Simultaneous liposomal delivery of quercetin and vincristine for enhanced estrogen-receptor-negative breast cancer treatment

Man-Yi Wong and Gigi N.C. Chiu

Breast cancers are either estrogen receptor-positive (ER^+) or negative (ER^-). ER^- breast cancers are clinically more aggressive and have fewer effective treatment options. Quercetin and vincristine are both active against ER^- breast cancers and exhibit synergism *in vitro*. However, the clinical use of quercetin is hampered by its low water solubility. In addition, optimal synergism can only be achieved at a particular ratio of the drugs. Therefore, the objectives of this study are to develop a liposomal formulation to solubilize quercetin, and to co-encapsulate and coordinate the release of quercetin and vincristine in their synergistic ratios to maximize anticancer activity. The optimal synergistic molar ratio of quercetin/vincristine was found to be 1 : 2 by in-vitro MTT assay. Quercetin liposomes were prepared by the film hydration method followed by extrusion, and vincristine was subsequently loaded into the core of the liposomes by remote loading with manganese sulfate and the ionophore A23187. The optimal liposome formulation co-encapsulating quercetin and vincristine comprised egg sphingomyelin/cholesterol/PEG_{2000} ceramide/quercetin (72.5 : 17.5 : 5 : 5 mol ratio). This formulation was physically stable, enhanced quercetin solubility 8.6 times, co-encapsulated quercetin and vincristine with efficiencies of 78.3 and 78.5%, respectively, and displayed coordinated release of both drugs to maintain the synergistic molar ratio. In-vitro MTT assays of this liposomal formulation showed significant synergism, with a combination index of 0.113 and a dose-reduction index value of 115 at ED_{50} for vincristine. Therefore, liposomal delivery represents a strategy to solubilize poorly soluble drugs and coordinate the release of two drugs in their synergistic ratio for optimal anticancer effect. *Anti-Cancer Drugs* 21:401–410 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: combination, drug delivery systems, drug interaction, drug therapy, liposomes, quercetin, vincristine

Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore

Correspondence to Dr Gigi N.C. Chiu, Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore
Tel: +65 6516 5536; fax: +65 6779 1554; e-mail: phacncg@nus.edu.sg

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Introduction

Breast cancer is the most common cancer worldwide [1], and the leading cause of cancer-related deaths in women [2]. Breast cancers can be classified as either estrogen receptor-positive (ER^+) or negative (ER^-), on the basis of the expression of estrogen receptors [3]. These receptors are located in the cytoplasm [4] and migrate to the nucleus after estrogen binding [5]. ER^- breast cancers are clinically more aggressive and have worse prognoses than ER^+ breast cancers [6]. In addition, there are also fewer effective treatments against ER^- tumors, as hormone therapies targeting the ER receptor are ineffective [7]. This highlights the need for continued research to develop and improve treatment regimens against ER^- breast cancer. In addition, current treatment regimes remain suboptimal owing to the narrow chemotherapeutic index of chemotherapeutic drugs, which limits the dose that can be administered. Hence, there is great interest in investigating methods to reduce the toxicity and increase the efficacy of chemotherapeutic drugs.

The efficacy of chemotherapeutic drugs can be increased by administering them in their synergistic ratio [8]. However, free drugs might not maintain the synergistic ratio after in-vivo administration owing to their different pharmacokinetic profiles. In contrast, drugs that are encapsulated in a drug delivery system have to be released for their biological effects to be exerted [9]. Therefore, an appropriately designed drug delivery system that coordinates drug release can be used to maintain the synergistic molar ratio of the two drugs *in vivo* to increase the efficacy of the anticancer treatment [8]. Currently, most of the work carried out on the use of drug delivery systems to coordinate drug release is focused on the combination of conventional amphipathic chemotherapeutic drugs, such as irinotecan with floxuridine [10], doxorubicin with vincristine [11], fludarabine with mitoxantrone [12], and cytarabine with daunorubicin [13] in liposomes. Of these, the liposomal combinations of irinotecan with floxuridine [14] and cytarabine with daunorubicin [15] are currently in phase II clinical trials.

The drug combination we are interested in is vincristine and quercetin. Vincristine is an alkaloid derived from the Madagascan periwinkle that inhibits microtubule formation in the mitotic spindle, leading to an arrest of the

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dividing cells at metaphase [16]. Although vincristine is currently used in its free form for the treatment of ER^- breast cancer [17–19], a drug carrier for vincristine can be used to modulate the dose-limiting neurotoxicity and increase the antitumor efficacy of vincristine, thereby increasing its therapeutic index [20]. Quercetin is a flavonoid with cytotoxic activities against colon [21], prostate [22], lung [23], and breast cancers [24] *in vitro*. In addition, quercetin has also been shown to be active against multidrug-resistant ER^- breast cancer cell lines [25], and has selective cytotoxic activity towards cancer cells without affecting normal cells [22,26]. Most importantly, it has been shown that quercetin could enhance the cytotoxicity of vincristine by reducing vincristine efflux from the cancer cells [27]. These findings form the basis of this study, which aims to co-encapsulate quercetin and vincristine in an appropriately designed delivery system.

Despite the promising in-vitro biological activity of quercetin, this compound has an unfavorable pharmacokinetic profile that limits its clinical use. Quercetin has low water solubility, which limits absorption [28]. In addition, quercetin has been shown to be extensively metabolized to its inactive form by the small intestine [29]; thus, ingestion of quercetin alone may not provide the concentrations that are needed for anticancer activity at the site of action [28]. Hence, the development of an appropriate carrier for quercetin presents the dual advantages of altering the pharmacokinetic profile of quercetin, making it amenable for clinical use, and coordinating the release of the anticancer drugs to maintain the synergistic ratio.

In the past, poly(D,L-lactide-*CO*-glycolide) nanoparticles have been developed to co-encapsulate quercetin and vincristine [30]. Although a high entrapment efficiency of 92.8% was achieved for vincristine, the entrapment efficiency of quercetin was only 32.7%, which is suboptimal [30]. In addition, both vincristine and quercetin were released rapidly, with around 70% of both drugs released in 24 h [30]. This rapid release from the carrier could potentially prevent the accumulation of the anticancer drugs in the tumor site owing to the enhanced permeability and retention (EPR) effect [31]. With the EPR effect, macromolecules with sizes larger than 40 kDa (such as liposomes entrapping the anticancer drug) accumulate preferentially in the tumor tissue owing to the abnormal architecture of the tumor blood vessels, which allows them to escape from the bloodstream and accumulate in the tumor interstitium. In contrast, when the drug is rapidly released from the carrier, the drug is unable to accumulate in the tumor interstitium through the EPR effect, as the small drug molecules quickly leak out from the tumor interstitium and back to the blood plasma [32]. Finally, the release of vincristine and quercetin was not coordinated, with quercetin being released more slowly than vincristine. This could lead to nonsynergistic molar ratios of the two compounds being released, potentially hampering therapeutic efficacy. Therefore, a more appropriate drug carrier for the delivery of the vincristine/quercetin combination is necessary.

Quercetin has previously been encapsulated in egg phosphocholine (EPC) liposomes with an efficiency of close to 100% [33], while vincristine had been encapsulated in either 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)/cholesterol liposomes (55 : 45 mol ratio) [34] or egg sphingomyelin (ESM)/cholesterol liposomes (55 : 45 mol ratio) [35], with encapsulation efficiencies close to 100%, showing improved in-vivo efficacy compared with free vincristine [20,35]. Nevertheless, the concurrent administration of individually encapsulated liposomal quercetin and liposomal vincristine could potentially lead to pharmacokinetic interaction between the formulations, changing the drug-release profile of the tumor [11], and the infusion-related adverse events associated with the administration of high lipid doses [36], as the administration of two liposome formulations would double the amount of lipid administered. These problems can be circumvented by the co-delivery of vincristine and quercetin in a liposomal formulation. In this study, vincristine was encapsulated in the aqueous liposomal core by remote loading using manganese sulfate and the A23187 ionophore [20], while quercetin was intercalated within the hydrophobic region of the lipid bilayer and thus solubilized by the same liposome carrier [33]. The overall aim of this study is therefore to develop a physically stable liposome formulation that allows the solubilization of quercetin, the efficient co-encapsulation of quercetin and vincristine, and the coordinated release of the two drugs such that synergism can be shown using a representative ER^- human breast cancer cell line, MDA-MB-231.

Materials and methods

Materials

All lipids were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, Virginia, USA). 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide, and chloroform were obtained from MP Biomedicals Asia Pacific (Singapore). All other materials were purchased from Sigma-Aldrich (St Louis, Missouri, USA).

In-vitro cytoxicity assays

In-vitro cytotoxicity was assessed by the MTT colorimetric cytotoxicity assay [37]. MDA-MB-231 human breast cancer cells were grown in RPMI 1640 media and added into 96-well cell culture plates at 5000 cells/well. They were incubated at 37°C with 5% carbon dioxide for 24 h for cell adherence to the cell culture plates. The cells were treated with serial dilutions of

single drugs (quercetin, vincristine) or drug combinations (quercetin and vincristine at molar ratios of 1 : 4, 1 : 2, 1 : 1, and 2 : 1) for 72 h. Subsequently, 50 µl of 1 mg/ml MTT reagent was added to each well. This was incubated with the cells for 4 h and aspirated. One hundred and fifty microliters of dimethyl sulfoxide was added to each well and the 96-well plates were shaken for 20 min to solubilize the cells. The plates were then read on a microplate spectrophotometer set at 570 nm. Cell survival at the end of treatment was calculated from the optical density readings as a percentage of the control. All assays were performed in triplicate.

Median-effect analysis for drug combinations

CalcuSyn (Biosoft, Ferguson, Missouri, USA), a software program based on the median-effect principle described by Chou and Talalay [38], was used for the drug combination interaction analysis. For studies on the combined effects of quercetin and vincristine, fixed ratios of the two drugs (1 : 4, 1 : 1, 2 : 1, and 1 : 2) were used. The CalcuSyn program determines whether the combined agents act in an additive, synergistic, or antagonistic manner by using the mean cell survival percentages from the MTT assay as a function of drug concentrations to generate a combination index (CI) value, which is defined as being synergistic (CI < 0.9), additive (CI = 0.9–1.1), or antagonistic (CI > 1.1). The dose-reduction index (DRI), which represents the magnitude of dose reduction for each drug when given in combination, compared with the concentration of a single agent that is needed to achieve the same effect level, was also assessed.

Liposome preparation

Liposomes were prepared with the thin film hydration method [39]. Briefly, the lipids were dissolved in chloroform whereas quercetin was dissolved in ethanol and mixed by vortexing. The preparation was subsequently dried under a stream of nitrogen gas, and the resulting lipid film was placed under vacuum to remove organic solvent. The dried lipid films were hydrated with 300 mmol/l of manganese sulfate (pH 3.4) for 1 h at 60°C. The resulting preparation was extruded 15 times at 60°C through one stacked 0.1 µm pore-size polycarbonate filter (Northern Lipids Inc., Vancouver, British Columbia, Canada) with an extruder apparatus (Northern Lipids Inc.). The resulting mean diameter of the liposomes was determined by quasi-elastic light scattering using the Zetasizer 3000HS operating at 633 nm.

pH gradient loading of vincristine

Vincristine was actively loaded into the liposomes using an ionophore-mediated proton gradient [40]. The divalent cation ionophore A23187 (0.5 µg/mg lipid) was incorporated into the liposomal bilayer after incubation at 60°C for 10 min. Subsequently, the efficiency of vincristine encapsulation by liposomes (drug-to-lipid weight ratio of 0.1 : 1) was determined as a function of time. Encapsulated drug was separated from free drug using a Sephadex G-50 mini spin column. Vincristine was quantified by measuring its absorbance at 297 nm [20] after solubilization with *n*-octyl glucoside, while quercetin was quantified at 376 nm after solubilization of the liposomes in ethanol [33].

Drug release of quercetin and vincristine

The drug release characteristics of this formulation were assessed by dialysing (3500 MWCO, Pierce) the liposomes against 2 l of 0.9% w/v sodium chloride for 72 h at 37°C. At 4, 6, 24, and 48 h, 3×50 µl aliquots were removed from the dialyser and analyzed for encapsulated quercetin and vincristine concentrations using the methods outlined in the earlier section.

Statistical analysis

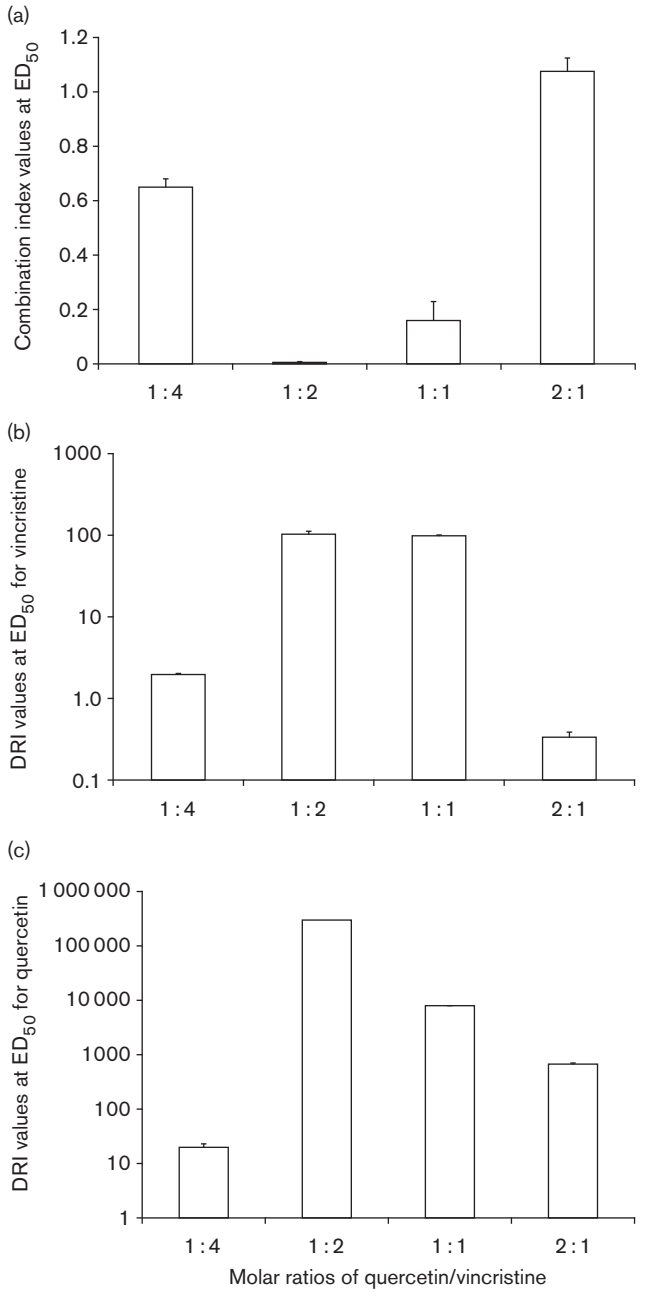
The statistical tests used include the *t*-test, repeated-measures, and one-way ANOVA test with the post-hoc Tukey test. This was analyzed with the NCSS 2004 software (Kaysville, Utah, USA) supplied by NCSS LLC. A P value of less than 0.05 was considered statistically significant.

Results

In-vitro activities of quercetin and vincristine

The optimal molar ratio to encapsulate quercetin and vincristine in the liposomes was determined by assessing the combination effects of the two drugs in the ER^- MDA-MB-231 breast cancer cell line. The data were analyzed with the median-effect principle at fixed molar ratios of quercetin/vincristine of 1 : 1, 2 : 1, 1 : 2, and 1 : 4. These ratios were selected on the basis of the expectation that the two drugs could be successfully encapsulated in the liposomes. Of these four ratios, the ratio with the optimal CI and DRI values would be used for liposome encapsulation. Figure 1a shows the CI values at the four molar ratios of quercetin/vincristine at ED_{50}. CI values of 0.9–1.1 indicate additive activity, CI values less than 0.9 indicate drug synergy and values more than 1.1 indicate antagonism [38]. The results showed that the ratio of 1 : 2 for quercetin/vincristine was the most optimal with a CI of 0.01, compared with 0.65 for the 1 : 4 quercetin/vincristine ratio, 0.16 for the 1 : 1 quercetin/vincristine ratio, and 1.07 for the 2 : 1 quercetin/vincristine ratio (Fig. 1a). In addition, Fig. 1b shows the DRI of vincristine at ED_{50}, which represents the magnitude of dose reduction of vincristine [41] when it is combined with quercetin at molar ratios of 1 : 4, 1 : 2, 1 : 1, and 2 : 1 in MDA-MB-231 breast cancer cells. The most optimal ratio was 1 : 2 for quercetin/vincristine. The DRI values of vincristine were 2, 105, 100, and 0.343 for quercetin/vincristine ratios of 1 : 4, 1 : 2, 1 : 1, and 2 : 1, respectively (Fig. 1b), whereas those of quercetin were 20, 3.0×10^5, 8014, and 690, respectively (Fig. 1c).

Fig. 1



(a) Combination index (CI) values at ED_{50} for quercetin/vincristine exposed to MDA-MB-231 breast cancer cells at molar ratios of quercetin/vincristine of 1 : 4, 1 : 2, 1 : 1, and 2 : 1. Each value represents the mean ± SEM from three independent experiments. CI values of 0.9–1.1 indicate additive activity, CI values of less than 0.9 indicate drug synergy, and values greater than 1.1 indicate antagonism. (b) The dose-reduction indices (DRI) at ED_{50} for vincristine when it was used in combination with quercetin at quercetin/vincristine molar ratios of 1 : 4, 1 : 2, 1 : 1, and 2 : 1 in MDA-MB-231 breast cancer cells. Each value represents the mean ± SEM from three independent experiments. DRI represent the magnitude of dose reduction. (c) The DRI at ED_{50} for quercetin when it was used in combination with vincristine at quercetin/vincristine molar ratios of 1 : 4, 1 : 2, 1 : 1, and 2 : 1 in MDA-MB-231 breast cancer cells. Each value represents the mean ± SEM from three independent experiments. DRI represent the magnitude of dose reduction.

Quercetin incorporation into egg sphingomyelin liposomes and stability studies

Although quercetin had been encapsulated in egg phosphocholine (EPC) liposomes [33], quercetin loading in egg sphingomyelin (ESM) liposomes was explored owing to the superior pharmacokinetic properties of vincristine encapsulated into ESM/cholesterol liposomes [42] compared with EPC/cholesterol liposomes [34]. The formulation comprising ESM/cholesterol/quercetin (50 : 45 : 5 mol ratio) was used initially. However, owing to the low encapsulation efficiency of quercetin (30.3%, Table 1) in the ESM/cholesterol/quercetin (50 : 45 : 5 mol ratio) liposomes, the proportions of ESM and cholesterol were changed to obtain the optimal liposomal formulation for quercetin loading. Table 1 shows the quercetin-loading efficiency in the presence of 0.0, 10.0, 15.0, 17.5, 20.0, and 45.0 mol% cholesterol, respectively. Overall, quercetin incorporation in the liposomes decreased in the presence of cholesterol. The percentage of quercetin loaded in the liposomes was 101.8, 93.6, 88.4, 81.5, 62.9, and 30.3% in the presence of 0.0, 10.0, 15.0, 17.5, 20.0, and 45.0 mol% cholesterol, respectively. The extent of solubilization of quercetin by liposomes was calculated on the basis of the concentration of quercetin in the liposomes divided by the solubility of quercetin in free buffer (80 μmol/l). All liposomal formulations could improve quercetin solubilization, ranging from 3.3 to 11.2 times (Table 1). Finally, the physical stability of these liposomes was monitored immediately after extrusion and storage for 7 days at 4°C after extrusion. Although the liposomes were of similar size after extrusion, the liposomes containing less than 45.0 mol% cholesterol showed an increase in size and polydispersity after storage at 4°C for 7 days (Table 2), suggesting liposome aggregation.

Past research has shown that the inclusion of PEG_{2000} lipids reduces liposome aggregation [43]. Although negatively charged DSPE-PEG_{2000} is normally used, it has been shown to increase the in-vivo leakage rates of vincristine [44]. Therefore, the neutral PEG_{2000} ceramide was used for the formulation in this study. Quercetin incorporation was found to be not significantly different after PEG_{2000} ceramide incorporation ($P > 0.05$) (Table 1). In addition, the physical stability of these liposomes was monitored immediately after extrusion and 7 days after extrusion. There was no significant change in the size and polydispersity of the liposomes (Table 3).

Vincristine loading into ESM/PEG_{2000} ceramide/cholesterol and ESM/PEG_{2000} ceramide/cholesterol/quercetin liposomes

In the past, vincristine has been loaded in liposomes containing a high concentration of cholesterol (45.0 mol%) [20,35]. However, owing to the low incorporation of quercetin in ESM/PEG_{2000} ceramide liposomes containing

Table 1 Quercetin loading efficiency (%) expressed as a function of the mol% cholesterol in the liposomes in the presence and absence of 5 mol% PEG_{2000} ceramide in ESM liposomes

	In the absence of 5 mol% PEG_{2000} ceramide		In the presence of 5 mol% PEG_{2000} ceramide	
Mol% of cholesterol	Quercetin loading (%)	Extent of solubilization (%)	Quercetin loading (%)	Extent of solubilization (%)
0.0	101.8 ± 1.8	11.2	101.4 ± 2.1	11.1
10.0	93.6 ± 6.8	10.3	97.2 ± 3.2	10.6
15.0	88.4 ± 1.7	9.7	89.3 ± 2.4	9.8
17.5	81.5 ± 3.0	8.9	78.3 ± 1.9	8.6
20.0	62.9 ± 1.5	6.9	54.4 ± 10.7	6.0
45.0	30.3 ± 2.0	3.3	25.7 ± 2.2	2.8

For formulations without 5 mol% PEG_{2000} ceramide, ESM/quercetin/cholesterol were in molar ratios of 95 : 5 : 0, 85 : 5 : 10, 80 : 5 : 15, 77.5 : 5 : 17.5, 75 : 5 : 20 and 50 : 5 : 45. For formulations with 5 mol% PEG_{2000} ceramide, ESM/quercetin/PEG_{2000} ceramide/cholesterol formulations were in molar ratios of 90 : 5 : 5 : 0, 80 : 5 : 5 : 10, 75 : 5 : 5 : 15, 72.5 : 5 : 5 : 17.5, 70 : 5 : 5 : 20 and 45 : 5 : 5 : 45. All formulations were formulated at 5 : 95 drug-to-lipid molar ratios. Each value represents the mean ± SEM from three independent experiments.
ESM, egg sphingomyelin.

Table 2 Physical stability of the ESM/cholesterol/quercetin liposomes immediately and 7 days after extrusion

	Immediately after extrusion		7 days after extrusion	
Mol% cholesterol	Size (nm)	Polydispersity	Size (nm)	Polydispersity
0.0	135.9 ± 14.2	0.135 ± 0.082	1120.8 ± 116.4	1.000 ± 0.001
10.0	131.5 ± 13.8	0.137 ± 0.062	1320.4 ± 110.2	1.000 ± 0.001
15.0	158.0 ± 26.5	0.470 ± 0.043	1034.6 ± 123.4	0.434 ± 0.024
17.5	141.5 ± 17.8	0.424 ± 0.134	1043.6 ± 236.4	0.463 ± 0.045
20.0	200.0 ± 34.5	0.637 ± 0.073	201.3 ± 143.6	0.634 ± 0.056
45.0	145.6 ± 22.3	0.195 ± 0.023	143.5 ± 29.7	0.200 ± 0.034

ESM/quercetin/cholesterol were in molar ratios of 95 : 5 : 0, 85 : 5 : 10, 80 : 5 : 15, 77.5 : 5 : 17.5, 75 : 5 : 20 and 50 : 5 : 45. All formulations were formulated at 5 : 95 drug-to-lipid molar ratios. Each value represents the mean ± SEM from three independent experiments.
ESM, egg sphingomyelin.

Table 3 Physical stability of the ESM/quercetin/PEG_{2000} ceramide/cholesterol liposomes immediately and 7 days after extrusion

	Immediately after extrusion		7 days after extrusion	
Mol% cholesterol	Size (nm)	Polydispersity	Size (nm)	Polydispersity
0.0	117.9 ± 12.3	0.123 ± 0.002	123.9 ± 12.3	0.132 ± 0.045
10.0	121.5 ± 16.4	0.100 ± 0.003	120.5 ± 13.2	0.181 ± 0.023
15.0	131.5 ± 14.6	0.153 ± 0.045	134.0 ± 15.6	0.112 ± 0.053
17.5	135.9 ± 12.0	0.161 ± 0.032	134.7 ± 13.2	0.143 ± 0.043
20.0	133.3 ± 13.5	0.137 ± 0.034	134.5 ± 14.5	0.146 ± 0.046
45.0	140.6 ± 12.6	0.172 ± 0.089	143.6 ± 16.8	0.187 ± 0.036

ESM/quercetin/PEG_{2000} ceramide/cholesterol were in molar ratios of 90 : 5 : 5 : 0, 80 : 5 : 5 : 10, 75 : 5 : 5 : 15, 72.5 : 5 : 5 : 17.5, 70 : 5 : 5 : 20 and 45 : 5 : 5 : 45. All formulations were formulated at 5 : 95 drug-to-lipid molar ratios. Each value represents the mean ± SEM from three independent experiments.
ESM, egg sphingomyelin.

45.0 mol% cholesterol, the effect of vincristine loading with varying cholesterol levels was explored. Table 4 shows the effect of cholesterol on vincristine loading. The maximum percentage of vincristine loaded in the liposomes was 25.5, 26.1, 55.0, 70.0, 95.8, and 90.8% in the presence of 0.0, 10.0, 15.0, 17.5, 20.0, and 45.0 mol% cholesterol, respectively. Therefore, vincristine loading is increased with higher cholesterol levels. In addition,

Table 4 Vincristine loading efficiency (%) expressed as a function of the amount of cholesterol for liposomes comprising of ESM/PEG_{2000} ceramide and varying ratios of cholesterol at 60°C

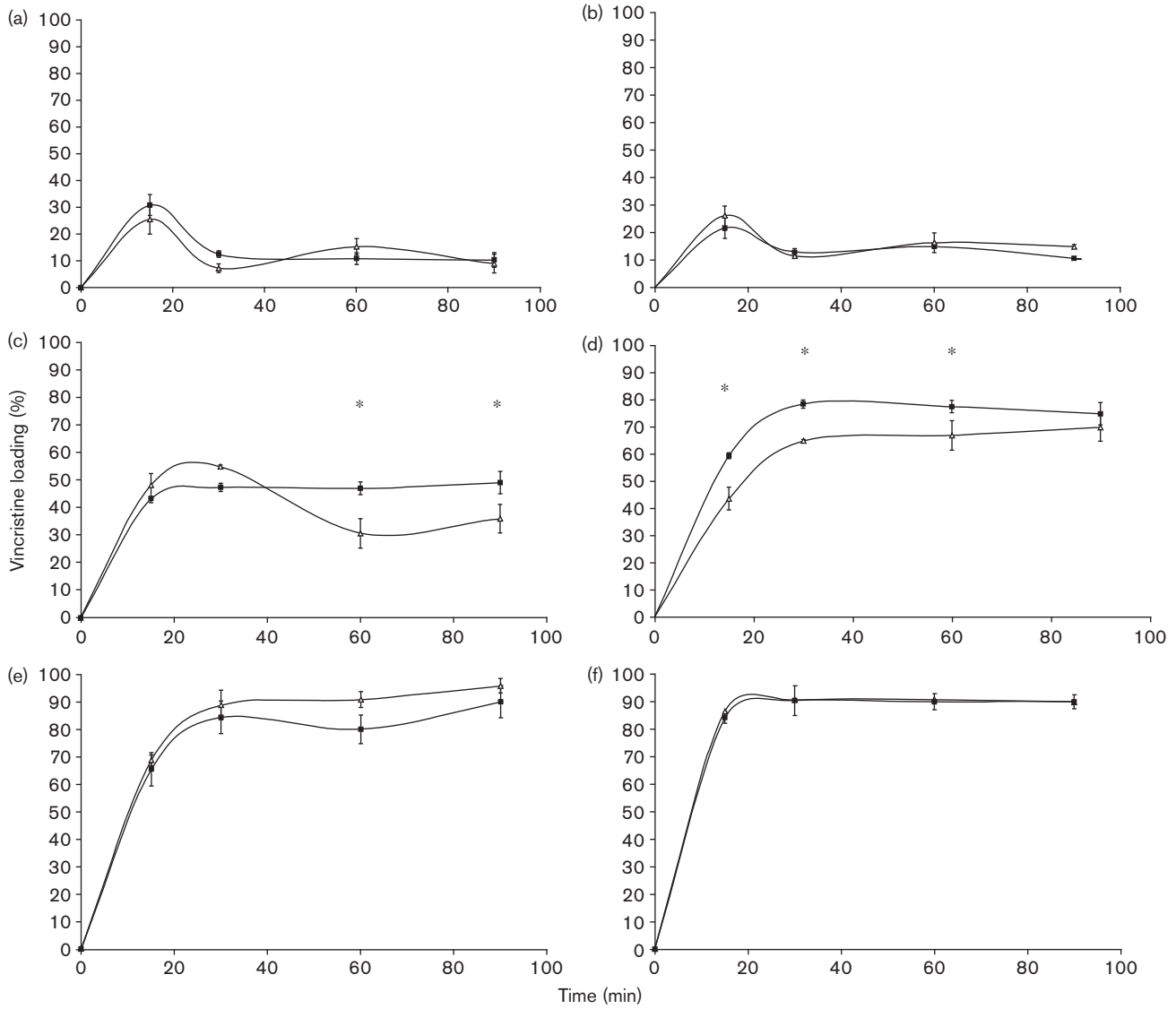
	Time (min)			
Mol% of cholesterol	15	30	60	90
0.0	25.5 ± 5.5	7.2 ± 1.6	15.3 ± 3.0	8.9 ± 3.5
10.0	26.1 ± 3.4	11.4 ± 0.8	16.2 ± 3.6	14.8 ± 0.7
15.0	48.3 ± 4.2	55.0 ± 0.5	30.7 ± 5.4	36.1 ± 5.2
17.5	43.7 ± 1.8	65.0 ± 2.9	67.0 ± 1.2	70.0 ± 1.4
20.0	68.7 ± 5.1	88.8 ± 5.1	90.8 ± 5.3	95.8 ± 2.4
45.0	86.5 ± 2.0	90.7 ± 5.4	90.8 ± 2.9	90.0 ± 2.6

ESM/PEG_{2000} ceramide/cholesterol were in molar ratios of 95 : 5 : 0, 85 : 5 : 10, 80 : 5 : 15, 77.5 : 5 : 17.5, 75 : 5 : 20 and 50 : 5 : 45. Each value represents the mean ± SEM from three independent experiments.
ESM, egg sphingomyelin.

vincristine loading declined over time for liposomes with cholesterol levels of 0.0, 10.0, and 15.0 mol%, but this was not observed for liposomes with higher cholesterol levels of 17.5, 20.0, and 45.0 mol%.

In addition to the amount of cholesterol, the presence of quercetin could also influence vincristine loading. This is because quercetin is incorporated in the lipid bilayer [33] and could alter the permeability of the liposomes. Hence, the effect of quercetin incorporation on vincristine loading was also explored by comparing the loading of vincristine in the presence and absence of quercetin (Fig. 2). Quercetin incorporation had no effect on vincristine loading at 0.0, 10.0, 20.0, and 45.0 mol% cholesterol (Fig. 2a, b, e and f). However, quercetin incorporation affected the vincristine-loading profile at 15 and 17.5 mol% cholesterol (Fig. 2c and d). In the absence of quercetin, vincristine loading peaked at 30 min at 59.3% but subsequently declined to 30.7% upon longer incubation at 15 mol% cholesterol (Fig. 2c). In contrast, this decline did not occur in the presence of quercetin. In addition, at 17.5 mol% cholesterol, the amount of vincristine loaded was higher in liposomes containing quercetin than in those without quercetin for the time points of 15, 30, and 60 min (Fig. 2d).

Fig. 2

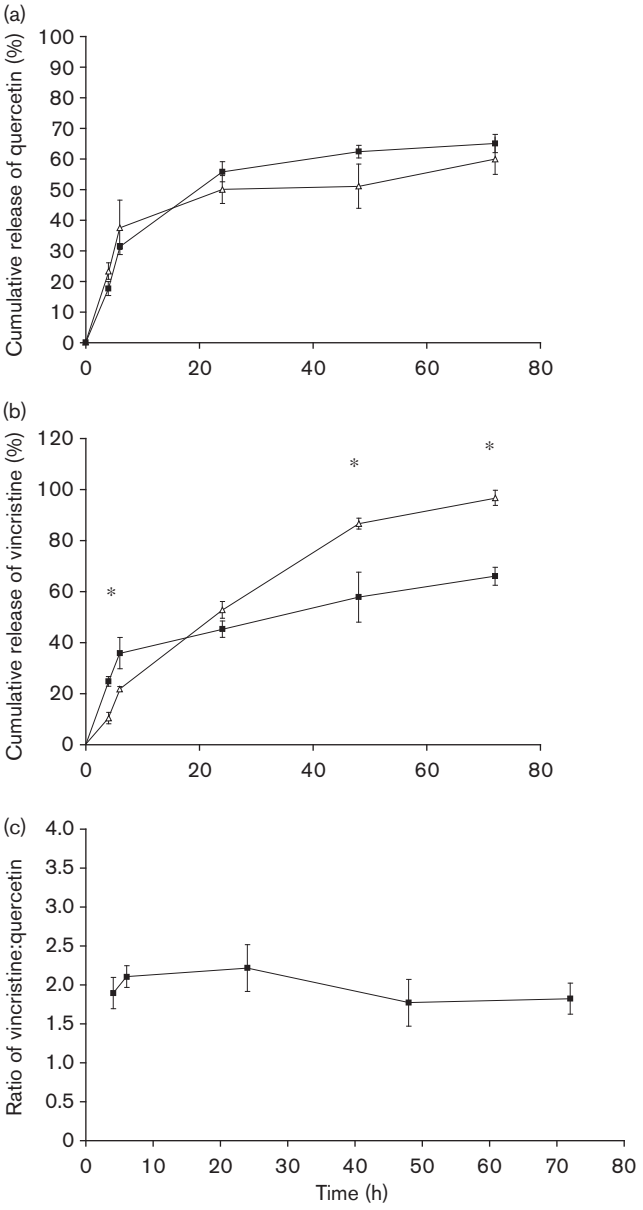


Comparison of vincristine loading efficiency (%) in the presence (■) and absence (△) of 5 mol% quercetin at varying cholesterol levels. (a) 0.0 mol% cholesterol. (b) 10.0 mol% cholesterol. (c) 15.0 mol% cholesterol. (d) 17.5 mol% cholesterol. (e) 20.0 mol% cholesterol. (f) 45.0 mol% cholesterol. *$P<0.05$. For formulations with quercetin, ESM/PEG_{2000} ceramide/quercetin/cholesterol were in molar ratios of 90 : 5 : 5 : 0 (Fig. 2a), 80 : 5 : 5 : 10 (Fig. 2b), 75 : 5 : 5 : 15 (Fig. 2c), 72.5 : 5 : 5 : 17.5 (Fig. 2d), 70 : 5 : 5 : 20 (Fig. 2e) and 45 : 5 : 5 : 45. (Fig. 2f). For formulations without quercetin, ESM/PEG_{2000} ceramide/cholesterol were in molar ratios of 95 : 5 : 0 (Fig. 2a), 85 : 5 : 10 (Fig. 2b), 80 : 5 : 15 (Fig. 2c), 77.5 : 5 : 17.5 (Fig. 2d), 75 : 5 : 20 (Fig. 2e) and 50 : 5 : 45. (Fig. 2f). Each value represents the mean ± SEM from three independent experiments. ESM, egg sphingomyelin.

Another factor that could influence the efficiency of vincristine loading is temperature. Hence, a comparison was made between vincristine loading at 60°C, which is above the phase transition temperature of ESM, and 37°C, which is below the phase transition temperature of ESM. Although vincristine loading at 37°C prevented the decrease in vincristine concentration over time for formulations containing low levels of cholesterol, the amount of vincristine loaded remained low (the maximal loading was approximately 30%). In addition, for liposomes with cholesterol levels of 20.0 and 45.0 mol%, the maximal amount of vincristine loaded was approximately 40%, much lower than 60°C.

Taking the quercetin incorporation and vincristine-loading data together, the best formulation to co-encapsulate quercetin and vincristine was the ESM/cholesterol/PEG_{2000} ceramide/quercetin 72.5 : 17.5 : 5 : 5 mol ratio. Hence, the physical stability of these liposomes was studied for 180 days at a storage temperature of 4°C.

Fig. 3



(a) In-vitro release profile of quercetin from liposomes loaded with quercetin only (△) and loaded with both vincristine and quercetin (■) at 37°C in 0.9% w/v sodium chloride determined with dialysis membrane. The liposome lipid composition consisted of egg sphingomyelin (ESM)/quercetin/PEG_{2000} ceramide/cholesterol (72.5 : 5 : 5 : 17.5 mol ratio). Each value represents the mean ± SEM from three independent experiments. (b) In-vitro release profile of vincristine from liposomes loaded with vincristine only (△) and loaded with both vincristine and quercetin (■) at 37°C in 0.9% w/v sodium chloride determined with dialysis membrane. The liposome lipid composition consisted of ESM/quercetin/PEG_{2000} ceramide/cholesterol (72.5 : 5 : 5 : 17.5 mol ratio). Each value represents the mean ± SEM from three independent experiments. *$P < 0.05$, repeated-measures test. (c) Ratio of vincristine/quercetin released over 72 h. The ratio of drug released was close to the initial loading vincristine/quercetin ratio of 2 : 1. The ratios were obtained by dividing the drug–lipid ratios of vincristine by that of quercetin. Each value represents the mean ± SEM from three independent experiments.

There was no significant change in the size and polydispersity of the liposomes over the monitoring period.

In-vitro drug release of quercetin and vincristine

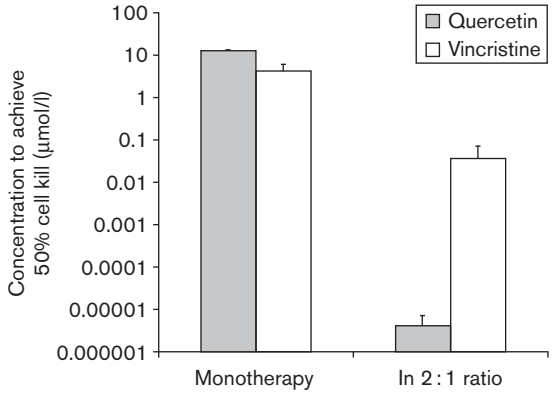
The in-vitro drug release profile of quercetin and vincristine was determined through membrane dialysis in 0.9% w/v sodium chloride. To determine whether the release of quercetin and vincristine from the co-encapsulated liposomes would be altered by the presence of the other substance, the in-vitro release profiles of quercetin and vincristine in co-encapsulated liposomes were compared with liposomes comprising quercetin and vincristine only. As shown in Fig. 3a, the release of quercetin was unchanged by the presence of vincristine ($P > 0.05$). In contrast, the release of vincristine was altered by the presence of quercetin (Fig. 3b). In the presence of quercetin, vincristine release slowed down to 57.7 and 65.9% in 48 and 72 h, respectively, compared with 86.5 and 96.6% in the absence of quercetin ($P < 0.05$). The data showed sustained release of quercetin and vincristine over 72 h and coordinated the release of both drugs, with the 2 : 1 ratio optimal for drug synergism maintained for the duration of the study (Fig. 3c).

Finally, the kinetics of drug release was determined by fitting the data into the most common models of drug release, namely, zero order, first order, and square root of time release. For all the preparations, the best fit was observed for first-order kinetics. The r^2 values for quercetin release for liposomes containing quercetin only and the co-encapsulated preparation were 0.89 and 0.83, respectively. The r^2 values for vincristine release for liposomes containing vincristine only and the co-encapsulated preparation were 0.85 and 0.87, respectively.

In-vitro cytotoxicity studies

To determine whether the empty liposome preparation contributed to the cytotoxicity of the cells, in-vitro cytotoxicity studies were conducted with empty liposomes on the MDA-MB-231 cells. The concentration of lipid tested was matched to the amount of lipid used in the formulation. At the concentrations used, there was no significant effect of the lipids on cell kill. Subsequently, liposomes encapsulating vincristine or quercetin alone or the drug combination were diluted serially and exposed to the ER^- MDA-MB-231 breast cancer cell line for 72 h. The drugs were diluted with serial dilutions. When vincristine and quercetin were co-encapsulated, the concentrations required to attain 50% cell kill were reduced by approximately 6 log-fold for quercetin and 2 log-fold for vincristine compared with monotherapy (Fig. 4). In addition, the CI was 0.113 (very synergistic) at ED_{50} and the DRI for vincristine was 115. Therefore, much lower doses of vincristine were needed when used in combination with quercetin compared with monotherapy. As vincristine is the agent with a dose-limiting

Fig. 4



Plot of quercetin and vincristine concentrations needed to achieve 50% cell kill. Data were obtained with the CalcuSyn software, which uses the median dose effect method developed by Chou and Talalay to determine the combination index. Each value represents the mean ± SEM from three independent experiments.

toxicity, the reduction in vincristine needed to attain the same cell kill is of great clinical significance, as it could improve the therapeutic index of the preparation.

Discussion

Although combination chemotherapy has been the mainstay of cancer treatment owing to the potential increase in efficacy and tolerability, not all drug combinations are beneficial [45]. Drugs may either counteract each other (antagonism), such that the effect of the drug combination is less than the sum of the activities of the individual drugs, or enhance each other's effect (synergy), such that the effect of the combination is more than the sum of the activities of the individual drugs [11]. An appropriately designed drug delivery system can be used to coordinate the release of drugs in their synergistic ratios, allowing for the drug combination to achieve its maximal therapeutic efficacy *in vivo*. We focused our attention on the combination of quercetin and vincristine primarily because quercetin exhibits selective cytotoxicity towards cancer cells [22,26] and could also increase the cytotoxic effect of vincristine by reducing vincristine efflux from cancer cells [27]. In addition, liposomes were selected as the drug delivery system to co-encapsulate both drugs owing to the presence of a hydrophilic liposome core and a hydrophobic lipid bilayer, which makes them suitable for both hydrophobic and amphipathic drugs.

We successfully developed a physically stable combination liposomal formulation that solubilized quercetin, efficiently co-encapsulated quercetin and vincristine, and coordinated the release of the two drugs such that synergism was shown *in vitro*. A drug delivery system that coordinates the release of quercetin and vincristine is crucial, as shown in Fig. 1a, where a slight change in the quercetin/vincristine mole ratio from 1 : 2 to 2 : 1 shifted the combination effect of the two drugs from synergism to antagonism. Therefore, the earlier formulation of PLGA nanoparticles, which failed to coordinate the release of quercetin and vincristine [30], may not exert its full antitumor potential owing to the narrow range in which synergism occurs.

The liposomal formulation we developed maintained the synergistic ratio of quercetin/vincristine 1 : 2 throughout the duration of study. This ratio was maintained for a sufficiently long period for synergism to be exerted, as shown by the CI value of 0.113, which is defined by Chou and Talalay [38] as showing significant synergism. In addition, the DRI value, which is a measure of dose reduction in a synergistic drug combination, was found to be 115 for vincristine. This means that the dose of vincristine needed to achieve the same cell kill was reduced by 115 times. Clinically, this could reduce the incidence of dose-dependent side effects associated with vincristine such as neurotoxicity, and could potentially allow for the same therapeutic effect to be attained with fewer side effects than monotherapy. In addition to reducing the side effects, the sustained release profile of vincristine and quercetin in liposomes would allow for the drugs to be maintained above the minimal effective concentrations for a longer time than the free drug. In view of the promising results, we are currently determining whether the liposomal formulation encapsulating quercetin and vincristine will maintain the synergistic ratio after in-vivo administration, and assessing the therapeutic efficacy of quercetin and vincristine co-encapsulated in liposomes compared with free quercetin, free vincristine, free quercetin, and vincristine in combination in a human breast cancer xenograft model in SCID mice.

The development of a liposomal formulation that coordinates the release of both quercetin and vincristine is challenging owing to the hydrophobic nature of quercetin and the amphipathic nature of vincristine, and the fact that they require different conditions to be efficiently incorporated into a carrier. Therefore, the optimization of formulation variables was necessary to co-encapsulate the two drugs. For example, quercetin incorporation and solubilization was found to be most efficient either in the absence or at low levels of cholesterol, possibly owing to the competition of cholesterol and quercetin for the same hydrophobic space in the lipid bilayer [33] and the reduced flexibility of the hydrocarbon chains of the lipids after the addition of cholesterol [46], which would interfere with quercetin penetration into lipid bilayer. In contrast, efficient and stable vincristine loading required at least 17.5 mol% cholesterol. This could be a result of the higher permeability of liposomes with low cholesterol levels

compared with liposomes with higher levels of cholesterol, leading to the leakage of manganese sulfate from the core of the liposomes and the elimination of the transmembrane pH gradient driving vincristine into the liposomes [47].

However, with 17.5 mol% cholesterol, the liposomes were physically unstable and increased in size and polydispersity, although this was not observed at higher cholesterol levels of 45.0 mol%. This could be a result of the ability of high levels of cholesterol to decrease the attractive van der Waals forces while increasing net repulsive forces, thereby reducing the tendency towards liposomal aggregation and fusion compared with lower cholesterol concentrations [48]. Although the inclusion of cholesterol could increase the physical stability of liposome, high cholesterol levels reduced quercetin loading and were also shown to reduce the retention of other drugs such as floxuridine [10]. This warrants alternative methods for increasing liposome physical stability. Therefore, PEG_{2000}-conjugated lipids, which confer physical stability through steric stabilization, were added to the formulation [43]. Although negatively charged DSPE-PEG_{2000} lipid is conventionally used, it has been shown to increase vincristine release from liposomes [44]. Therefore, the neutral PEG_{2000} ceramide lipid was used instead. In addition to preventing liposome aggregation, the inclusion of PEG_{2000} ceramide had no adverse effect on quercetin loading. In addition, there was no significant change in the size and polydispersity of the liposomes over 24 weeks. Therefore, PEG_{2000} ceramide lipids could be used instead of cholesterol to stabilize liposomes in the presence of physical aggregation without affecting the incorporation of hydrophobic drugs in the lipid bilayer.

We have shown for the first time that the incorporation of quercetin could alter the loading and release of vincristine. Quercetin incorporation not only increased vincristine loading but also reduced vincristine release from the liposomes, possibly through the formation of intermolecular hydrogen bonds that rigidify the liposomal membrane [49], reducing the permeability of the liposomal membrane to vincristine during loading and release. It is worthwhile to note that despite this, the kinetics of drug release remained first-order, with the maintenance of the synergistic ratio of quercetin and vincristine release over 72 h. This is crucial in maximizing the therapeutic activity of the drug combination. The alteration of drug release by quercetin is anticipated to apply to other membrane-permeable amphipathic drugs similar to vincristine. Therefore, in addition to changing lipid composition [20], the formation of drug precipitates [50] and changes in pH gradient [51], which are traditional methods of altering drug release, and the incorporation of quercetin or other compounds that can form hydrogen bonds represent a new avenue to alter drug-loading and release profiles. In contrast, the observation that vincristine loading had no significant effect on quercetin release is expected, as vincristine is incorporated in the aqueous core of the liposomes [35] and is unlikely to interfere with the release of quercetin in the lipid bilayer [33].

In conclusion, we developed a novel drug delivery carrier that co-encapsulated two drugs exhibiting synergism, coordinated the drug release profiles of quercetin and vincristine, and prolonged the exposure times for both drugs. The fixed ratio was maintained for a sufficiently long period for synergism to be exerted as shown by the in-vitro data, allowing optimal anticancer activity to be attained. Our drug delivery system represents a paradigm shift from current chemotherapy dosing based on the maximal tolerated dose, which fails to determine whether the drug levels are at their optimal synergistic level, preventing optimal benefit from being attained. In-vivo studies in mice to compare the pharmacokinetics and therapeutic efficacy of liposomal and free drugs are in progress to further characterize this formulation.

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