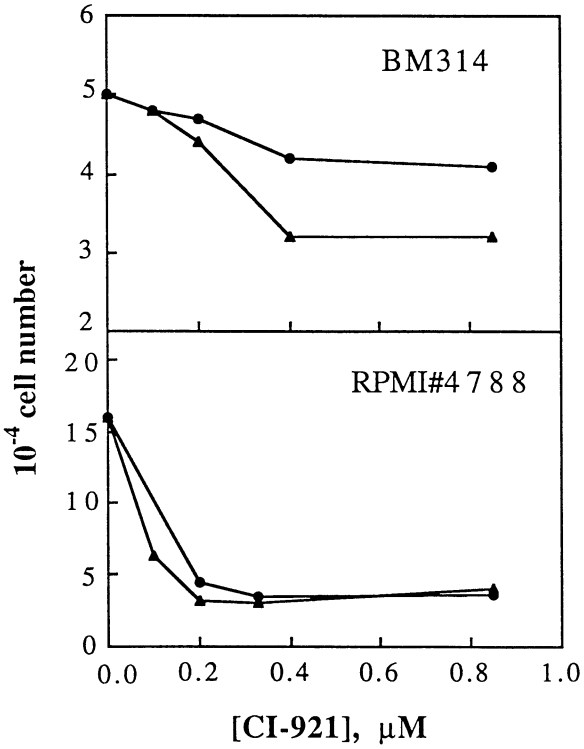


Fig. 3. Antitumor activity of CI-921. The deprotonated drug encapsulated in GMB-CHP coated liposomes, ●, and in immunoliposomes, ▲, were incubated with BM-314 cells (upper plot) and RPMI#4788 cells (lower plot) for 3 days at 37 °C. The initial cell number was 3×10^4.

phenylquinoline-(**5**), and phenylbenzimidazolecarboxamides (**6**) from a variety of liposomes (uncoated, with 10% cholesterol, and coated with pullulan anchored by a cholesterol moiety in the lipid bilayer).

Comparative Antitumor Effect of Liposome Encapsulation of CI-921. The cytotoxicity of CI-921, which was presented to BM-314 and RPMI#4788 tumor cell lines in vitro as either the free drug in its protonated form or the deprotonated drug encapsulated into MLV, is compared in Fig. 2. There was a minor difference in effectiveness in the latter case but no difference in the former.

The deprotonated drug was also encapsulated into GMB-CHP-coated liposomes and into immunoliposomes, and the results for the two cell lines are compared in Fig. 3. In the case of BM-314 cells, the immunoliposome-encapsulated drug was less effective, while for RPMI#4788 there was no significant difference.

Discussion

Our initial interest was to determine whether egg phosphatidylcholine liposomes could be used as a carriers for the cytotoxic drugs amsacrine (**1**), CI-921 (**2**), and acridinecarboxamide (**3**). We had thought that acridinecarboxamide would be the most promising compound for encapsulation because of its high solubility in water (>17 mg ml^{-1}) compared with amsacrine (0.12 mg ml^{-1} in water, 2—3 μg ml^{-1} in saline) and CI-921 (0.72 mg ml^{-1} in water, 23 μg ml^{-1} in saline). Surprisingly, LUV containing the acridinecarboxamide, prepared by the reversed-phase evaporation method, were found to encapsulate only 0.2 to 0.4 percent of the compound. This unexpected result arose from drug induced damage of the liposomal membrane and therefore this compound proved to be unsuitable for encapsulation within egg phosphatidylcholine liposomes. By analogy, therefore, these membrane destructive drugs would be expected to cause some membrane toxic effects even on normal cells in vivo.

Because of their low solubility in water, both amsacrine and CI-921 were unsuitable for encapsulation into the aqueous phase. Furthermore, attempts to encapsulate them into the liposome showed that amsacrine was not detectable in the liposomal fraction and the concentration of CI-921 was less than 0.4 μg ml^{-1}. However, when the pH was raised and the drugs were presented to the liposome in their neutral form, there was a significant increase in the concentration of CI-921 incorporated within the lipid bilayers of MLV. Coating the liposomes with cholesterol substituted pullulan (CHP-50-1.9) increased the drug content of CI-921 more than ten fold.

Of the three compounds investigated, only CI-921 was suitable for encapsulation into liposomes, and then only when applied in its neutral form and when the surface of the liposomes was coated with polysaccharide. When the deprotonated drug was encapsulated in pullulan coated liposomes (results not shown) it appeared to be more effective than the free drug against BM-314 cells, but less effective for RPMI#4788-cells. Anti-CEA bearing immunoliposomes (which encapsulated deprotonated CI-921) were also less effective than the free drug, tested against RPMI#4788 and BM-314 cells, even though Sunamoto et al.[13] have shown that these immunoliposomes are very effectively endocytosed by target (BM-314) cells which are CEA-producing. The cytotoxicity of empty egg-PC liposomes and of anti-CEA monoclonal antibodies, at the concentrations employed herein, was negligible (data not shown).

Since the ability of antitumor agents to lyse a cell membrane may be an important parameter in the design of more effective chemotherapeutic agents, we investigated in more detail the ability of four tricyclic carboxamide DNA-intercalating agents, differing in the aromaticity of their tricyclic chromophore, to interact with egg phosphatidylcholine membranes. In order that these lipid-bilayer structures could act as more effective mimics of a cell membrane they were modified by adding cholesterol therein and by coating them with polysaccharide anchored by a cholesterol moiety.

The results shown in Fig. 1 suggest that cholesterol containing liposomes acquired increased resistance against physicochemically induced damage. High concentrations of both the parent phenylquinoline and phenylbenzimidazole were needed to lyse the cholesterol containing liposomal membrane compared with the concentrations required for the conventional and polysaccharide-coated vesicles.

Unexpectedly, the polysaccharide-uncoated liposomes showed greater resistance to lysis compared with the cholesterol containing vesicles if the lysing drug were the parent anthracene (**4**). However, only ten mol percent cholesterol to phospholipid had been added into the membrane and it is possible that at higher concentrations of cholesterol the liposomal membrane may have shown improved stability. Unfortunately, by increasing the ratio of cholesterol to phospholipid above that of ten percent, one runs the risk of altering the structure of the liposomal membrane to such an extent that the bilayer is damaged. Similar results to these present ones were found by O'Connor et al.[20] who reported that incorporation of cholesterol into the liposomes did not affect the ability of some bile salts to lyse the membrane. In fact, in the case of chenodeoxycholic acid, even the addition of fifty mol percent cholesterol to phospholipid did not significantly alter the magnitude of carboxyfluorescein-release from the liposomal membrane.

Previously, Sunamoto et al. had assembled a cell wall-like coat on the outer surface of liposomes using the modified polysaccharides, *O*-palmitoylpullulan

and *O*-palmitoylamylopectin and showed that such liposomes were more tolerant against permeability of water soluble drugs[21] and changes in ionic strength, pH, osmotic pressure as well as enzymatic lysis by lipases or lipoxygenases.[10] However, unlike these previous experiments the present study dealt with the cytotoxic agents which were injected into the external media rather than being encapsulated within the aqueous interior. Under these conditions, the liposomes coated with pullulan-derivative showed the greater susceptibility to lysis. Interestingly, the present results reported on the cytotoxic agents are similar to those obtained by O'Connor et al.[20] for lysis of polysaccharide-coated liposomes by bile salts. They found that coating the surface of SUV with *O*-palmitoylpullulan led to faster release of carboxyfluorescein for all the bile salts investigated. Coating the liposomes with polysaccharides made the vesicles more hydrophobic and resulted in enhanced lysis by the bile salts.[20]

It seems probable, therefore, that the cytotoxic agents interact more favorably with the polysaccharide-coated vesicles than with the unmodified or cholesterol containing liposomes because of the increased hydrophobicity of the interface. Furthermore, it seems that the increased hydrophobicity of the neutral drugs themselves plays a major role in determining lysis. The relative hydrophobicity values[22] of the carboxamides of the parent phenylbenzimidazole, acridine, anthracene, and phenylquinoline are −0.26, −0.20, −0.02, and 0.01, respectively (determined by liquid-liquid chromatography[22,23] compared with the values of log(1/10% lysis) (i.e. the log of the inverse of the concentration (molar) of drug needed to achieve 10 percent release of carboxyfluorescein from SUV after 5 min at 36.5 °C) equal to 3.54, 3.73, 3.80, and 4.49, respectively.

An investigation of the lysing ability of an extended series of phenylquinolines and phenylbenzimidazoles has been reported separately.[23]

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