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Preparation and in vitro synergistic anticancer effect of Vitamin K3 and 1,8-diazabicyclo[5,4,0]undec-7-ene in poly (ethylene glycol)-diacyllipid micelles

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Abstract

Polymeric micelles consisting of poly(ethylene glycol)-distearoyl phosphoethanolamine conjugates (PEG-DSPE) loaded with Vitamin K3 (VK3) to 0.2 mg of drug/mg of carrier and with 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) to 0.06 mg of drug/mg of carrier were prepared. These micelles were stable for as long as 6 months during storage at 4 °C and did not change their size or release the incorporated drugs. Co-encapsulation of VK3 and DBU into PEG-DSPE micelles resulted in synergistic anticancer effects against both murine and human cancer cells in vitro. The synergism may be explained by the fact that the presence of DBU promotes the escape of drug-loaded micelles from the endosomes of cancer cells directly into the cytoplasm as demonstrated by fluorescent microscopy.

Keywords: Polymeric micelles; PEG-PE; Anticancer drugs; Vitamin K3; 1,8-Diazabicyclo[5,4,0]undec-7-ene; Synergistic anticancer effect

1. Introduction

The synthetic Vitamin K3 (2-methyl-1,4-naphthoquinone, VK3) has been repeatedly reported to inhibit the growth of various cancer cell types both in vitro and in vivo (Nutter et al., 1991; Carr et al., 2002; Markovits et al., 2003). The mechanism of the VK3 cytotoxicity has not been completely understood, although two possible explanations for this phenomenon have been considered. The first was VK3-mediated oxidative stress (Thor et al., 1982; Ross et al., 1986; Brown et al., 1991; Nutter et al., 1992), observed in liver cells during the synthesis of prothrombin. VK3 was believed to enter the "redox cycle" with a resultant formation of the reactive O_2^- and the subsequent death of hepatocytes. However, the presence of this mechanism in human cancer cells has not yet been established. The second, now believed to be the principal mechanism for the antitumor action of the VK3, was the involvement of the VK3 in the direct arylation of cellular thiols resulting in the depletion of glutathione and the inhibition of sulfhydryl-dependent proteins in cancer cells (Ross et al., 1985, 1986; Rossi et al., 1986; Wilson et al., 1987; Gant et al., 1988; Nishikawa et al., 1995).

Although VK3 exhibits a broad spectrum of anticancer activity against rodent and human cancer cells, its effects are somewhat lower compared to those of

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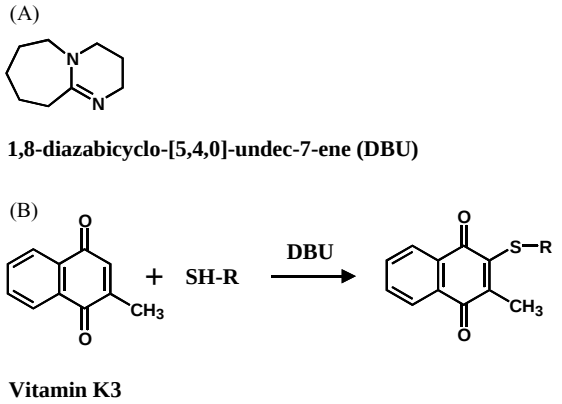

Fig. 1. DBU—the structure (A) and the use in the synthesis of the VK3 thioethers (B).

other anticancer agents of the quinone structure, such as doxorubicin or daunorubicin. To find more potent VK3 derivatives, many types of VK3 analogues have been synthesized and evaluated (Wang et al., 2001; Chen et al., 2002; Kim et al., 2003). Thus, Cpd 5, a thioether analog of VK3, demonstrated greater anticancer effects than VK3 and has become a most attractive VK3 analogue (Ekesi, 1999; Tamura et al., 2000; Kar et al., 2003). However, as a thioether of VK3, Cpd 5 cannot perform the direct arylation of cellular thiols, which suggests that the mechanism of the anticancer activity of Cpd 5 is, at least in part, different from that of VK3.

Earlier, 1,8-diazabicyclo[5,4,0]undec-7-ene (see Fig. 1) was used to accelerate the formation of thioether analogs of VK3, such as Cpd 5 (Nishikawa et al., 1995). We hypothesized that the arylation of cellular thiols by the VK3 and the acceleration of thioether formation by DBU could result in synergistic anticancer effects, when VK3 and DBU are delivered inside cancer cells simultaneously. With this in mind, we prepared polymeric micelles loaded with both VK3 and DBU and investigated their action on various cancer cell lines in vitro.

Micelles, spontaneously formed aggregates of amphiphilic molecules, are widely considered to be promising as drug delivery systems (Kwon and Kataoka, 1995; Jones and Leroux, 1999; Torchilin, 2001). Particularly, the polymeric micelles prepared with poly(ethylene glycol)-phosphatidylethanolamine conjugates (PEG-PE) are convenient and stable long-circulating carriers for many poorly soluble drugs (Trubetskoy and Torchilin, 1995; Lukyanov et al., 2002; Gao et al., 2002, 2003; Torchilin et al., 2003; Zhang et al., 2003). PEG-PE micelles can simultaneously entrap VK3 and DBU, since the VK3 is a sparingly water-soluble agent and can be easily loaded into the hydrophobic micelle cores. Although the DBU is a relatively water-soluble polar amine, it can still be associated with micelles by electrostatic attraction force because the PEG-PE is a type of a negatively charged surfactant.

Here, we describe the preparation, characterization, and the in vitro synergistic anticancer effects of PEG-PE conjugate micelles loaded with both VK3 and DBU (MIC-VK3-DBU).

2. Materials and methods

2.1. Materials

Vitamin K3 and pyrene were purchased from Sigma (St. Louis, MO). 1,8-Diazabicyclo[5,4,0]undec-7-ene was purchased from Aldrich Chemical Company (Milwaukee, WI). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-3000] (PEG-DSPE) and phosphatidylethanolamine lissamine rhodamine B (Rh-PE) were from Avanti Polar Lipids (Alabaster, AL). Cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD). Cell culture media and supplements were purchased from CellGro (Kansas City, MO). All other reagents and buffer solution components were analytical grade preparations.

2.2. Preparation of MIC-VK3-DBU

To prepare MIC-VK3-DBU, PEG-DSPE, VK3 and DBU (15/3/1, w/w) were dissolved in ethanol. The ethanol was removed using a rotary evaporator and a thin film of the above listed components was formed. An appropriate volume of saline was mixed with the film to obtain a final PEG-DSPE concentration of 15 mg/ml. Upon the addition of the saline, MIC-VK3-DBU formed spontaneously. Free VK3 and DBU were separated from MIC-VK3-DBU by HPLC using a size-exclusion Shodex® column (Shoko Co., Ltd., Japan). The separation was performed at a PEG-DSPE

concentration of 15 mg/ml, which is at least 500-fold higher than PEG-DSPE critical micelle concentration (Lukyanov et al., 2002). This insured that the loss of PEG-DSPE monomers, which are always in dynamic equilibrium with the micelles, was negligible. The mobile phase was a 5 mM HEPES buffer, pH 7.4. The flow rate was 1.0 ml/min. The MIC-VK3-DBU preparation was sterilized by filtration through a sterile syringe-driven filter unit with a pore size of 100 nm (Millipore Co., Bedford, MA). Finally, the dispersion of MIC-VK3-DBU was aliquoted into brown glass ampoules (10 ml per ampoule), and the ampoules were filled with nitrogen and sealed.

2.3. Characterization of MIC-VK3-DBU

2.3.1. Loading efficiency

The loading efficiencies of VK3 and DBU were expressed as the percentage of VK3 and DBU (of initial quantities) associated with micelles in the dispersion of MIC-VK3-DBU. Concentrations of constituents were determined by high-performance liquid chromatography (HPLC) assay. A D-7000 HPLC system equipped with a diode array detector (Hitachi, Japan) was used. VK3 and DBU were detected at 265 and 210 nm, respectively (Hu et al., 1995). Calibration curves were obtained using individual solutions of VK3 and DBU in HPLC quality water with concentrations of 9.3×10^{-2} to 5.8×10^{-3} mg/ml and 1.6–0.1 mg/ml, respectively.

2.3.2. Particle size analysis

The analysis was performed using a Coulter® N4-Plus Submicron Particle Sizer (Coulter Corporation, Miami, FL). The micelle suspensions under investigation were diluted with the de-ionized distilled water until a concentration providing a light scattering intensity of 5×10^4 to 1×10^6 counts/s was achieved. The particle size distribution of all samples was measured in triplicate.

2.3.3. Micelle surface charge measurement

Zeta-potentials of micelle formulations were measured by Zeta Phase Analysis Light Scattering (PALS) with an Ultra-Sensitive Zeta Potential Analyzer instrument (Brookhaven Instruments, Holtsville, NY). The micelle suspensions were always diluted with the de-ionized distilled water to have a signal intensity within the limits required by the instrument. The zeta-potential of each sample was determined eight times.

2.3.4. Stability

Three lots of MIC-VK3-DBU were stored in a dark place at 4 °C for 6 months. The stability of the micelles was monitored by the changes in particle size and drug concentration in MIC-VK3-DBU samples during the storage period. Particle size analysis and the determination of the released VK3 and DBU after their separation from remaining micelles by HPLC was performed as described above.

2.4. Cell cultures

Murine Lewis lung carcinoma (LLC), human mammary adenocarcinoma (BT-20), and human ovarian carcinoma (A2780) were maintained in DMEM (LLC), RPMI 1640 (BT-20) or EMEM (A2780) cell culture medium at 37 °C, 5% CO_2. Cell culture media were supplemented with FBS to 10%, Na pyruvate to 1 mM, L-glutamine to 1 mM, penicillin to 50 U/ml, and streptomycin to 50 μg/ml.

2.5. In vitro anticancer effects

The in vitro anticancer effect of MIC-VK3-DBU was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method (Ni et al., 1998). Briefly, LLC, BT-20, or A2780 cells were plated at 2×10^4 cells per well density in 96-well plates (Corning, Inc., Corning, NY). After 24 h incubation at 37 °C, 5% CO_2, the medium was replaced with a medium containing MIC-VK3-DBU or controls consisting of free VK3 and VK3-loaded DBU-free PEG-DSPE micelles (MIC-VK3). After an additional 24 h incubation at 37 °C, 5% CO_2, the medium containing VK3 formulations was replaced with phosphate-buffered saline containing 0.1 mg/ml MTT, and the cells were incubated at 37 °C, 5% CO_2. The absorbance of the degraded MTT at 492 nm was periodically measured using an ELISA reader (Labsystem Multiskan MCC/340, Finland) until the signal intensity of control untreated cells reached a value of 0.7–1 units of absorbance (typically, 3 h in our case). The IC_{50} values were determined as the concentrations of tested materials, at which cell samples developed the absorbance of 50% of that of untreated

control cells (i.e. 50% growth inhibition) as estimated from the dose–response curves.

2.6. Fluorescence microscopy of endocytosis for micelles by BT-20 cells

Micelles-to-cell interaction was studied with BT-20 cells using MIC-VK3-DBU and MIC-VK3 fluorescently labeled with a 1% Rh-PE (w/w). Adherent BT-20 cells were grown on glass cover slips placed into six-well tissue culture plates. After the cells reached a confluence of 60–70%, the cells were washed twice with Hank's buffer and treated with 1% solution of bovine serum albumin (BSA) in EMEM medium. After 1 h incubation at 37 °C, 5% CO_2, the BSA-containing EMEM was replaced with Rh-PE-labeled MIC-VK3-DBU or DBU-free MIC-VK3 micelles diluted in the medium to a PEG-DSPE concentration of 3.5×10^{-3} mg/ml. After 0.5, 1 and 4 h incubation at 37 °C, 5% CO_2, the cover slips were washed three times with cold saline, and mounted individually cell side down on clean glass slides using a fluorescence-free Trevigen® mounting medium (Trevigen, Gaithersburg, MD). Mounted slides were studied with a Nikon Eclipse E400 microscope (Nikon, Japan) in the fluorescence mode using an Rh/TRITC filter.

2.7. Statistical analysis

Statistical differences in average particle sizes, zeta potentials, and IC_{50} values of different micelle formulations were analyzed using the Student's *t*-test. Calculation were performed using a computer program, Origin (Microcal Software, Inc., Northampton, MA).

3. Results and discussion

3.1. Characterization of MIC-VK3-DBU

The loading efficiencies for VK3 and DBU (see structures in Fig. 1) into MIC-VK3-DBU were 98 ± 0.2% and 87 ± 5%, respectively, which corresponded to incorporation of approximately 197 μg of VK3 and 58 μg of DBU per 1 mg of micelle-forming PEG-DSPE. The VK3, as a water-insoluble agent, is expected to be located in the hydrophobic core of the micelle. As a polar amine, DBU may be located on the border between the polar shells of the micelles (PEG chains) and the hydrophobic cores of the micelles.

Table 1
Properties of various micellar preparations

Micelles	Size ± S.D. (nm)	Zeta-potential ± S.D. (mV)
PEG-DSPE micelles	8 ± 2	−25 ± 1
MIC-VK3	15 ± 4	−18 ± 2
MIC-VK3-DBU	45 ± 10	−1.5 ± 1

The average particle size, surface charge, and particle size distribution of MIC-VK3-DBU and some related micelles are shown in Table 1 and Fig. 2. The average particle diameter of the plain micelles composed of PEG-DSPE was approximately 8 nm. When loaded with VK3, the diameter of micelles was enlarged to 15 nm. When loaded with both VK3 and DBU, the size was further enlarged to 45 nm.

The plain micelles were negatively charged with a zeta-potential of approximately −25 mV. The incorporation of the VK3 into the plain micelles caused a statistically significant ($P < 0.01$) but still not very high increase in their zeta-potential to approximately −18 mV. However, the size of VK3-containing micelles increased rather significantly, presumably because of the expansion of the hydrophobic core due to dissolution there of a substantial amount of the water-insoluble VK3 ($P < 0.05$). On the other hand, DBU, as a positively charged polar amine, is likely to be kept within the micelles due to strong electrostatic interactions with negatively charged PEG-DSPE. As a result, the negative charge of PEG-DSPE is partially neutralized. Naturally, the incorporation of the DBU in PEG-DSPE micelles resulted in a sharp increase of the zeta-potential (zeta-potential of MIC-VK3-DBU is about −1 mV). A significant increase in the micelle diameter (from 15 to 45 nm) was also observed upon DBU incorporation, presumably due to the micelle corona expansion after the DBU loading due to hindered folding of PEG chains.

Micelles loaded with both agents were stable during storage at 4 °C. The data presented in Table 2 demonstrate that when MIC-VK3-DBU were stored at 4 °C in the dark for 6 months, the particle size as well as DBU and VK3 load of the micelles did not change significantly (although a small decrease in the VK3

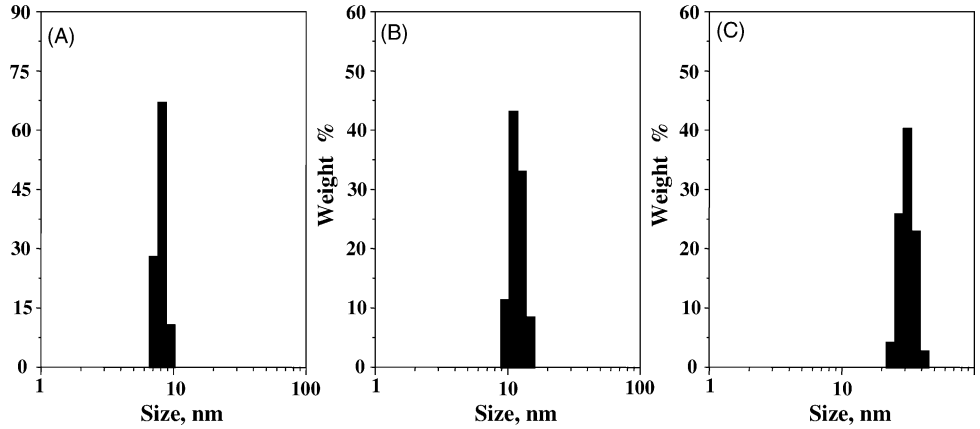

Fig. 2. Particle size distributions of plain PEG-DSPE micelles (A); MIC-VK3 (B); and MIC-VK3-DBU (C).

Table 2
Stability of MIC-VK3-DBU upon storage at 4 °C in darkness

Storage time (months)	Particle size ± S.D. (nm)	Drug concentration ± S.D. (mg/ml)	
		VK3	DBU
0	45 ± 10	2.2 ± 0.1	0.67 ± 0.02
1	46 ± 14	2.0 ± 0.2	0.63 ± 0.28
3	50 ± 12	1.9 ± 0.1	0.64 ± 0.24
6	51 ± 19	1.9 ± 0.3	0.69 ± 0.13

quantity was observed, probably, because of VK3 decomposition).

3.2. In vitro anticancer effects

The cytotoxicities of MIC-VK3-DBU and its individual constituents towards the cancer cell lines are shown in Table 3. The results obtained clearly demonstrate that the incorporation into PEG-DSPE micelles did not change the in vitro cytotoxicity of the VK3. Micelles containing only DBU (MIC-DBU) showed no detectable cytotoxicity in our experiments at the highest DBU concentration tested (3.33 μg/ml) with all types of cancer cells. However, when DBU was associated with VK3-containing PEG-DSPE micelles, the cytotoxicicity of resulting MIC-VK3-DBU were significantly greater ($P < 0.01$) than those of both free VK3 and MIC-VK3 at the equal VK3 concentration. Thus, when associated within the same micelle, VK3 and DBU clearly showed synergistic in vitro anticancer effects. It has been reported earlier that VK3 could arylate SH-groups, particularly in the presence of DBU (Nishikawa et al., 1995). Therefore, the mechanism of the synergistic anticancer action of VK3 and DBU possibly involves the DBU-mediated acceleration of the arylation of the SH-groups of some important proteins in cancer cells by the VK3 when both are located in close proximity within the same mixed micelle. The substantial increase in the micelle zeta-potential upon the incorporation of DBU may also play a certain role by facilitating the micelle uptake b negatively charged cancer cells (Marino et al., 1994). Among the three types of cancer cells tested, BT-20 was the most sensitive to the action of MIC-VK3-DBU.

Table 3
In vitro anticancer effect of various VK3 preparations

Cancer cell line	Preparation ID_{50} (μM of VK3)		
	VK3	MIC-VK3	MIC-VK3-DBU
LLC	5.9	5.7	3.3
BT-20	4.6	3.6	1.9
A2780	10.9	10.5	4.2

3.3. Fluorescence microscopy of endocytosis of micelles by BT-20 cells

The patterns of endocytosis of fluorescent Rh-PE-labeled MIC-VK3-DBU and MIC-VK3 by BT-20 cells were compared (see Fig. 3). The results obtained show that within first 30 min of incubation of micelles with BT-20 cells, MIC-VK3-DBU or MIC-

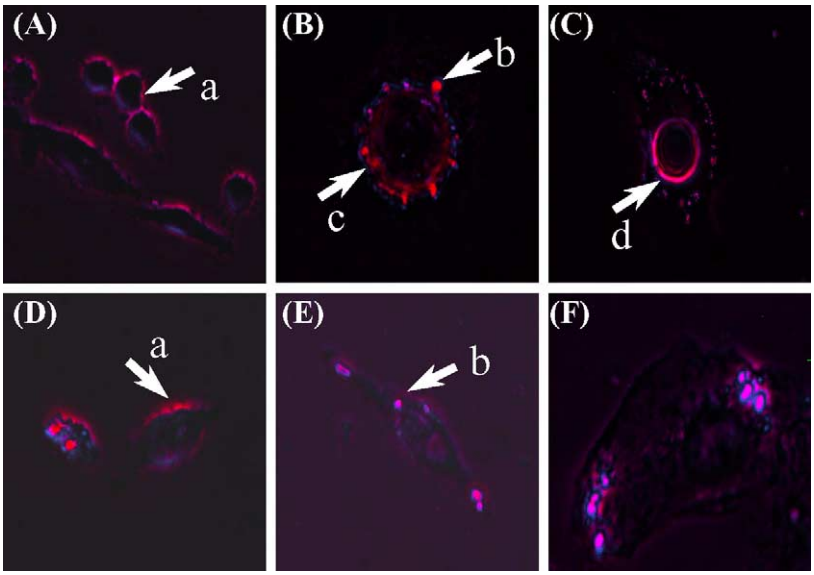

Fig. 3. Fluorescent microscopy of the interaction of Rh-PE-labeled micelles with BT20 cells. MIC-VK3-DBU: co-incubation for 0.5 h (A); 1 h (B); 4 h (C). MIC-VK3: co-incubation for 0.5 h (D); 1 h (E); 4 h (F). The binding of micelles to the cell surface (a); the formation of endosomes (b); the endosomal escape (c); the accumulation in the perinuclear space (d).

VK3 attached to the cell's outer surface (Fig. 3A and D, fluorescent structures on the cell surface labeled as "a"). No endocytosis was observed at this point. One hour later, however, it was clearly seen that both MIC-VK3-DBU and MIC-VK3 were endocytosed and could be seen in the endosomes of the cell (Fig. 3B and E, fluorescent dots inside cells labeled as "b"). In addition, some broader structures with less compact fluorescence could be found in the cytoplasm of BT-20 cells incubated with MIC-VK3-DBU but not with MIC-VK3 (Fig. 3B, structures labeled as "c"). Four hours later, a significant decrease of the fluorescence associated with endosome-trapped micelles was observed within the cells incubated with MIC-VK3-DBU. Instead, an accumulation of the fluorescence in the perinuclear space was observed (Fig. 3C, fluorescence labeled as "d"). However, no similar structures or fluorescence accumulation on the nuclear membrane were found in the BT-20 cells incubated with MIC-VK3 (Fig. 3F). These results suggest that DBU induced a certain destabilization of endosomes, which led to the escape of drug-loaded micelles directly into the cytoplasm. One can assume that the enhanced arylation of the SH-groups of some proteins by the VK3 in the presence of the DBU could be involved not only in the anti-cancer effect but also in the endosome destabilization, if an extensive chemical modification of protein endosomal components takes place. This phenomenon may provide another possible explanation of the synergistic anticancer action of the combination of VK3 and DBU encapsulated into PEG-DSPE micelles.

Further work is now in progress to investigate the exact mechanism of the enhanced anticancer action of MIC-VK3-DBU and evaluate its in vivo anticancer potential.

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