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Packaging the Alkaloids of Cinchona Bark in Combination with Etoposide in Polymeric Micelles Nanoparticles

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***Abstract*—**Today, cancer remains one of the major diseases that lead to death. The main obstacle in chemotherapy as a main cancer treatment is the toxicity to normal cells due to Multidrug Resistance (MDR) after the use of anticancer drugs. Proposed solution to overcome this problem is the use of MDR efflux inhibitor of cinchona alkaloids which is delivered together with anticancer drugs encapsulated in the form of polymeric nanoparticles. The particles were prepared by the hydration method. The characterization of nanoparticles was particle size, zeta potential, entrapment efficiency and *in vitro* drug release. Combination nanoparticle size ranged 29-45 nm with a neutral surface charge. Entrapment efficiency was above 87% for the use quinine, quinidine or cinchonidine in combination with etoposide. The release test results exhibited that the cinchona alkaloids release released faster than that of etoposide. Collectively, cinchona alkaloids can be packaged along with etoposide in nanomicelles for better cancer therapy.

***Keywords*—**Cinchona alkaloids, etoposide, MDR efflux inhitor, polymeric nanomicelles.

I. Introduction

CANCER is a disease with high number of sufferers in the world. The World Health Organization (WHO) has long predicted that cancer would overtake heart disease as the leading cause of death. About 7.6 million people died on cancer in 2008, and about 12.4 million new cases are diagnosed each year. In addition, the WHO projected by 2030, cancer deaths will rise beyond numbers 11 million people [1]. Chemotherapy is the primary treatment of choice for select cancers. However, conventional anticancer treatments are nonspecific to target killing of tumor cells, may induce severe systemic toxicity, and produce drug resistant phenotypic growth [2]. It has been demonstrated that chemotherapeutic drugs were encapsulated into a carrier system to improve specificity of drugs so that toxic effect on normal cells could be reduced. However, they did not provide optimal results to suppress resistance problems. [3].

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One membrane transporter proteins responsible for the occurrence of multidrug resistance (MDR) in chemotherapy are ABC transporter (adenosine triphosphate-binding cassette). ABC transporter can pump out active substances that have been diffused into cancer cells, leading to a decrease in the concentration of the active substances in cancer cells. It is known that there are three known transporter responsible for the mechanism of resistance, the P-glycoprotein (P-gp), MDR-associated protein (MRP1) and breast cancer resistance protein (BCRP) [4]. P-gp is overexpressed on tumor cells and plasma membrane responsible for the process of efflux few chemotherapeutics such as doxorubicin, etoposide and paclitaxel [5]. It was demonstrated that inhibition of the activity of P-gp was enhanced the accumulation of chemotherapeutics in cancer cells. Some P-gp inhibitors have known among others verapamil, cyclosporine [6] and quinidine [5].

A potential strategy to solve drug resistant issue is combination therapy by administering chemotherapeutic and MDR modulator [7]. It was reported that doxorubicin was combined with verapamil in liposomes and the combination of doxorubicin and P-gp inhibitors (GG918) which were prepared in novel polymer-lipid hybrid nanoparticle (PLN) [8]. Combination of doxorubicin and verapamil were also prepared and they were incorporated in transferrin-modified liposomes. It was demonstrated that the lowest IC50 on the K562 cells (sensitive-cells to doxorubicin) was derived from single administration of doxorubicin. While the lowest IC50 in K562/DOX cells (doxorubicin resistant cells) was generated from administration of combination doxorubicin and verapamil which were encapsulated in liposomes and in the form of unencapsulated [8]. These results indicated that administration of combination doxorubicin and verapamil increased citotoxicity of doxorubicin to the resistant cancer cells. Combination of doxorubicin and inhibitor P-gp (GG918) are in novel polymer-lipid hybrid nanoparticles (PLN) [9]. It was reported that co-administration of GG918 increased the uptake of doxorubicin on the doxorubicin resistance cells. The highest uptake of doxorubicin, the highest cytotoxicity and the longest suppression of cancer cells proliferation was obtained from doxorubicin combined with GG918 in the liposomes.

Cinchona alkaloids reported to have the ability as a P-gp efflux inhibitor. It was reported that the alkaloid cinchona as conceived by quinine, quinidine, cinchonine and cinchonidine can improve the accumulation of doxorubicin in cancer cells

[10]. Giving cinchonine intravenously in mice showed increased accumulation of doxorubicin in some cancer cells (mouse colon cells and leukemic cells K562/ADM DHD/K12/PROb) [10]. In the study, mice that had been injected doxorubicin-induced cancer and cinchonine delivered separately. This can lead to resistance and toxic to normal cells. A better concept to be offered in this research is to incorporate both anticancer drugs and compounds inhibiting P-gp efflux into the same carrier as nanoparticles for delivery to the cell. This study investigated the packaging of MDR-efflux inhibitor of the alkaloid cinchona bark and chemotherapeutics of etoposide in a nanoparticle system with polymeric micelles. When chemotherapeutic agent is packaged with MDR-efflux inhibitor, both substances would be delivered to the same target and significantly reduce resistant side effect of inhibitor P-gp.

II. Materials and Methods

A. Materials

Poloxamer (Synperonic PE-P84), quinidine and etoposide were purchased from Sigma-Aldrich Pte. Ltd (Singapore). Quinine, cinchonine, cinchonidine, proanalytical ethanol and glacial acetic acid, sodium acetic, potassium dihydrogen phosphate and sodium hydroxide were purchased from PT. Merck Tbk (Jakarta, Indonesia). Acetonitrile was purchased from JT. Baker (Singapore). Other materials used in this study were proanalytic grade.

B. Methods

1. Preparation of single cinchona alkaloids-loaded nanoparticles

Single drug-loaded nanoparticles were prepared by the hydration method. At this stage, formulation of the nanoparticles was prepared for each MDR-efflux inhibitor used such as quinine, quinidine, cinchonine and cinchonidine. Poloxamer and drugs densities were varied to obtain the best optimization formula. Initially, the cinchona alkaloids and poloxamer were independently dissolved in ethanol. Range of varied densities prepared for quinine, quinidine, cinchonine, cinchonidine and poloxamer were 0.1-0.3% w/v, 0.1-0.3% w/v, 0.1-0.15% w/v, 0.1-0.4 5 w/v and 1-10% w/v, respectively. The alkaloid quinine was mixed with poloxamer solution until homogeneous. The ethanol was evaporated until it formed a thin film layer. These thin layers were further subjected to a vacuum desicator for overnight to completely remove traces of solvent. Thin-film layers were hydrated using deionized water with temperature of 50 ± 1 ˚C. The hydration process was allowed for 10 minutes and stirred with the rate of 500 rpm speed for 1 hour. The temperature was maintained at 50±1˚C during stirring process. Nanoparticle suspension was then filtered through the membrane with pores size of 0.2 µm (Minisart) to eliminate unencapsulated drug.

2. Preparation of combination of the alkaloid quinine and etoposide -loaded nanoparticles

Four types of combination drug-loaded nanoparticles, quinine-etoposide, quinidine-etoposide, cinchonine-etoposide and cinchonidine-etoposide were prepared by the hydration method. Ethanol was used to dissolve the cinchona alkaloids, etoposide and poloxamer. Firstly, etoposide and poloxamer were initially prepared with concentrations of 0.015 and 10% w/v, respectively. The quinine and quinidine alkaloids were prepared with concentration of 0.3 % w/v, whereas the alkaloid cinchonine and cinchonidine were prepared with a concentration of 0.1 % w/v. The solutions of cinchona alkaloids, etoposide and poloxamer were homogeneously mixed. The ethanol was evaporated until it formed a thin film layer. These thin layers were further subjected to a vacuum desicator for overnight to completely remove traces of solvent. Thin-film layers were hydrated using deionized water with temperature of 50 ± 1 ˚C. The hydration process was allowed for 10 minutes and stirred with the rate of 500 rpm speed for 1 hour. The temperature was maintained at 50±1˚C during stirring process. Nanoparticle suspension was then filtered through the membrane with pores size of 0.2 µm (Minisart) to eliminate unencapsulated drug

3. Physicochemical characterization of nanoparticles

The particle size, size distribution, polydispersity index and zeta potential of each sample were determined using Delsa Nano C particle analyzer (Beckman Coulter). After the nanoparticles in glass tube was sonicated for approximately 10 min in a bath-type sonicator (Branson Ultrasonics 5510) and resuspended, sample was poured into a cuvette and examined using this instrument.

4. Determination of entrapment efficiency of etoposide and or the alkaloid quinine in the nanoparticles

Entrapment efficiency of single drug-loaded nanoparticles was performed by direct method. The amount of etoposide, quinine/quinidine or cinchonine/cinchonidine was measured in the supernatant by UV spectrofotometry at wavelength 284, 331, or 289 nm, respectively. Determination of entrapment efficiency of combination-drug loaded nanoparticles was performed using the method of high performance liquid chromatography (HPLC). Stationary phase used in this method was 5 µ Kromasil Phenomenex columns 100 A C18 with dimensions 250 x 4.6 mm. Mobile phase used was a mixture of acetonitrile and acetate buffer pH 4 (35: 65 v/v) with a flow rate of 1 mL/min. The detector used was ultra violet lamp at a wavelength of 254 nm. Samples were diluted with deionized water and then injected into the HPLC system. Entrapment efficiency was calculated using the following equation: percent entrapment = (amount of substance in the nanoparticle)/(initial dose of substance) x 100%

5. Evaluation of in vitro release of etoposide and the alkaloids of cinchona

Release test of active substances from combination of etoposide and the cinchona alkaloids-loaded nanoparticles was carried out by diffusion method using dialysis membrane which has a molecular weight cut off 12,000 dalton. The doses applied for etoposide, quinine, quinidine, cinchonine and cinchonidine were equivalent to 0.293, 5.548, 5.279, 0.556 and 1.963 mg, respectively. The nanoparticles were incubated in an orbital

shaker with constant temperature of 37 ± 0.5°C. The dialysis bag that has contained nanoparticles was put into 200 mL medium solution of phosphate buffer pH 6. Samples were stirred at a rate of 75 rpm for 18 hours. At certain time intervals (0.25, 0.5, 0.75, 1, 2, 3, 6, 9, 12 and 18 hours), samples were taken 5 mL of test solution, then replaced it with a 5 mL of fresh medium. Each sample was filtered using membrane filters 0.2 µm, then analyzed using the HPLC method as explained above. All the experiments were performed in triplicate.

III. Results and Discussion

Based on the previous report showing that separately administration of cinchonine alkaloids were extensively enhanced accumulation of doxorubicin in some cancer cells, we attempted to package the alkaloids of cinchona bark together with etoposide in polymeric micelles nanoparticles for reducing toxicity problem on the normal cells.

Poloxamer polymers can form micelles in an aqueous medium above criticical micelles concentration (CMC). Poloxamer micelle formation occurs spontaneously and is influenced by polymer concentration and temperature system [11]. The selection of poloxamer type was based on the poloxamer classification which has an inhibition effect on P-glycoprotein (P-gp) [12]. Poloxamer used in this study was classified in the second group that has an excellent P-gp inhibitory activity.

In this study, selected nanoparticles form was polymeric micelles type. This nanomicelle is suitable for substances that are hydrophobic because the core of the micelles formed is hydrophobic. Thus, the encapsulation of hydrophobic would be excellent. As for the hydrophilic substances, liposomes would be better because the core liposome could be adjusted to hydrophilic [13]. In this work, the alkaloids of cinchona bark and etoposide were hydrophobic, so that the use of polymeric micelles of poloxamer was selected for the formulations.

Polymeric micelles have several characteristics which have a size of about 20-100 nm. By the use of poloxamer, it has hydrophilic surface properties so that it can circulate longer in the blood circulation [13]. Thus, polymeric micelles are suitable for cancer drug delivery systems that are passively accumulate to the intended target. With a very small size, nanoparticles can enter and penetrate into the pores formed on the surface membrane of cancer cells. While the nature of the hydrophilic surface allows to avoid taken up by the reticuloendothelial system, so that the nanoparticles can circulate longer in the blood circulation.

In the preparation of single drug-loaded nanoparticles, formula designed to produce quinine nanoparticles had entrapment efficiency above 90% except for densities of quinine 0.3% (w/v) and poloxamer 3% (w/v). This micelles produced 69.19% entrapment efficiency. This is due to a small density of poloxamer which was unable to produce a sufficient number of micelles to encapsulate large amounts of quinine. When the density of poloxamer was increased to 5% (w/v) and 7% (w/v), percent entrapment quinine was increased to 92.28% and 92.66%, respectively. Additionally, quinine with the density of 0.1% (w/v) was added to the water without poloxamer. As a result, quinine was dissolved in the water in limited amount approximately 24.31%. While poloxamer with the density of 1% (w/v), entrapment efficiency of quinine was drastically enhanced approximately 92.43%. It is likely that an increase in the solubility of quinine were highly significant with the addition of poloxamer as much as 1% (w/v).

TABLE I
Characteristics of Single Drug-Loaded Nanoparticles

Formula	Composition (%w/v)	Size (nm)	PI	Entrapment (%)
F1	0.1Q + 1P	37.05	0.04	92.43
F2	0.1Q + 3P	34.80	0.26	100.43
F3	0.1Q + 5P	34.45	0.40	93.29
F4	0.2Q + 3P	31.15	0.39	90.37
F5	0.3Q + 3P	30.80	0.35	69.19
F6	0.3Q + 5P	35.50	0.35	92.28
F7	0.3Q + 7P	36.80	0.17	92.66
F8	0.1Qd + 1P	33,00	0.06	29.97
F9	0.1Qd + 3P	37.23	0.23	91.88
F10	0.1Qd + 5P	34.07	0.17	91.43
F11	0.3Qd + 7P	35.47	0.35	48.73
F12	0.3Qd + 10P	33.47	0.44	92.40
F13	0.1C + 1P	N.D.	-	6.44
F14	0.1C + 3P	43.73	0.02	9.87
F15	0.1C + 5P	32.27	0.33	14.18
F16	0.1C + 10P	33.03	0.12	21.76
F17	0.15C + 10P	33.70	0.32	16.86
F18	0.1Cd + 1P	N.D	-	28.00
F19	0.1Cd + 3P	54.83	0.56	33.32
F20	0.1Cd + 5P	31.50	0.34	96.43
F21	0.15Cd + 7P	39.17	0.35	29.58
F22	0.15Cd + 10P	36.27	0.14	90.58
F23	0.2Cd + 10P	32.00	0.33	57.63
F24	0.4Cd + 7P	38.37	0.30	16.06
F25	0.4Cd + 10P	36.97	0.15	24.58
F26	0.015E + 5P	39.88	0.06	89.40
F27	0.015E + 7P	38.33	0.22	94.17
F28	0.015E + 10P	37.95	0.16	106.83

Q : Quinine Qd : Quinidine C : Cinchonine Cd : Cinchonidine
P : Poloxamer E : Etoposide N.D. : not detected

In the preparation of single quinidine-loaded nanoparticles, when the densities of quinidine of 0.1% (w/v) and poloxamer 1% (w/v), the entrapment was limited 29.97%. However, when the densities of poloxamer increased to 3% (w/v) and 5% (w/v), an increase in trapping ratio was very significant to more than 90%. When performed the experiment by increasing the density of quinidine up to 3% (w/v) and poloxamer 7% (w/v), entrapement efficiency was obtained approximately 48.73%. This level is considered low because many portions were unencapsulated. To improve entrapment, density of poloxamer was increased to 10% (w/v). As a result, the entrapment efficiency was significantly increased to 92.40%. The small percent of entrapment caused by a lack of concentration poloxamer impacted on the lack of micelles formed to

TABLE II
CHARACTERISTICS OF COMBINATION DRUG-LOADED NANOPARTICLES

No.	Composition (% w/v)	Size (nm)	PI	ζ (mV)	Entrapment (%)	
					Cinchona alkaloids	Etoposide
F29	10P + 0.3Q + 0.015E	29.15	0.24	-0.74	92.47	95.98
F30	10P + 0.3Qd + 0.015E	34.38	0.20	-2.74	87.98	90.71
F31	10P + 0.1C + 0.015E	44.30	0.45	-2.80	27.78	105.11
F32	10P + 0.1Cd + 0.015E	33.45	0.27	-3.43	98.16	97.32

encapsulate active substances. With increasing concentration of poloxamer, increasing the number of micelles were formed so that the space to encapsulate active substances increased.

In the case of the use of cinchonine, its entrapment efficiency in single-loaded nanoparticles was enhanced with concomitant increased in the density of poloxamer used. However, the highest entrapment efficiency was extremely limited approximately 21.76%. This value was considerably low comparing to other cinchona alkaloids. These results indicated that with increasing densities of poloxamer were used, the percent entrapment of substances into the micelles was enhanced. The particle size of the nanoparticles formed cinchonine was around 32-44 nm with a polydispersity index below 0.5. When cinchonidine was encapsulated in the polymeric micelles, encapsulation efficiency gradually increased by the use of higher density of poloxamer. This result was in line with the results of other alkaloids cinchona in which the surface of hydrophobic alkaloid was shifted to hydrophilic one by the contribution of poloxamer. In general, factors that influenced entrapment efficiency of single-loaded drug nanoparticles were the densities of active substances and polymer. It is likely that the high density of poloxamer micelles resulted in large numbers so that the space for the encapsulation of active substances into the micelle core increased. In terms of particle size, there was no significant effect between elevated densities of poloxamer and the resulting particle size.

Zeta potential of combination cinchona alkaloids and etoposide in nanoparticles range from-0.74 to-3,43 mV (Table II). These results indicated that the surface of the nanoparticles was relatively neutral charge. This is due to the used polymer was non-ionic. Encapsulation efficiency for trapping cinchona alkaloids or etoposide was greater than 87% except on cinchonine were limited approximately 27.78%. This result is in line with an entrapment efficiency of a single cinchonine-loaded nanoparticle in which trapping percentage of cinchonine was also extremely low. Thus, more optimization is needed to prepare nanoparticles containing etoposide in combination with cinchonine alkaloid.

Test release of active substances was performed by diffusion method using dialysis bag. Dialysis bag used in the study had a pore size or commonly called by MWCO (molecular weight cut off) of 12 kDa. This means those only substances that have a molecular weight less than 12 kDa, which diffuses and out through the dialysis bag. The medium used in the release test was phosphate solution in a pH of 6. This medium was chosen since the release of the drug target was in intracellular having a pH of about 6 or commonly referred to with the term early endosome. In the earlier time of release process, the release of quinine was more dominant compared with etoposide. After 6 h, quinine was released approximately 99.68%, while etoposide was released about 49.79% (Fig. 1). Release etoposida reached more than 90% achieved at 12 h. It is suggested that it needed a much longer time to release all of the etoposide in nanoparticles compared to that of the quinine. The release profile of combination etoposide and quinidine were nearly identical to the release profile of substances on quinine-etoposide combination formula. At the beginning of the release process, adstringent quinidine released more dominant compared with etoposide. After 6 h, the release of quinidine was 98.80%, while etoposide was released approximately 47.44%. Etoposide achieved a release of 100% in the next 18 h. Almost the same patterns with the pattern release of the combination quinine-etoposide and quinidine-etoposide, were occurred for the release of substances from the combination cinchonine-etoposide and cinchonidine-etoposide. After 6 h, the released of cinchonine and cinchonidine were approximately 90% and followed the release of etoposide was approximately 90% after 18 h.

Etoposide was released slower than the release of the alkaloid of cinchona bark. It is presumably that the differences hydrophobicity between the cinchona alkaloids and etoposide. The difference of this hydrophobicity influence on the position of each of the substances in the nanoparticles. The nature of core the micelle poloxamer is hydrophobic. Thus, substances that is highly hydrophobic properties would tend to tend to occupy an inner position of the core in the micelle structure. Dependent on hydrophobicity of the structure, it is likely that etoposide were more hydrophobic than the cinchona alkaloids. Therefore, etoposide would be disposed to the inside of the core structure of the micelle. This led to the release of etoposide from the polymeric micelles became slower.

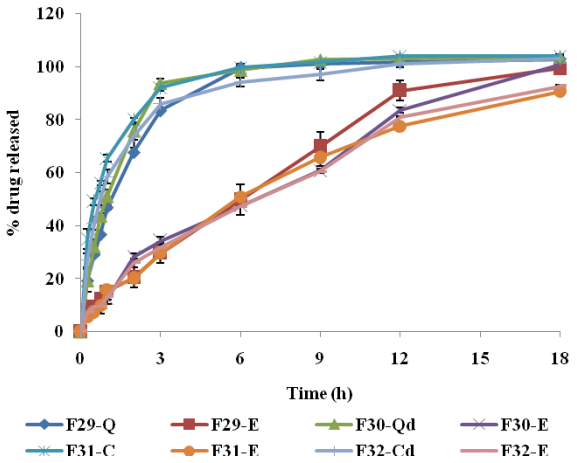

Fig. 1 The cinchona alkaloids and etoposide release profiles from nanoparticles system

It was previously reported that selection of the four cinchona alkaloids was performed to find the best effect on the inhibition of P-gp. In this case, cinchona alkaloids were firstly administered a few hours before therapy with anticancer. It is suggested that cinchona alkaloids should previously work to inhibit the activity of P-gp [10]. It was then followed by the hemotherapy drug to work to inhibit the cell growth. It has also been demonstrated that phase 1 clinical trials was performed against cinchonine to inhibit cancer resistance to chemotherapy [14]. In this study, the initial therapy used was intravenous administration of cinchonine prior to the delivery of anticancer drugs. Based on the reported data, it was summarized that the cinchona alkaloids should be worked out in advance to suppress the activity of P-gp. It was then proceeded with anticancer activity so that P-gp is not able to pump out a given activity because anticancer has been inhibited. This is analogous to the release of test results of active substances for the combination formula nanoparticles produced in this research. The alkaloids cinchona was released faster, then it was followed by the release of etoposide. However, this needs to be demonstrated through its effectiveness in vivo assay activity for all of the resulting formula.

IV. Conclusion

The increase of hydrophilic moiety of poloxamer on the nanoparticles enhanced encapsulation efficiency of etoposide and the cinchona alkaloids tested into the micelles. The alkaloid of cinchona bark can be packaged into the same structural nanoparticlec ontaining etoposide as a MDR-efflux inhibitor with high entrapment efficiency, particularly for quinine, quinidine and cinchonidine. In addition, the hydrophobicity and disposition in the micelles may influence the release rate of the contents. Collectively, these results indicate that adequate density control of the constituents forming nanoparticles is an important factor in designing the prominent particles for anticancer therapy.

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