รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาฤทธิ์ต่อเซลล์มะเร็งในตับร่วมกันของด๊อกโซรูบิซินและพาคลิเทคซอลที่ถูกปล่อยจากระบบส่งยาแบบฉีดที่ผลิตจากพอลิเมอร์

โดย รศ.ดร.นรเศรษฐ์ ณ สงขลา

มิถุนายน 2560 ที่เสร็จโครงการ

สัญญาเลขที่ RSA5680027

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รศ.ดร.นรเศรษฐ์ ณ สงขลา มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และมหาวิทยาลัยมหิดล

กิตติกรรมประกาศ

โครงการวิจัยนี้ได้รับทุนอุดหนุนจากทุนพัฒนานักวิจัย โดยได้รับงบประมาณประจำปี พ.ศ. 2558 จำนวน 176,666.00 บาท ระยะเวลาทำวิจัย ตั้งแต่ มิถุนายน 2558 ถึง พฤศจิกายน 2558

Abstract

Project Code : RSA5680027

Project Title : Combination chemotherapy of doxorubicin and paclitaxel against human liver cancer cells by injectable polymeric drug delivery system

Investigator : Assoc. Prof. Dr. Norased Nasongkla Mahidol University

E-mail Address : norased.nas@mahidol.ac.th

Project Period : 0.5 year

Doxorubicin (DOX) and paclitaxel (PTX)-encapsulated polymeric depot provided controllable drug release for liver cancer treatment. The excess of initial burst release of drug-loaded polymeric depot is remaining problem of drug efficiency. To diminish the initial burst release of polymeric depot, the variation of poly(lactic-*co*-glycolic acid) (PLGA) weight ratio of protonated and deprotonated DOX in the depots was investigated. The results showed that high level of initial burst release was occurred at 100% protonated DOX within 24 hours (86.6 $\pm$ 5.3%) and was reduced to 48.5 $\pm$ 2.5, 41.8 $\pm$ 1.5, 35.1 $\pm$ 2.4, 27.3 $\pm$ 2.9, and 22.0 $\pm$ 2.1 % when protonated DOX ratio was reduced to 98.75, 97.5, 95, 90, and 85%, respectively. This data indicated that 15% deprotonated DOX could reduce initial burst release. Moreover, at lower concentration of 85% protonated DOX-loaded depot could exhibit cytotoxic effect on HepG2 cells. In addition, the releases of DOX and PTX were increased by time-dependent manner at 20% PLGA and 30% drug loading, interestingly, the release of DOX-depot was obviously increased

within 2 hours but was taken long period by PTX-depot. For combined DOX-PTX-loaded polymeric depots, ratio of drug at 25:1 and 35:1 of DOX:PTX presented high ratio of DOX:PTX release as 20:1. Preliminary study of synergistic effect of DOX and PTX on HepG2 cell, the effective ratio of DOX:PTX was shown at 5-50:1 with pre-incubated for 6 hours, 18 hours and 2 days. From this data, we evaluated the effect of combined DOX-PTX-loaded depot on HepG2 cells and the results revealed that was cytotoxicity from combined drug release at 18:1 of DOX:PTX after pre-incubation for 1 and 3 days, and also presented synergistic effect with CI value 0.0050 ± 0.0002 and 0.0032 ± 0.0002, respectively. Thus, this exploration could be advantage for further development of liver cancer treatment.

Keywords : Doxorubicin, Paclitaxel, Synergistic effect, Polymeric depots

บทคัดย่อ

รหัสโครงการ RSA5680027

ชื่อโครงการ การศึกษาฤทธิ์ต่อเซลล์มะเร็งในตับร่วมกันของด๊อกโซรูบิซินและพาคลิเทคซอลที่ถูกปล่อยจากระบบส่งยาแบบฉีดที่ผลิตจากพอลิเมอร์

ชื่อนักวิจัย รศ.ดร.นรเศรษฐ์ ณ สงขลา มหาวิทยาลัยมหิดล

E-mail Address : norased.nas@mahidol.ac.th

ระยะเวลาโครงการ 0.5 ปี

การใช้ระบบส่งยาแบบฉีดที่ผลิตจากพอลิเมอร์ในการขนส่งยาด๊อกโซรูบิซินและพาคลิเทคซอลสามารถช่วยควบคุมการปลดปล่อยยาเพื่อใช้ในการรักษามะเร็งตับได้ ซึ่งปัญหาสำคัญของระบบส่งยาดังกล่าวคือปริมาณยาที่ถูกปลดปล่อยออกมาในปริมาณมากในช่วงแรก ทำให้ประสิทธิภาพของยาลดลง ดังนั้นเพื่อเป็นการลดปริมาณยาที่ถูกปลดปล่อยออกมาในช่วงแรก ทางผู้วิจัยจึงได้ทำการทดสอบหาอัตราส่วนน้ำหนักที่เหมาะสมของยาด๊อกโซรูบิซินชนิดโปรโตเนตและดีโปรโตเนต โดยใช้พอลิแลคติกโคไกลโคลิกเอซิด (poly(lactic-*co*-glycolic acid), PLGA) จากการศึกษาพบว่าการใช้ยาด๊อกโซรูบิซินชนิดโปรโตเนต 100% จะมีปริมาณยาที่ถูกปลดปล่อยออกมาในช่วงแรกในปริมาณสูงภายในเวลา 24 ชั่วโมง (ปริมาณยาที่ถูกปลดปล่อยออกมาคิดเป็นร้อยละ 86.6 $\pm$ 5.3) และปริมาณยาที่ถูกปลดปล่อยจะลดลงเมื่อลดอัตราส่วนของยาด๊อกโซรูบิซินชนิดโปรโตเนตเป็น 98.75, 97.5, 95, 90 และ 85% (ปริมาณยาที่ถูกปลดปล่อยออกมาคิดเป็นร้อยละ 48.5 $\pm$ 2.5, 41.8 $\pm$ 1.5, 35.1 $\pm$ 2.4, 27.3 $\pm$ 2.9 และ 22.0 $\pm$ 2.1 ตามลำดับ) จากข้อมูลดังกล่าวจึงสามารถสรุปได้ว่าการใช้ยาด๊อกโซรูบิซินชนิดดีโปรโตเนต 15%

สามารถช่วยลดปริมาณยาที่ถูกปลดปล่อยออกมาในช่วงแรก นอกจากนี้การใช้ยาด๊อกโซรูบิซินชนิดโปรโตเนต 85% ยังเหนี่ยวนำให้เกิดความเป็นพิษต่อเซลล์มะเร็งตับ (HepG2) ได้ด้วย ในการศึกษาการปลดปล่อยยาด๊อกโซรูบิซินและพาคลิเทคซอลโดยใช้อัตราส่วนพอลิเมอร์ร้อยละ 20 และปริมาณยาร้อยละ 30 พบว่าอัตราการปลดปล่อยของยาสัมพันธ์กับระยะเวลา โดยยาด๊อกโซรูบิซินจะถูกปลดปล่อยออกมามากภายใน 2 ชั่วโมง ส่วนยาพาคลิเทคซอลจะใช้เวลานานกว่าในการปลดปล่อยยาออกมา ทั้งนี้ในการศึกษาผลร่วมกันของยาสองชนิดคือด๊อกโซรูบิซินและพาคลิเทคซอลในระบบส่งยาแบบฉีดที่ผลิตจากพอลิเมอร์พบว่าการใช้ยาด๊อกโซรูบิซินและพาคลิเทคซอลที่อัตราส่วน 25:1 และ 35:1 ให้ผลการปลดปล่อยยาด๊อกโซรูบิซินและพาคลิเทคซอลที่อัตราส่วน 20:1 ซึ่งจากการศึกษาเบื้องต้นของยาด๊อกโซรูบิซินและพาคลิเทคซอลในเซลล์มะเร็งตับ พบว่าอัตราส่วนยาด๊อกโซรูบิซินและพาคลิเทคซอล 5-50:1 หลังจากบ่มยาทั้งสองชนิดเป็นเวลา 6 ชั่วโมง 12 ชั่วโมง และ 2 วัน มีผลการเสริมฤทธิ์กันของยาทั้งสองชนิดในการต้านมะเร็งตับ จากผลดังกล่าวจึงนำไปสู่การทดสอบผลร่วมกันของยาด๊อกโซรูบิซินและพาคลิเทคซอลที่ถูกปลดปล่อยจากระบบส่งยาแบบฉีดที่ผลิตจากพอลิเมอร์ โดยปริมาณยาด๊อกโซรูบิซินและพาคลิเทคซอลที่ถูกปลดปล่อยในอัตราส่วน 18:1 หลังจากบ่มยาเป็นเวลา 1 วัน และ 3 วัน พบว่ามีผลเหนี่ยวนำความเป็นพิษต่อเซลล์มะเร็งตับและยังมีผลการเสริมฤทธิ์กันของยาทั้งสองชนิด จากการแสดงของค่า CI เท่ากับ 0.0050 ± 0.0002 และ 0.0032 ± 0.0002 ผลการศึกษาครั้งนี้สามารถเป็นประโยชน์ต่อการศึกษาและพัฒนาการรักษามะเร็งตับต่อไป

คำหลัก: ด๊อกโซรูบิซิน, พาคลิเทคซอล, การเสริมฤทธิ์กันของยา, ระบบส่งยาแบบฉีดที่ผลิตจากพอลิเมอร์

Introduction

Currently, liver cancer is one of most leading cause of death globally. Owing to its poor prognosis and response of chemotherapy, the tumor recurrence can be occurred in patients [1, 2]. However, the systemic chemotherapy is remaining conventional treatment by specific action on cancer cells. The common cancer chemotherapeutic agents have been used including cis-platinum, interferon, 5-fluorouracil, doxorubicin (DOX), and paclitaxel (PTX). DOX, a highly active anthracycline antibiotic, is used as first-line treatment of liver cancer [3, 4]. DOX is act as a topoisomerase II inhibitor which can inhibit cancer cell growth and causing DNA adduct formation. Remarkably, there are principal adverse effects of DOX in human body such as cardiotoxicity, bone marrow suppression, hyperuricemia, and leukopenia [5]. Paclitaxel (PTX) is widely used for several cancer therapy such as lung, ovary breast and liver cancers. The anticancer activity of PTX is shown by interfering the disintegration of microtubule resulting in induction of cell cycle arrest and cell death [6]. Nonetheless, the limitations of PTX comprised of poor water solubility, high protein binding (89 - 98%) and provide some side effects such as leukopenia, nausea, vomiting, diarrhea, and hypertension.

In order to reduced adverse effect of single drug using, the combination of cancer chemotherapeutic drugs were broadly used with the synergistic response of drugs. Methotrexate, vincristine, 6-mercaptopurine (6-MP) and prednisone so-called POMP regimen was used in acute lymphoblastic leukaemic patients and it was able to induce long-term remissions [7]. Otherwise, DOX is selectively used as combination chemotherapy with several chemotherapeutic agents (**Table 1**).

However, these drug combinations could provide unexpected results with low response rates (0-25%) and no prolonged survivals [8, 9]. To improve drug efficacy, the new combination of DOX with other drugs have been conducted in clinical trials but still low response rates (**Table 2**).

Table 1. Selected clinical trials for common combination of doxorubicin

Drug	Cancer types	Response rate	Comments	Phase	Ref
doxorubicin + cisplatin	metastatic HCC	2.7%CR, 16.2%PR, 17.2%SD 64.5%PD	- modest antitumor activity - tolerable adverse effects	2	Lee J et al. [10]
doxorubicin + cisplatin + capecitabine	metastatic HCC	24%PR, 20.6%SD	- modest antitumor activity - tolerable adverse effects	2	Park S et al. [11]
PIAF	inoperable HCC	26%PR, no CR, 28%SD, 30%PD	- active in HCC - improved operability - moderate toxicity	2	Leung T et al. [12]
PIAF	biliary tract cancer	21.1%	- recommend for gallbladder carcinoma, not for cholangiocarcinoma - significant toxicity	2	Patt Y et al. [13]
PIAF	unresectable HCC	PIAF (20.9%) doxorubicin (10.5%)	- no statistical difference - treatment-related toxicity.	3	Yeo W et al. [14]

PIAF: doxorubicin + cisplatin + 5-fluorouracil + interferon-α, PR: partial response, CR: complete response, SD: stable disease, PD: progression of disease

Table 2. Selected clinical trials for combination of doxorubicin and other drugs

Drug	Cancer types	Response rate	Comments	Phase	Ref
Doxorubicin + sorafenib	inoperable HCC	4%PR, no CR, 63%SD, 31%PD	- well tolerated and more effective than doxorubicin alone	1	Richly H et al. [15]
Doxorubicin + sorafenib	advanced HCC	4%PR	- greater median time to progression, overall survival, and progression-free survival	2	Abou-Alfa G et al. [16]
Doxorubicin + topotecan	HCC	13.9%, no CR	- significant toxicity with topotecan - ineffective with advanced HCC	2	Wall J et al. [17]

PR: partial response, CR: complete response, SD: stable disease, PD: progression of disease

The combination of DOX and PTX have been studied in HCC therapy. It has been proven to be in solid tumors [18, 19] and granted high response rates in breast cancer therapy [20, 21]. The study of synergistic effect of DOX and PTX on HepG2 cells were recently investigated and available as in **Table 3**.

Table 3. Effect of combination of DOX and PTX in HepG2 cell line

Doxorubicin (ug/ml)	Paclitaxel (ug/ml)	Drug delivery system	CI	Ref
0.5	0.01	free drug	Synergy	Jin C et al. [22]
96.66	71.16	double emulsion (mPEG-PLGA)	Synergy	Wang H et al. [23]

The results of dual drug study of DOX and PTX by Jin C et al. found that at this drug concentration could show effective response in high single dose but in short period. From this point, the multiple doses given should be a selective approach to achieve highest therapeutic efficacy in HCC therapy.

However, there are some drug limitations that affect to drug efficiency including nonselective biodistribution, poor bioavailability and solubility, and low specificity. The polymeric drug delivery systems (DDS) is selected to be an alternative approach by control the drug transportation in cells. General, polymeric drug carriers have been developed in numerous forms such as liposomes [24], micelles [25], albumin particle [26], dendrimer [27], nanoparticles [28, 29], etc. In this study, an injectable polymeric depot was formulated as spherical depot. This form of depot was considered that could directly deliver drugs to targeted cancer cells by maximizing the local drug concentration and minimizing the systemic toxicity. Moreover, another advantages of local injectable depot are reduction of surgical site and the ease of preparation. In previous study, SN-38 was successfully loaded into these depots with high encapsulation efficiency (> 98%) and it is able to protected almost 100% of encapsulated SN-38 from converting to an inactive form for more than 45 days which can present the cytotoxic effect against human glioblastoma cell line (U-87MG) [30]. Furthermore, it has been studied in animal model and found that SN-38-loaded depots exhibited antitumor efficacy by sustained higher level of SN-38 inside tumors [31, 32]. From these investigations could verify that the using of polymeric depot is used for deliver therapeutic agents into animal cells and tissues. These valuable data hence lead us to use this local polymeric depot for other anticancer drugs.

In this study, we investigated the effect of dual drug-loaded polymeric depots, DOX and PTX, on HepgG2 cells for liver cancer treatment. To accomplish the goal of synergistic of drugs, the ratio of polymeric depot and drug loading were explored to obtain the best conditions. Moreover, the pre-incubation times of drug were varied to 1 day and 3 days. DOX and PTX-encapsulated polymeric depots were formulated by the self-solidifying process with controlling drug loading contents and drug release profile. The cytotoxicity was then evaluated.

Part 1

Preparation and fabrication of DOX and paclitaxel-loaded polymeric depots as injectable drug delivery system

Materials and Methods

Materials

PLEC 57 kDa (18.8% lactide) and PLGA 28.8 kDa (75% lactide) were synthesized from BioNEDD lab, Mahidol University. Doxorubicin hydrochloride (DOX) was obtained from Vesino Industrial Co., Ltd., Tianjin, China. Dimethyl sulphoxide, dichloromethane, ethyl acetate, and methanol were purchased from RCI Labscan Ltd. (Milwaukee, WI, USA). Formic acid was purchased from Carlo Erba Reagents (Milan, Italy). Paclitaxel (Taxol) was purchased from SHAANXI SCIPHAP BIOTECHNOLOGY CO., LTD. Tetraglycol (Glycofurol) and other reagents were purchased from Sigma Aldrich.

Cell supplementary

The human hepatocellular carcinoma cell line (HepG2, JCRB1054) was purchased from the Japanese Collection of Research Bioresources (JCRB, Japan). Cells were seeded in Dulbecco's Minimum Essential Medium (Biochrom, Germany) supplemented with 10 % fetal bovine serum (PAA Laboratories GmbH, Austria), 100U/ml of penicillin and 100 μg/ml of streptomycin (PAA Laboratories GmbH, Austria) in a humidified atmosphere incubator at 37 ^{o}C and 5 % CO_2.

Methods

Preparation of hydrophobic DOX

Protocol 1

40 mg of hydrophilic doxorubicin was weighed into centrifuge tube (size of 50 mL) and then dissolved by 10 mL of distilled water (final conc. = 4 mg/mL). After that, DOX solution was vortexed and sonicated until complete dissolved. The solution was frozen at 4 $^{\circ}$C for 10-15 minutes. The pH of this solution was adjusted to 9.6-9.9 using NaOH and HCl (this step was done on ice). The adjusted solution was freeze for 10-15 minute and then centrifuged at 4 $^{\circ}$C, 4000 rpm for 5 minutes. The supernatant was done by step 3-5 for 2 times and the sediment was washed with distilled water twice. Finally, all of the supernatants were collected and freeze-dried.

Protocol 2

100 mg of hydrophilic doxorubicin was weighed into centrifuge tube (size of 50 mL) and then dissolved by 15 mL of distilled water (final conc. = 6.67 mg/mL). DOX solution was vortexed and sonicated until complete dissolved. The solution was freeze at 4 $^{\circ}$C for 10-15 minutes. The cold solution was vortexed and the pH of the solution was adjusted to 10.5-10.6 using 0.2M NaOH and HCl. It should be noted that the pH adjustment was done during the spiral wave was occurred.The adjusted solution was freeze for 10-15 minute and then centrifuged at 4 $^{\circ}$C, 4000 rpm for 3 minutes. The supernatant was done by step 3-5 for 2 times and the sediment was washed with distilled water twice. All of the supernatants were collected and freeze-dried.

Purification of hydrophobic DOX

Protocol 1

Dried powder of hydrophobic DOX was dissolved in 30 mL of THF, vortexed and sonicate until completely dissolved. The solution was centrifuged at 4 $^{\circ}$C, 4000 rpm for 3 minutes. The supernatant was collected and freeze whereas step 1-2 was done with the sediment again.

Protocol 2

Dried powder of hydrophobic DOX was dissolved in 20 mL of THF and stirred for 1 hour. The solution was centrifuged at 4 $^{\circ}$C, 4000 rpm for 3 minutes. Step 1-2 was done with the sediment for 8 times. The THF was evaporated from the supernatant. All of the sediments were collect and kept at -20 $^{\circ}$C.

Stability of hydrophobic doxorubicin

TLC method was used to study the stability of hydrophobic DOX compared with DOX hydrochloride (commercial). Silica gel coated on a plate was used as a stationary phase, a polar part. Less polar parts which were used as mobile phases were 4.1 mL distilled dichloromethane, 1.2 mL pure methanol, 0.1 mL formic acid 85%, and 0.05 mL distilled water. Mobile phases were contained in the small vial which its size was similar to 50 mL beaker, and filter paper was put into vial to provide the saturated solvents vapor. Various type of DOX in aqueous solution (hydrophilic), THF (hydrophobic) were immediately spotted on TLC plate after preparation and put it into the closed vial. After the solvents near the top, TLC plate was removed from vial and the separated samples were noticed.

Calibration curve of DOX

1 mg of hydrophobic and hydrophilic DOX were weighed and dissolved in 10 mL of THF and PBS, respectively. The solution was then diluted using THF and PBS at various concentrations. DOX solution was determined using UV-visible spectrophotometer at 480 nm. Finally, DOX concentration and absorbance were plotted to evaluated a linear relationship.

Calibration curve of paclitaxel

1 mg of paclitaxel was weighed and dissolved in 10 mL of PBS: MeOH (1:1, v/v).The solution was then diluted using PBS: MeOH (1:1, v/v) at various concentrations. DOX solution was determined using UV-visible spectrophotometer at 480 nm. DOX concentration and absorbance was plotted to evaluated a linear relationship.

Preparation of hydrophilic & hydrophobic dox-loaded depots

PLEC (57 kDa and LA content at 18.8 %) was dissolved with glycofurol (GF) with an assisting of a high temperature. After that, various ratios of hydrophilic to hydrophobic DOX was varied as shown in **Table 4**. Polymer concentration and total drug loading content were fixed at 25 and 15%, respectively. For the *in vitro* release study, different formulations (Table 1) of polymeric solution (PLEC-glycofurol-hydrophihlic&hydrophobic DOX) were mixed by a vortex. The depots were prepared by a direct injection of these solution using 1 mL syringe into 10 mL of phosphate buffer saline (PBS, pH 7.4) contained in a vial. The depot weights were controlled at 23-27 mg. This procedure was also used for the preparation of DOX-loaded PLGA as well.

Table 4. Various ratio hydrophobic DOX: hydrophilic DOX (% by mole)

No.	Hydrophobic DOX	Hydrophilic DOX
1	1.25	98.75
2	2.5	97.5
3	5	95
4	10	90
5	15	85

***In vitro* release study**

The vials were placed in an incubator (Wisecube®) with a rotating speed of 90 rpm at 37 °C. At a certain time (0.5, 1, 2, 3, 6 hours, 1, 2, 4 days, 1, 2, 3 weeks, and every 2 weeks), the buffer in vials were periodically replaced with 10 mL of PBS and kept in the incubator. Preliminary, DOX concentration was determined by UV-visible spectroscopy (Evolution 600 model, Thermo Scientific) at 480 nm. The encapsulation efficiency of DOX-loaded depots was calculated by the following equation. The release profiles were obtained by plotting the amount of released against time.

$$\text{Encapsulation efficiency } \% = \frac{\text{Drug in the depot (mg)}}{\text{Drug used before the depotformation (mg)}} \times 100$$

98.75:1.25, 97.5:2.5, 95:5, 90:10 and 85:15 % of hydrophilic: hydrophobic DOX

Cytotoxicity study

Cell viability of HepG2 was evaluated by medium extraction method. HepG2 cells (1000 cells/ml) were seed in 96-well plates and maintained for 2 days before the experiment. Blank, 85 or 100 % hydrophobic DOX-loaded depots was formed in the sterilized vial containing 12 ml of PBS and incubated at 37 ^{o}C and 90 rpm of orbital shaking for 3 days. The released DOX was collected (90 μL) and diluted with 10X culture medium (10 μL) before replacing with the culture medium in 96-well plate. After 3 days of co-incubation, the testing medium was removed and washed by PBS. Cell viability of HepG2 was measured by MTT standard assay (Vybrant® MTT cell proliferation assay kit, Molecular Probes: V-13154). Briefly, MTT working solution was added into each well and incubated for 3 hours before dissolving formazan with DMSO. The formazan concentration that was related to cell viability was determined by microplate reader (Metertech M965+, Taiwan) at the wavelength of 570 nm.

Results and discussions

Preparation of hydrophobic DOX

Hydrophobic DOX could be prepared using protocol 1 and 2 as previously described. Yield (%) of each protocols were presented in **Table 5**. Only 5.2 % of hydrophobic DOX were achieved using protocol no.1. Therefore, protocol 2 was developed in order to increase % yield of protocol 1. In protocol 2, the concentration of NaOH and HCl solution were controlled at 0.2 M. Besides, the concentration of hydrophilic DOX solution was controlled at 6.67 mg/mL. When adjusting pH, it should be importantly noted that the solution must be vortexed until the spiral happened. At pH 10.5-10.6, there were a lot of sediment comparing with pH9.6-9.9. However, the color of the adjusted solution should be dark red and should not be changed to purple or dark purple. The % yield of hydrophobic DOX (dried powder) was increase to 69.8 % by using protocol 2.

Table 5. Yield (%) of hydrophobic DOX from different protocols

Protocol	Yield (%)
Preparation 1	5.2
Preparation 2	69.8
Purification 1	11.1
Purification 2	59.3

Purification of hydrophobic DOX

Due to lots of impurity in dried powder of hydrophobic DOX, it was further purified by dissolved in THF as described in purification protocol 1 and 2. The supernatant which was well dissolved were classified as purified hydrophobic DOX while the other sediments were either hydrophilic or inefficient DOX. Protocol 1 provided only 11.1 % yield of purified hydrophobic DOX while protocol 2 provided much higher % yield. We found that hydrophobic DOX used 0.5 – 1 hour for dissolving. Even though, there still had some of hydrophobic DOX left in a sediment therefore these sediments should be repeat dissolved with THF until the color of supernatant turn to be lighter. As a result, % yield of protocol 2 was increased to 59.3 % which was acceptable.

Stability of hydrophobic DOX

The stability of supernatant, sediment from preparation and purification of hydrophobic DOX were immediately tested by TLC after preparation. Hydrophilic DOX (commercial) in distilled water was used as control as shown in Fig.1 (left lane). The supernatant and sediment at various pH were spot as shown in Fig.2-14. It should be noted that TLC plate was strained with iodine and looked under UV black light. There was only one spot of hydrophilic DOX occurred at the middle of TLC plate. On the other hands, it can be implied that spot at Rf = 0 presented an inefficient DOX. For a qualified hydrophobic DOX, the spot should not occur at Rf more than the hydrophilic one and should not have a spot at Rf = 0. We found that, our hydrophobic DOX had some small amount of inefficient DOX mixed together in the same batch.

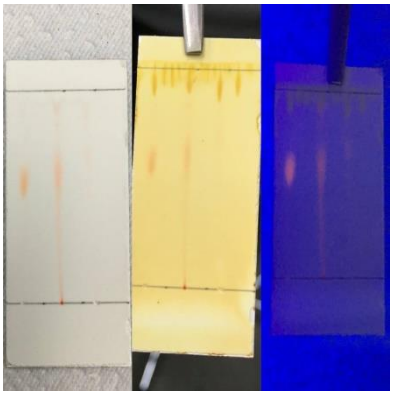


Figure 1. TLC results of the commercial DOX, supernatant at pH 9.6 and supernatant at pH 9.9 were presented from left to right lane of TLC plate.

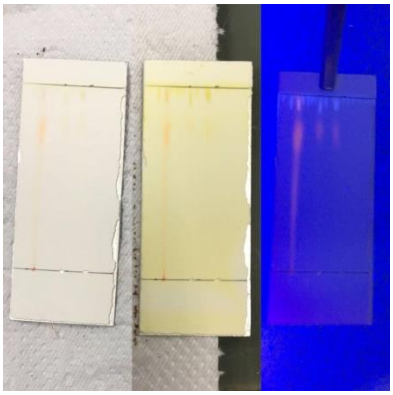


Figure 2. TLC results of the sediment at pH 9.9, 9.9, and 10.2 were presented from left to right lane of TLC plate.

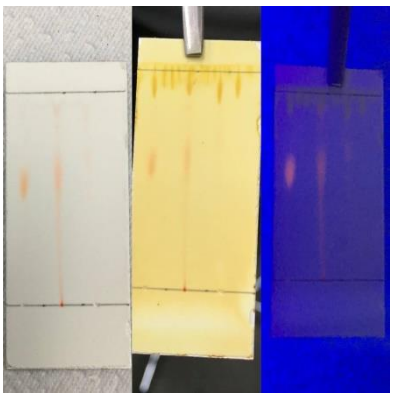


Figure 3. TLC results of the sediment at pH 9.9, 10.03, and 10.11 were presented from left to right lane of TLC plate.

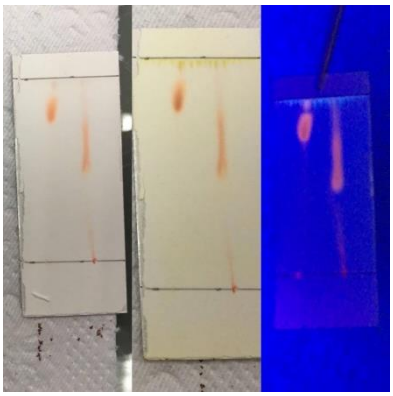


Figure 4. TLC results of the commercial DOX and sediment at pH 10.02 were presented from left to right lane of TLC plate.

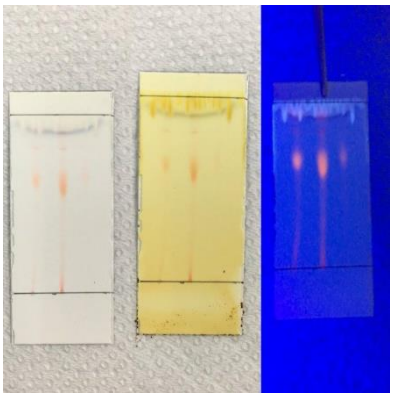


Figure 5. TLC results of the sediment at pH 9.8, 9.9, and 10.3 were presented from left to right lane of TLC plate.

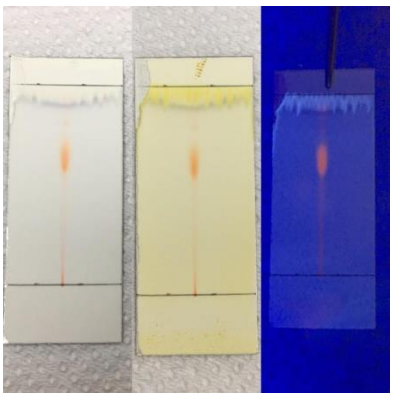


Figure 6. TLC results of the sediment from preparation protocol 2 at pH 10.5-10.6

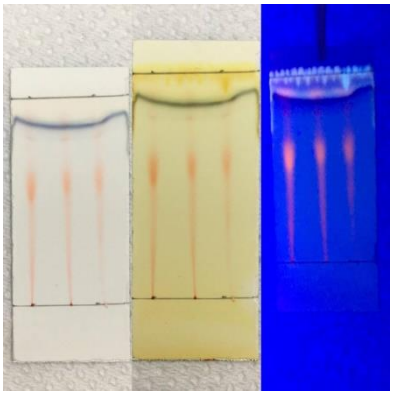


Figure 7. TLC results of the sediment at pH 10.3, 10.5, and 10.6 were presented from left to right lane of TLC plate.

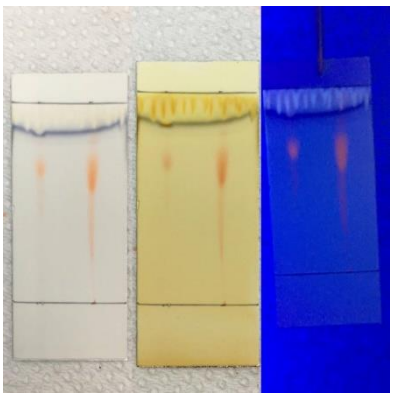


Figure 8. TLC results of the sediment at pH 10.5-10.6 vial from 1 and 2 were presented from left to right lane of TLC plate.

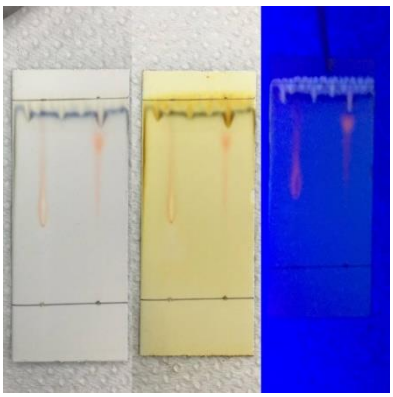


Figure 9. TLC results of the commercial DOX and sediment at pH 10.6 were presented from left to right lane of TLC plate.

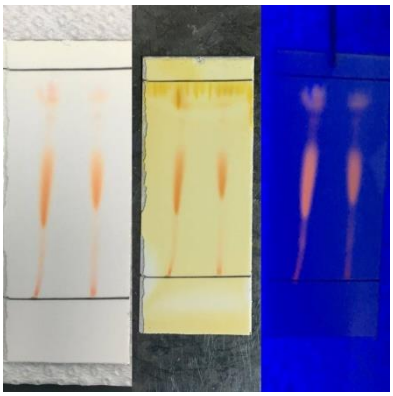


Figure 10. TLC results of hydrophobic DOX in THF batch 1, 2 (lot 4 May 2016) were presented from left to right lane of TLC plate.

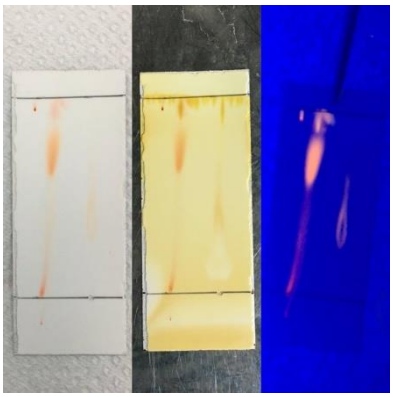


Figure 11. TLC results of hydrophobic DOX in THF batch 3 (lot 4 May 2016) and its sediment which could dissolved in water were presented from left to right lane of TLC plate.

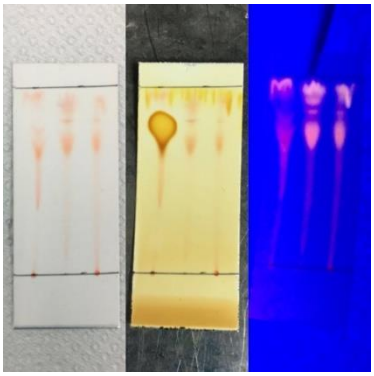


Figure 12. TLC results of sediment in DMSO, THF and Chloroform were presented from left to right lane of TLC plate.

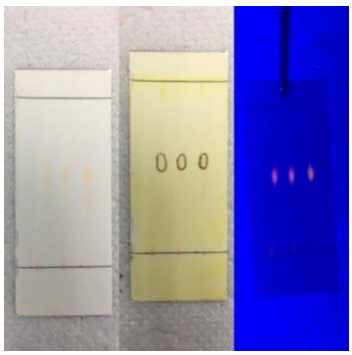


Figure 13. TLC results of triplicate sediment at pH 4.7 were presented from left to right lane of TLC plate.

Moreover, the stability of hydrophobic DOX at various time points were also evaluated using TLC. In this case, the dried power of hydrophobic DOX was kept at -20 ^{O}C for 5 days. At day 5, the spot at Rf = 0 was increase comparing with day 0 as shown in Fig 14. This can be implied that DOX is unstable and gradually changed into another form.

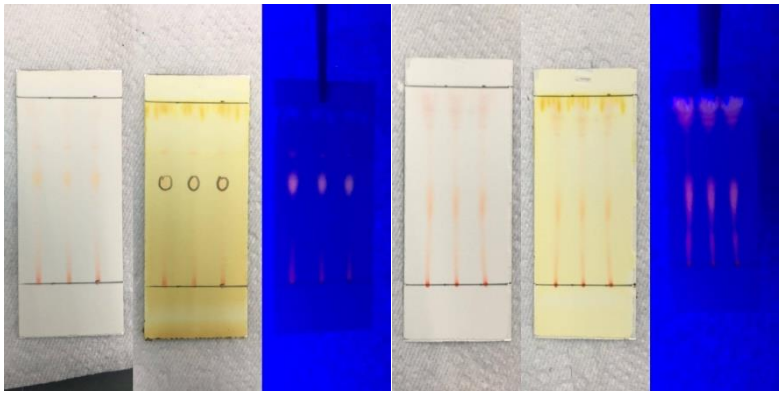


Figure 14. TLC results of triplicate hydrophobic DOX in THF at day 0 (Left) and Day 5 (Right)

Calibration curves using UV-visible spectrophotometry

Both hydrophobic and hydrophilic DOX, and paclitaxel presented a linear relationship between concentrations and absorbance. The slope and R^2 were presented in Fig 15-17. These calibration curves were further used for drug release study.

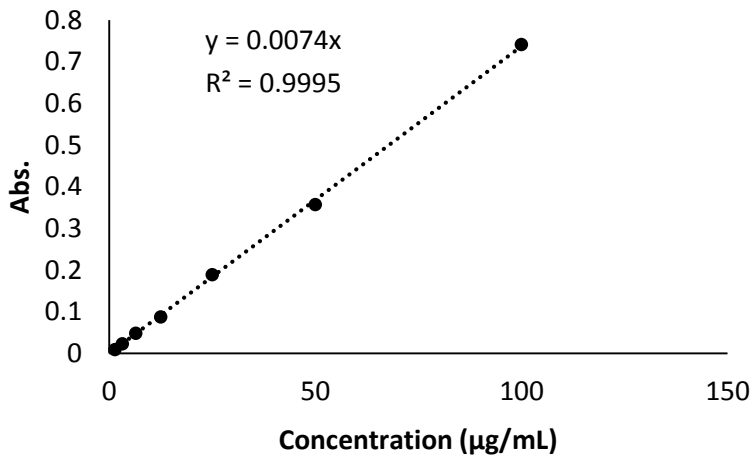


Figure 15. Calibration curve of hydrophobic DOX in THF

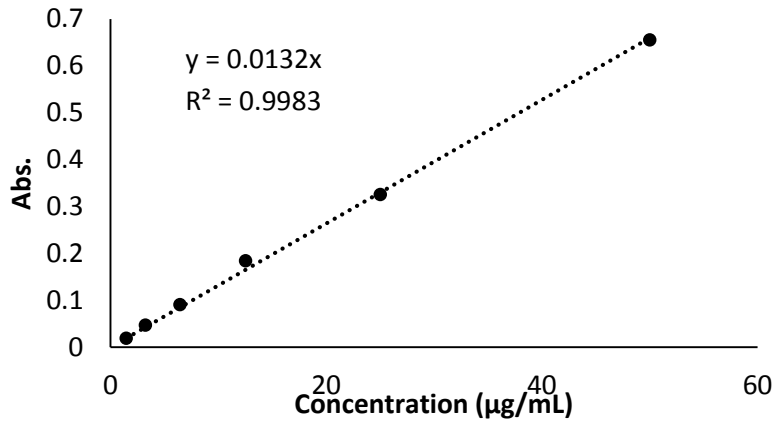


Figure 16. Calibration curve of hydrophilic DOX in PBS

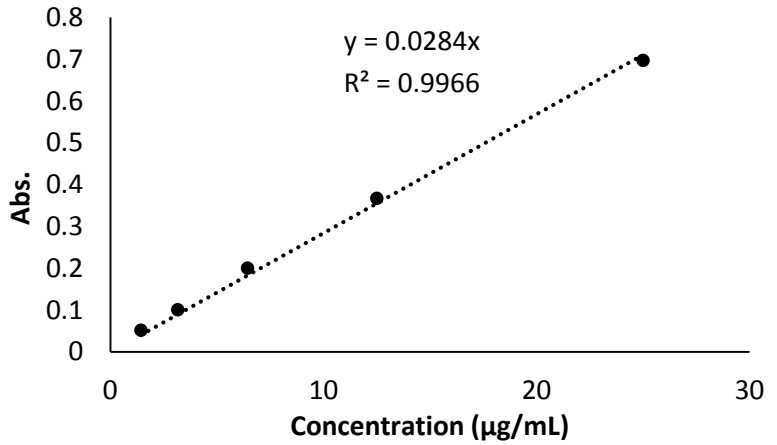


Figure 17. Calibration curve of paclitaxel in PBS:MeOH (1:1, v/v)

In vitro release of DOX from polymeric depots

In case of PLEC depots, the total drug loading contents and polymer concentration were fixed at 15 and 25% because of it high encapsulation efficiency, release profile, and easy formation. The release of DOX in the pattern of milligram and percentage cumulative release were shown in Fig 18. We found that the highest release of DOX was at 95:5, 90:10, 97.5:2.5, 85:15 and 98.75:1.25 % of hydrophilic: hydrophobic DOX (weight ratio), respectively. The results could be divided into 2 groups which were the ratio of hydrophobic DOX that

less than 5% and more than that. In case of hydrophobic DOX that less than 5%, it was found that the higher amount of hydrophobic DOX was, the more DOX release out from depot. In contrast, at more than 5% hydrophobic DOX, DOX release was decrease when hydrophobic DOX was added proportionally. For PLGA depots, the concentration of PLGA and drug loading content were 15%. The release of DOX in the pattern of milligram and percentage cumulative release were shown in Fig 19. We found that the highest release of DOX was at 98.75:1.25, 97.5:2.5, 95:5, 90:10 and 85:15 % of hydrophilic: hydrophobic DOX (weight ratio), respectively. The results could be concluded that DOX release was decrease when hydrophobic DOX was increased. Introducing hydrophobic DOX into the depot could increase the hydrophobic property of DOX inside the depot. Therefore lower amount of DOX was release out from depot. However, in every conditions, DOX release within 1-2 days at 40 % cumulative release.

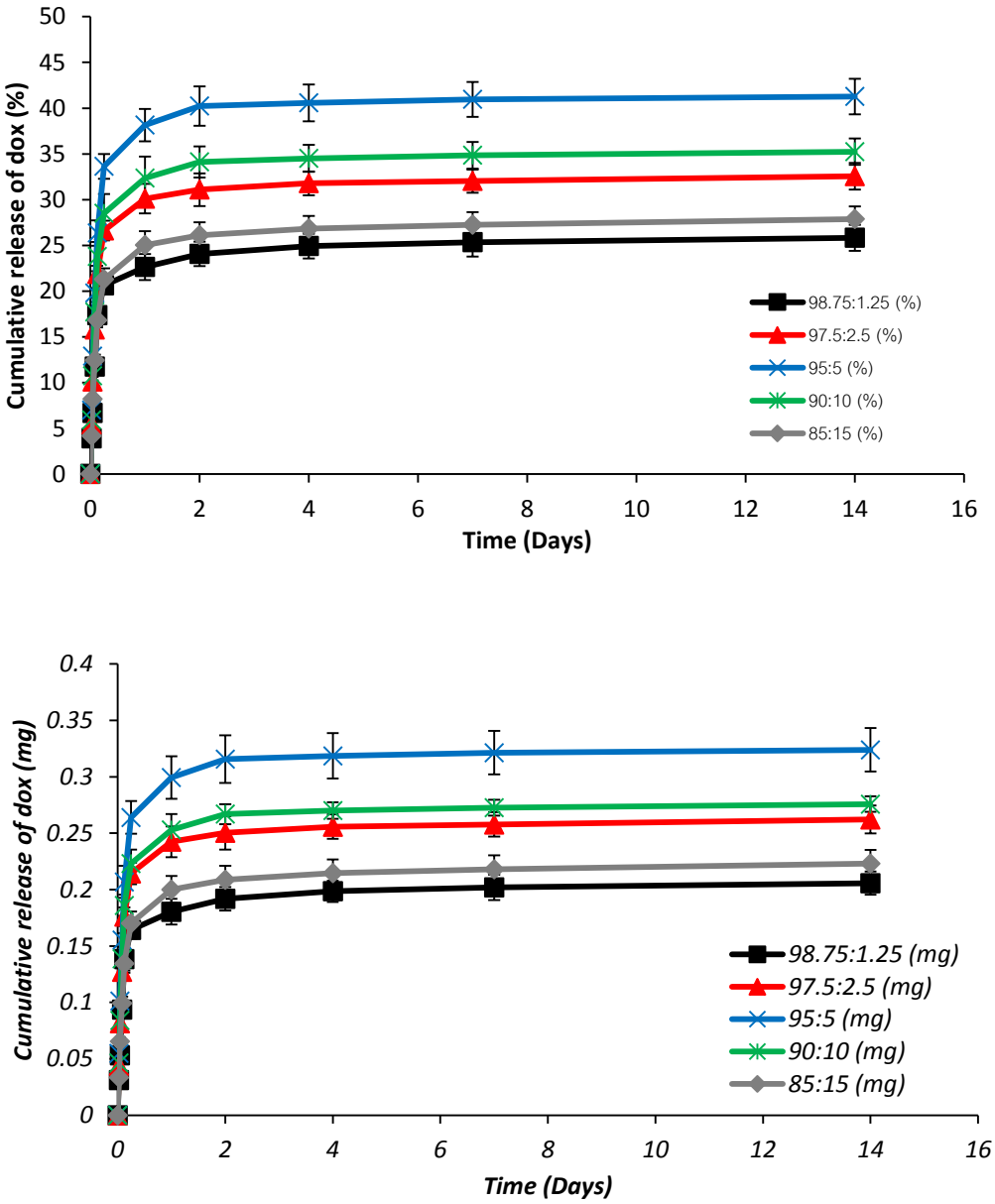


Figure 18. DOX release from PLEC depots at different mole ratio of hydrophilic and hydrophobic DOX; (Top) Cumulative released of DOX in milligram; (Bottom) Cumulative percent released of DOX. It should be noted that polymer concentration of every conditions was fixed at 25 % w/v and the total drug loading was fixed at 15 %.

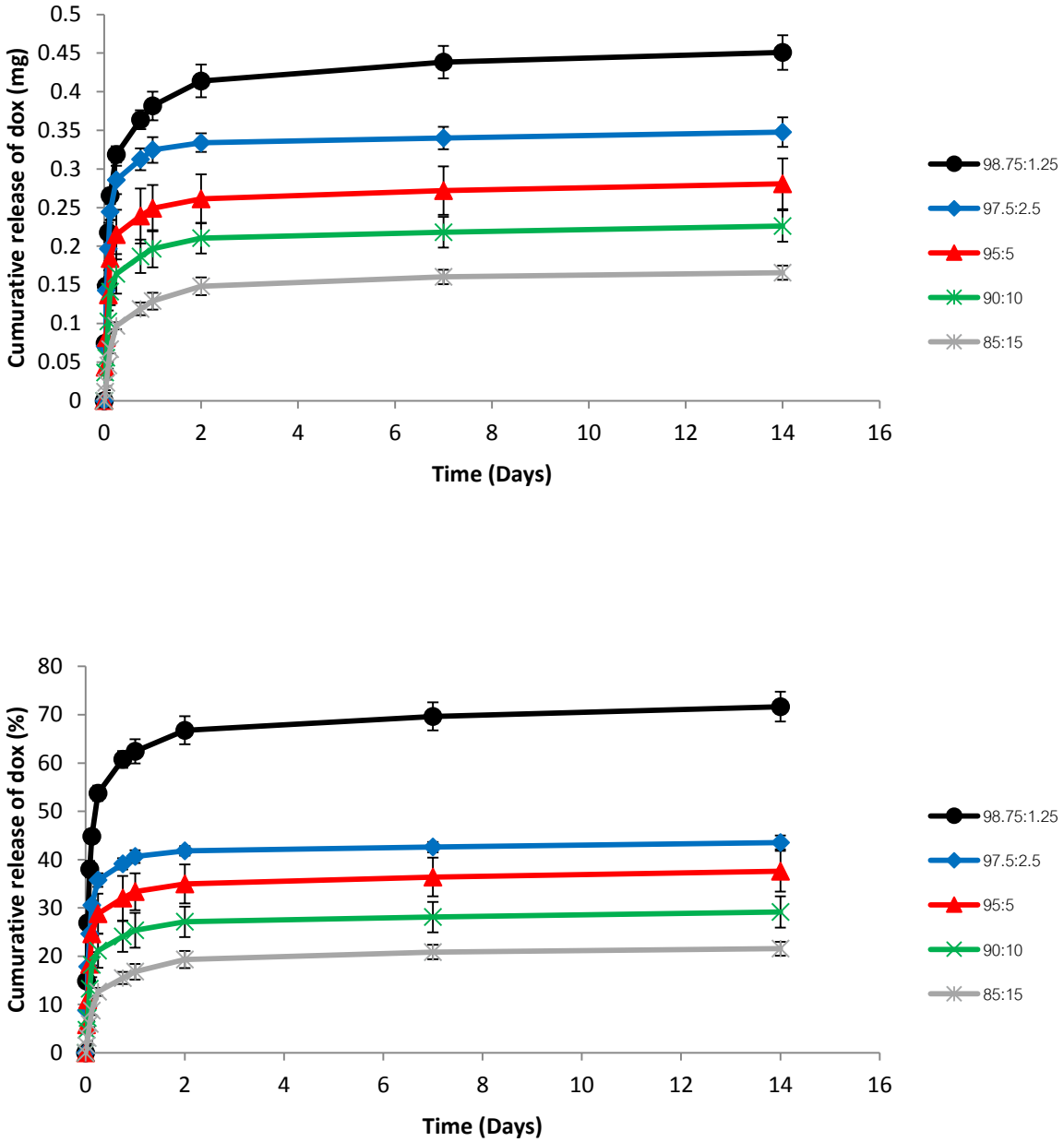


Figure 19. DOX release from PLGA depots at different mole ratio of hydrophilic and hydrophobic DOX; (Top) Cumulative released of DOX in milligram; (Bottom) Cumulative percent released of DOX. It should be noted that polymer concentration of every conditions and the total drug loading were fixed at 15 %.

In vitro cytotoxicity

To evaluate the cytotoxicity activity of the DOX-loaded depots, human hepatocellular carcinoma (HepG2) cell were incubated with extracted salt buffer solutions (Figure 20A) prepared from blank, 100 %

hydrophobic DOX, and 85 % hydrophobic DOX-loaded depots. In this work, phosphate buffer saline (PBS) was used as an extracted solution due to culture medium containing fetal bovine serum might oxidize DOX resulting in inactive form. After 24 and 72 hours of incubation, the extracted solutions were seeded to monolayer HepG2 cells and measured their activity by MTT assay. All extracts especially from 100DOX and 85DOX-loaded depots were able to inhibit the proliferation of the HepG2 cells as shown in **Table 6**. Whereas the blank depot was over 80 % cell viability with no significant change in the cell morphology. The viability around 80 % was a result from toxicity of GF that could be observed on HepG2 cells membrane. GF might dissolve some glycoprotein that is a major component of cell membrane so that cell membrane was a bit swollen comparing with control, Figure 20B. Here, the proliferation at this range was acceptable for blank depot as a nontoxic implantation. The released DOX within 24 hours from 100 % hydrophobic DOX and 85 % hydrophobic DOX-loaded depots were measured at 26.8 and 16.1 μg/ml. Those numbers were still higher than the half maximal inhibitory concentration or IC_{50} of DOX (6.15 ug/ml). It meant that those extracted medium were able to treat HepG2 cell as shown in Figure 20. These data are of interest, as it suggests that 85DOX-loaded depot were able to reduce DOX release at the level that was capable to treat HepG2 cells.

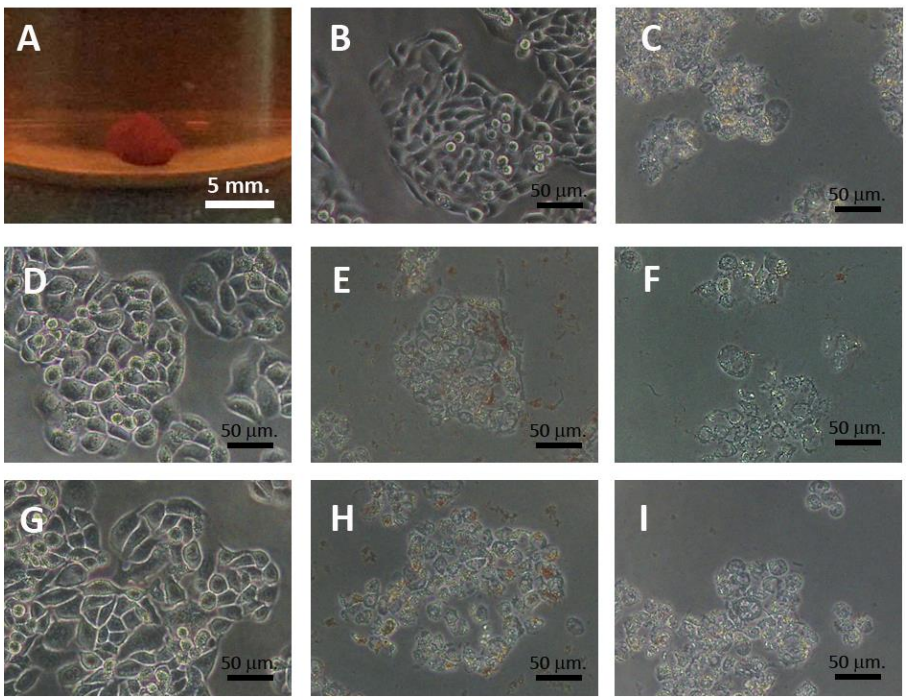


Figure 20. Photographs of (**A**) 85DOX-loaded depot at 1 day post formulation, (**B**) control HepG2 cells, and cell morphology of HepG2 cells treated with extracted medium from blank, 100 % hydrophobic DOX, and 85 % hydrophobic DOX-loaded depots changes for 1 day (**D-F**) and 3 days (**G-I**). Data represent the mean ± standard deviation (n = 3).

Table 6. Result of *in vitro* cytotoxicity of extracted medium or free DOX against HepG2 cells.

Conditions	Cell viability (%)	
	1 day co-incubation in PBS	3 days co-incubation in PBS
Blank depot	81.81 ± 1.38	85.45 ± 5.05
100 % hydrophobic DOX-loaded depot	5.85 ± 1.11	6.27 ± 1.02
85 % hydrophobic DOX-loaded depot	7.44 ± 0.72	7.27 ± 0.78
Free DOX*(16.1 μg/ml)	3.31 ± 1.13	

All data represent the mean ± standard deviation.

*Free DOX sample was freshly prepared by dissolving and diluting DOX in culture medium. HepG2 cells were incubated with Free DOX medium for 3 days before determining the cell viability.

Conclusion

In the DOX and PTX-loaded polymeric depot system, burst release of DOX was too much higher comparing to paclitaxel. Accordingly, this experiment aimed to reduce that burst release of DOX and prolong its release. Hydrophobic doxorubicin was successfully prepared and purified with more than 50% yield. The stability of hydrophobic DOX was evaluated by TLC immediately after preparation and 5 days when stored at -20 $^{\circ}$C. This hydrophobic DOX was introduced into the depot in order to increase the hydrophobicity resulting in either reducing burst release or sustain a release. Therefore, the effect of ratios of hydrophobic and hydrophilic DOX that loaded in the depot on release were examined. The hydrophobic DOX was introduced into the depot in order to reduce the burst release. We found that the more hydrophobic DOX in the depot, the less release of DOX. In the further experiment, this hydrophobic DOX will be added in to DOX and PTX depot. Moreover, another substance will be selected as a candidate for control release of DOX. The dosage that

released at the first 24 hours of 85 % hydrophobic DOX -loaded depot was able to treat HepG2. By controlling of protonated/deprotonated mass ratio to reduce the initial burst release of DOX, DOX -loaded depot may provide a promising for cancer treatment.

Part 2

In vitro release study of doxorubicin and paclitaxel in depots

Chemical and materials

PLGA 28 kDa (75% lactide) was synthesized from BioNEDD lab, Mahidol University. Doxorubicin hydrochloride (DOX) was obtained from Vesino Industrial Co., Ltd., Tianjin, China. Paclitaxel (PTX) was purchased from SHAANXI SCIPHAP BIOTECHNOLOGY CO., LTD. Tetraglycol (Glycofurol) and other reagents were purchased from Sigma Aldrich.

Methods

Standard calibration curve of DOX and PTX

1 mg of DOX and PTX were weighed and dissolved separately in 10 mL of MeOH : PBS (4:1 v/v), respectively. The solution was dissolve homogeneous and then diluted using MeOH / PBS at various concentrations. DOX solution was determined using UV-visible spectrophotometer (Evolution 600 model, Thermo Scientific) at 230 and 480 nm and PTX was determined only at 230 nm. Finally, concentration and absorbance of DOX and PTX were plotted to evaluate a linear relationship.

Preparation of DOX and PTX in depots

In this study, the percentage of PLGA polymer was used at 20% (w/v) which is easily to form the depot. Notably, the 20% (w/v) polymer comprises of 20 mg of polymer in 100 µL of glycofurol (equation 1). The drug loading was used at the maximum loading, 30% (w/w). For dual drug loading of DOX and PTX, the amount of drugs were calculated in equation 2 separately. This drug release study was evaluated at various ratio of DOX and PTX at 10:1, 25:1, and 35:1 (w/w). According to equation, the amount of DOX and PTX loading were showed in **Table 7**.

$$Polymer\ (\%) = \frac{Polymer(mg)}{Glycofurol(\mu L)} \times 100 \quad (1)$$

$$Drug\ Loading(\%) = \frac{drug(mg)}{drug(mg)+polymer(mg)} \times 100 \quad (2)$$

Table 7. Amount of DOX and PTX in depots

DOX:PTX	Amount of drug loading (μg)		Depot weight (mg)
	DOX	PTX	
10 : 1	2192.7 ± 9.8	219.3 ± 1.0	38.6 ± 0.2
25 : 1	2241.1 ± 153.0	89.6 ± 6.1	37.3 ± 2.5
35 : 1	2335.9 ± 30.1	66.7 ± 0.9	38.5 ± 0.5

Briefly, PLGA was dissolved in glycofurol and stirred homogenously. Drugs were then added into polymer solution and heat at 50 ^{o}C. After mixing, the drug solution was injected into 10 mL of phosphate buffer saline, pH 74 using 22G needle. The depots were weight controlled at 30-35 mg in spherical shape. All depots were incubated at 37 ^{o}C.

***In vitro* release study of DOX/PTX from PLGA depots**

The vials were placed in an incubator with a rotating speed of 90 rpm at 37 °C. At a certain time (0.5, 1, 2, 3, 6 hours and 1 day), the buffer in vials were periodically replaced with 10 mL PBS. The concentration of drugs were determined using UV-visible spectrophotometer at 230 nm and 480 nm. DOX and PTX concentration in solution was calculated using standard calibration curve. The release profiles were obtained by plotting the amount of drug released at various time.

Results and Discussion

In vitro release of DOX/PTX from PLGA depots

The total drug loading contents and polymer concentration were fixed at 30 and 20% which showed high encapsulation efficiency, release profile, and ease formation. The release profiles of DOX and PTX were demonstrated by percentage of drug release. The result showed that the releases of DOX and PTX were increased by time-dependent. For DOX releasing, it rose within 2 hours and then stabilized (Figure 21). The highest release of DOX was presented as 20% at ratio of DOX:PTX as 10:1. On the other hand, PTX was continuously released after 2 hours (Figure 22) and at ratio of DOX:PTX as 10:1 was also shown the highest release percentage of PTX, 50% (Figure 22).

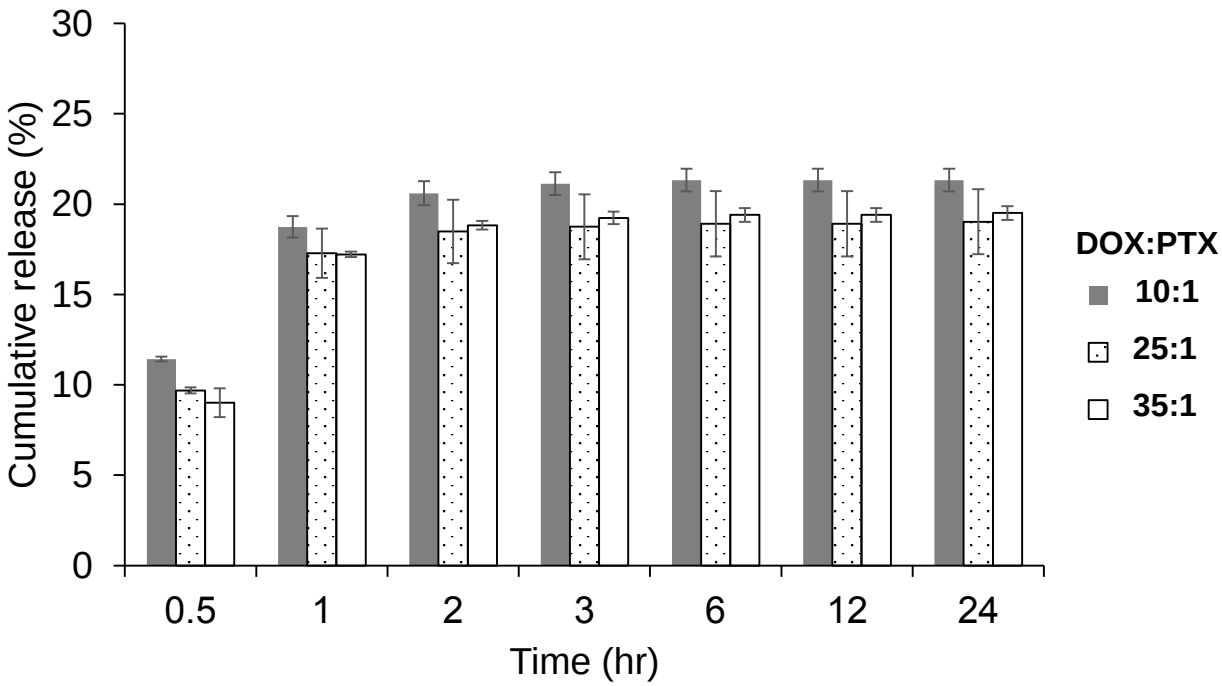


Figure 21. Cumulative drug (%) release of DOX in 1 day

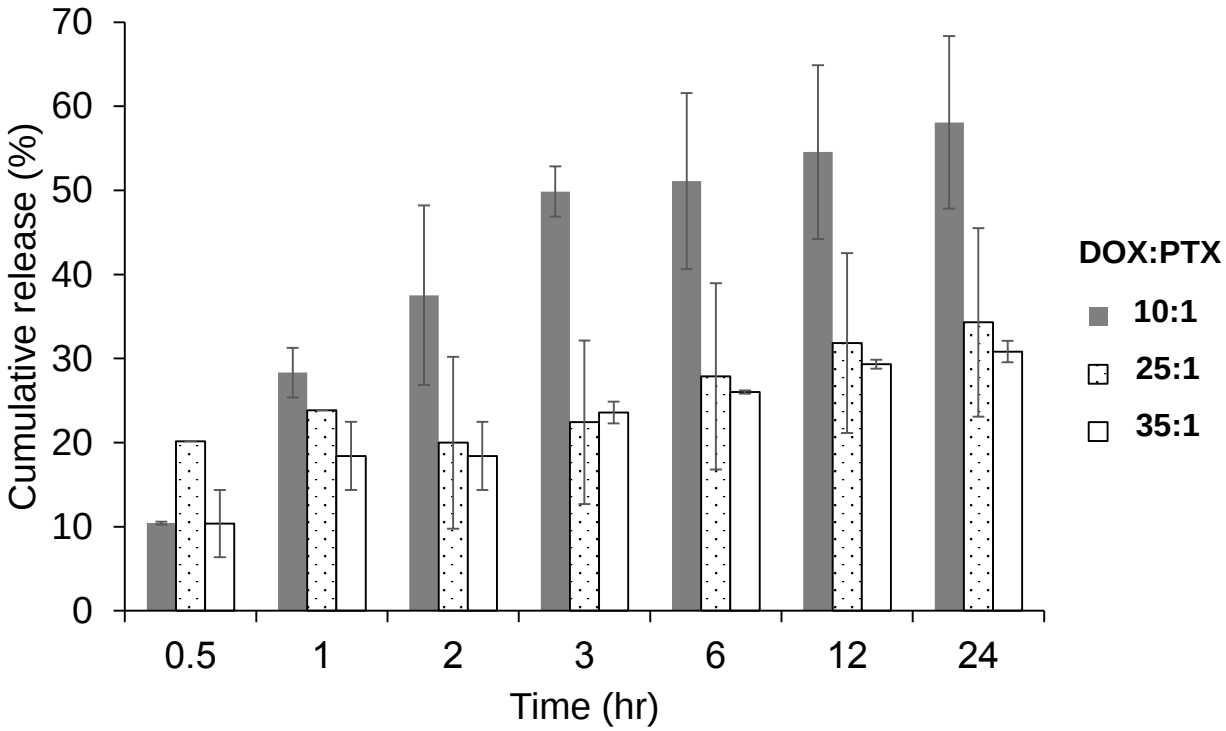


Figure 22. Cumulative drug (%) release of PTX in 1 day

To study release profile of dual drug ratio, the DOX:PTX ratio was varied as 10:1, 25:1 and 35:1 (w/w). At 10:1 ratio, drug release was decreased after 30 minutes and drop to 5:1. For 25:1 and 35:1 ratios, drug releases were diminished after 2-hour incubation with over 20:1 drug release ratio. These results indicated that the ratio of drug release from depot was slightly lower than the initial drug loading.

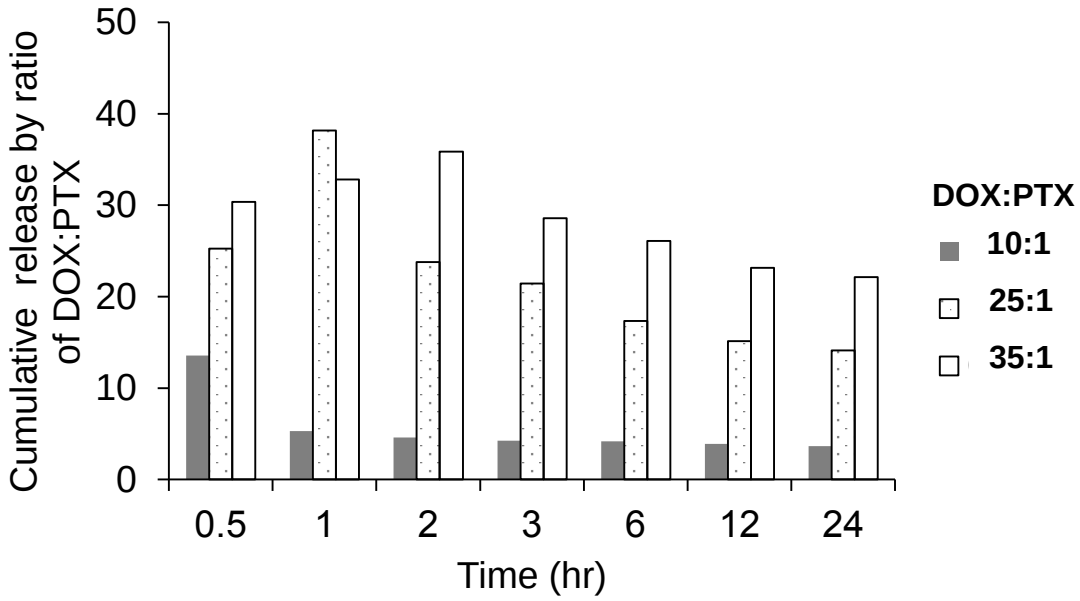


Figure 23. Cumulative release by ratio of DOX and PTX release in 1 day

Part 3

The synergistic effect of doxorubicin and paclitaxel on HepG2 cells

Methods

3.1 The synergistic study of free DOX and PTX

3.1.1 Preparation of single DOX and PTX for standard curve

DOX 2 mg was dissolved in 1 ml deionized water, while 4 mg of PTX was dissolved in 100 µl DMSO, mixed with DMEM, 5% FBS. Drugs were filtered and diluted to several concentrations as 0.001,0.005, 0.01,0.05, 0.1, 0.25, 0.5, 1, 10, 100, 1000 µg/ml of DOX and 0.0001, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 40 µg/ml of PTX. According to the released times of DOX and PTX from depots, drug solutions were individually incubated in culture medium at 37°C for 3h, 6h, 18h, 1d, 2d, 3d and 7d (pre-incubation).

3.1.2 Preparation of combined DOX and PTX

Previous study reported that the therapeutic efficacy of DOX was reduced by serum protein in cell culture medium (Wagner BA, 2007). Thus, in this study DOX was suspended in DMEM without serum after dissolved in deionized water. For PTX, it was used in a solution of 10% serum DMEM. After filtration by 0.45 µm membrane filter nylon, each drug was diluted and combined together to various concentrations at ration 1:1 (0.05, 0.1, 0.25 µg/ml of DOX and 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20 µg/ml of PTX). These combined drug solutions were pre-incubated at 37°C for several times as 3h, 6h, 18h, 1d, 2d, 3d and 7d and then tested in HepG2 cells.

3.2 The synergistic study of DOX and PTX depots

3.2.1 Preparation of DOX and PTX for standard curve

DOX was weighted and dissolved in sterile PBS and adjust to 6.25, 12.5, 25 and 50 µg/ml by serial dilution. PTX was prepared at concentration of 0.625, 1.25, 2.5 and 5 µg/ml in sterile PBS. Both of DOX and

PTX solutions were pre-incubated at 37°C for 6h, 18h 1d and 2d, and sterile PBS was also pre-incubated at the same condition.

3.2.2 Preparation of DOX and PTX depots

The combined drug depot formation was prepared by mixing of DOX and PTX with PLGA in glycofurol. After dissolved by heat, the mixture was injected into sterile PBS and each depot's weight was controlled to be at 30-35 mg (**Figure 24**). The depots were pre-incubated at 37°C for 1d and 3d without burst release removal. After pre-incubation, the drug releases were measured the concentrations using UV-Vis spectrophotometer and release solutions were analyzed by cytotoxicity test.

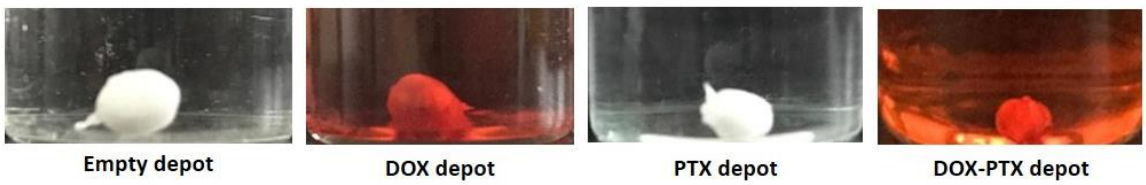


Figure 24. Characteristic of drug-loaded polymeric depot

3.3. Cell culture

Human hepatocellular carcinoma cell line (HepG2) was obtained from JCRB Cell bank, Japan and was maintained in DMEM (Biochrom AG, Germany) supplemented with 10% fetal bovine serum (Merck, USA) and 1% penicillin-streptomycin (AppliChem, Germany) at 37°C in a humidified atmosphere with 5% CO_2 and cells were subcultured every 2-3 days. At 80% confluent, cells were removed by trypsin-EDTA (0.25%) (Thermo Fisher Scientific, USA) and evaluated cell viability using 0.2% trypan blue and hemocytometer. Before performing cytotoxic test, cells were plated in 96 well-plate at $3x10^3$ cells/well at 37°C, 5% CO_2 for 48 hours.

3.4 Cytotoxic study of drugs

HepG2 cells were previously washed by PBS and the drug solutions (single drug, combined drug and drug release) were then added to cell at 100 µl/well. Cells were co-incubated with drugs at at 37°C, 5% CO_2 for 72 hours. Before test, cells were prepared by PBS washing and added 12 mM MTT solution 110 µl/well. After incubation for 4 hours, the supernatant was removed (85 µl) and added 100 µl DMSO. The solution was then measured at wavelength 570 nm using spectrophotometer (M965 MATE, Metertech Inc.).

3.5 Evaluation of drug synergistic

The synergistic effect of drug combination was estimated as the combination index (CI) of both drugs as following:

$$CI = [D_1]/[D_x]_1 + [D_2]/[D_x]_2$$

CI is combination index. D_1 is doxorubicin concentration at x% cell viability. D_{x1} is doxorubicin concentration with combination at x% cell viability. D_2 is paclitaxel concentration at x% cell viability. D_{x2} is paclitaxel concentration with combination at x% cell viability. If CI is less than 1 then there is synergistic effect for both drugs and become antagonism when CI value is more than 1.

Results and discussion

Effect of pre-incubated single DOX/PTX on HepG2 cells

The cytotoxicity of single DOX or PTX with pre-incubate in 5% serum DMEM for different times including 3h, 6h, 18h, 1d, 2d, 3d and 7d were analyzed. The results showed that high concentrations of DOX or PTX presented high cytotoxic capacity by decreased cell viability. For prolong period of pre-incubation, the cytotoxic of DOX was diminished as shown by increase the value of 50% inhibitory concentration (IC50), but there was increasing cytotoxicity at 1-3 days pre-incubation. The trend of PTX was a similar pattern of DOX excluding 1- and 7-days pre-incubation (**Table 8**). From this study, the cytotoxic efficacy of DOX or PTX was presented as a time-dependent of at short period of pre-incubation for 3h, 6h and 18h.

Table 8. The 50% inhibitory concentration (IC50) of single DOX at 3h, 6h, 18h, 1d, 2d, 3d and 7d.

Pre-incubation times	IC50 of DOX (μg/ml)	IC50 of PTX (μg/ml)
3 hours	0.05	0.03
6 hours	0.23	0.02
18 hours	0.95	0.07
1 day	0.80	0.02
2 days	0.20	0.06
3 days	0.20	0.08
7 days	4.00	0.01

Values are expressed as mean ± S.D.

Effect of pre-incubated combined DOX and PTX on HepG2 cells

In this investigation, the combination of DOX and PTX exhibited the different effects on HepG2 cells at several pre-incubation times. **Table 9** demonstrated the combination index (CI) of DOX and PTX at separated pre-incubation times and drug concentrations as shown by DOX/PTX ratios. At 3-hours pre-incubation, the synergism (CI < 1) was shown at low ratio of DOX/PTX (at 0.05 and 0.1 μg/ml of DOX). For 6- and 18-hours pre-incubation demonstrated the synergistic effect at all drug ratio (0.01-250:1 of DOX:PTX). Whereas, there was a narrow range of drug ratio that generate synergism at pre-incubation for 1 and 3 days, but at ratio 0.2-250:1 for 2-days pre-incubation. After 7-days pre-incubation, it presented the synergism at wide range of drug ratio. These recent data revealed that the synergistic of combined DOX and PTX was particularly occurred at appropriated pre-incubation times for 6 hours, 18 hours and 2 days (**Figure 25**).

Table 9. Combination index of combined DOX and PTX at different pre-incubation times.

Pre-incubation times	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI
3h	0.05	0.001	50	1.42 ± 0.19	0.1	0.001	100	1.68 ± 0.51	0.25	0.001	250	1.78 ± 0.62
		0.01	5	1.44 ± 0.23		0.01	10	1.79 ± 0.93		0.01	25	2.21 ± 0.43
		0.05	1	1.41 ± 0.18		0.05	2	0.63 ± 0.31		0.05	5	1.52 ± 0.56
		0.1	0.5	1.12 ± 0.11		0.1	1	0.39 ± 0.08		0.1	2.5	1.73 ± 0.50
		0.5	0.1	1.35 ± 0.20		0.5	0.2	0.11 ± 0.01		0.5	0.5	2.01 ± 0.37
		1	0.05	1.27 ± 0.19		1	0.1	0.12 ± 0.01		1	0.25	3.08 ± 1.53
		5	0.01	0.25 ± 0.07		5	0.02	0.18 ± 0.01		5	0.05	2.11 ± 0.77
		10	0.005	0.22 ± 0.01		10	0.01	0.27 ± 0.01		10	0.025	0.90 ± 0.19

Values are expressed as mean ± S.D.

Table 9. Combination index of combined DOX and PTX at different pre-incubation times (cont.).

Pre-incubation times	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI
6 hours	0.05	0.001	0.02	0.07 ± 0.01	0.1	0.001	0.01	0.11 ± 0.01	0.25	0.001	0.004	0.02 ± 0.00
		0.01	0.2	0.06 ± 0.01		0.01	0.1	0.12 ± 0.01		0.01	0.04	0.01 ± 0.00
		0.05	1	0.08 ± 0.01		0.05	0.5	0.13 ± 0.02		0.05	0.2	0.03 ± 0.00
		0.1	2	0.07 ± 0.01		0.1	1	0.13 ± 0.01		0.1	0.4	0.01 ± 0.00
		0.5	10	0.17 ± 0.10		0.5	5	0.14 ± 0.02		0.5	2	0.01 ± 0.00
		1	20	0.30 ± 0.18		1	10	0.15 ± 0.02		1	4	0.03 ± 0.00
		5	100	0.30 ± 0.17		5	50	0.16 ± 0.02		5	20	0.05 ± 0.00
		10	200	0.53 ± 0.35		10	100	0.21 ± 0.01		10	40	0.10 ± 0.00

Values are expressed as mean ± S.D.

Table 9. Combination index of combined DOX and PTX at different pre-incubation times (cont.).

Pre-incubation times	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI
18 hours	0.05	0.001	0.02	0.22 ± 0.10	0.1	0.001	0.01	0.01 ± 0.00	0.25	0.001	0.004	0.01 ± 0.00
		0.01	0.2	0.02 ± 0.01		0.01	0.1	0.01 ± 0.00		0.01	0.04	0.01 ± 0.00
		0.05	1	0.02 ± 0.00		0.05	0.5	0.01 ± 0.00		0.05	0.2	0.01 ± 0.00
		0.1	2	0.05 ± 0.01		0.1	1	0.01± 0.00		0.1	0.4	0.01 ± 0.00
		0.5	10	0.03 ± 0.00		0.5	5	0.02± 0.00		0.5	2	0.01 ± 0.00
		1	20	0.60 ± 0.08		1	10	0.06 ± 0.02		1	4	0.02 ± 0.00
		5	100	0.96 ± 0.50		5	50	0.18 ± 0.09		5	20	0.05 ± 0.01
		10	200	1.02 ± 0.05		10	100	0.32 ± 0.16		10	40	0.10 ± 0.01

Values are expressed as mean ± S.D.

Table 9. Combination index of combined DOX and PTX at different pre-incubation times (cont.).

Pre-incubation times	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/ [PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX] /[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX] /[PTX]	CI
1 day	0.05	0.00025	200	0.23 ± 0.05	0.1	0.00025	400	0.02 ± 0.01	0.25	0.00025	1000	0.14 ± 0.07
		0.0005	100	0.02 ± 0.00		0.0005	200	0.45 ± 0.08		0.0005	500	0.00 ± 0.00
		0.001	50	0.01 ± 0.00		0.001	100	1.01 ± 0.00		0.001	250	0.00 ± 0.00
		0.01	5	40.01 ± 17.37		0.01	10	4.22 ± 1.66		0.01	25	0.00 ± 0.00
		0.05	1	3.67 ± 1.15		0.05	2	1.01 ± 0.59		0.05	5	0.00 ±0.00
		0.1	0.5	36.46 ± 10.03		0.1	1	1.43 ± 0.10		0.1	2.5	0.01 ± 0.00
		0.5	0.1	16.67 ± 4.90		0.5	0.2	118.93 ± 36.20		0.5	0.5	0.04 ± 0.01
		1	0.05	19.22 ± 5.02		1	0.1	69.75 ± 27.14		1	0.25	0.06 ± 0.00
		5	0.01	45.25 ± 16.70		5	0.02	136.67 ± 37.85		5	0.05	14.42 ± 2.70
		10	0.005	61.24 ± 12.55		10	0.01	7.63 ± 1.39		10	0.025	43.87 ± 13.33

Values are expressed as mean ± S.D.

Table 9. Combination index of combined DOX and PTX at different pre-incubation times (cont.).

Pre-incubation times	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX] /[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX] /[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX] /[PTX]	CI
2 days	0.05	0.00025	200	8.06 ± 4.50	0.1	0.00025	400	5.17 ± 0.01	0.25	0.00025	1000	4.36 ± 2.15
		0.0005	100	4.17 ± 2.02		0.0005	200	0.43 ± 0.04		0.0005	500	10.39 ± 0.04
		0.001	50	0.11 ± 0.03		0.001	100	0.04 ± 0.00		0.001	250	0.00 ± 0.00
		0.01	5	0.00 ± 0.00		0.01	10	0.01 ± 0.00		0.01	25	0.04 ± 0.02
		0.05	1	0.00 ± 0.00		0.05	2	0.90 ± 0.48		0.05	5	0.15 ± 0.02
		0.1	0.5	0.60 ± 0.29		0.1	1	0.24 ± 0.03		0.1	2.5	0.30 ± 0.03
		0.5	0.1	1.61 ± 0.96		0.5	0.2	0.55 ± 0.27		0.5	0.5	1.13 ± 0.18
		1	0.05	102.99 ± 22.27		1	0.1	5.38 ± 2.04		1	0.25	2.31 ± 0.67
		5	0.01	10.59 ± 2.88		5	0.02	9.30 ± 3.56		5	0.05	4.34 ± 2.78
		10	0.005	14.51 ± 1.55		10	0.01	47.80 ± 19.41		10	0.025	1.60 ± 0.24

Values are expressed as mean ± S.D.

Table 9. Combination index of combined DOX and PTX at different pre-incubation times (cont.).

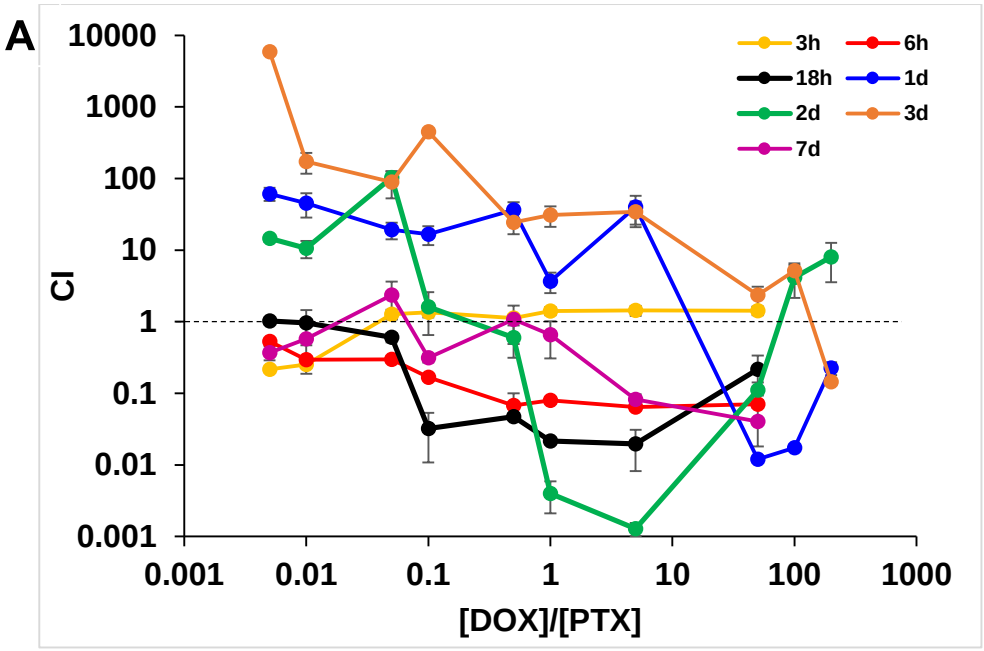
Pre-incubation times	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX] /[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX] /[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX] /[PTX]	CI
3 days	0.05	0.00025	200	0.14 ± 0.01	0.1	0.00025	400	0.56 ± 0.08	0.25	0.00025	1000	0.43 ± 0.10
		0.0005	100	5.17 ± 1.37		0.0005	200	8.45 ± 2.08		0.0005	500	1.66 ± 0.45
		0.001	50	2.36 ± 0.73		0.001	100	1.17 ± 0.15		0.001	250	0.97 ± 0.35
		0.01	5	34.35 ± 13.43		0.01	10	10.54 ± 0.64		0.01	25	1.65 ± 0.43
		0.05	1	30.86 ± 9.83		0.05	2	33.18 ± 18.74		0.05	5	5.09 ± 3.61
		0.1	0.5	24.27 ±7.62		0.1	1	4.89 ± 1.43		0.1	2.5	28.85 ± 9.79
		0.5	0.1	450.20 ± 11.87		0.5	0.2	157.00 ± 19.23		0.5	0.5	163.93 ± 24.04
		1	0.05	89.34 ± 36.66		1	0.1	62.39 ± 24.44		1	0.25	10.33 ± 4.97
		5	0.01	172.13 ± 55.76		5	0.02	186.55 ± 81.67		5	0.05	50.88 ± 38.84
		10	0.005	5911.19± 114.43		10	0.01	32.75 ± 9.43		10	0.025	100.68 ± 54.13

Values are expressed as mean ± S.D.

Table 9. Combination index of combined DOX and PTX at different pre-incubation times (cont.).

Pre-incubation times	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI	[DOX] (μg/ml)	[PTX] (μg/ml)	[DOX]/[PTX]	CI
7 days	0.05	0.001	50	0.04 ± 0.02	0.1	0.001	100	0.02 ± 0.01	0.25	0.001	250	8.56 ± 4.31
		0.01	5	0.08 ± 0.01		0.01	10	0.25 ± 0.15		0.01	25	6.19 ± 3.55
		0.05	1	0.66 ± 0.35		0.05	2	0.01 ± 0.00		0.05	5	1.15 ± 0.76
		0.1	0.5	1.08 ± 0.60		0.1	1	0.03 ± 0.00		0.1	2.5	0.09 ± 0.02
		0.5	0.1	0.31 ± 0.05		0.5	0.2	0.45 ± 0.12		0.5	0.5	0.02 ± 0.01
		1	0.05	2.35 ± 1.27		1	0.1	0.46 ± 0.18		1	0.25	3.86 ± 1.88
		5	0.01	0.58 ± 0.28		5	0.02	0.27 ± 0.16		5	0.05	38.84 ± 7.87
		10	0.005	0.37 ± 0.08		10	0.01	0.30 ± 0.13		10	0.025	68.01 ± 15.56

Values are expressed as mean ± S.D.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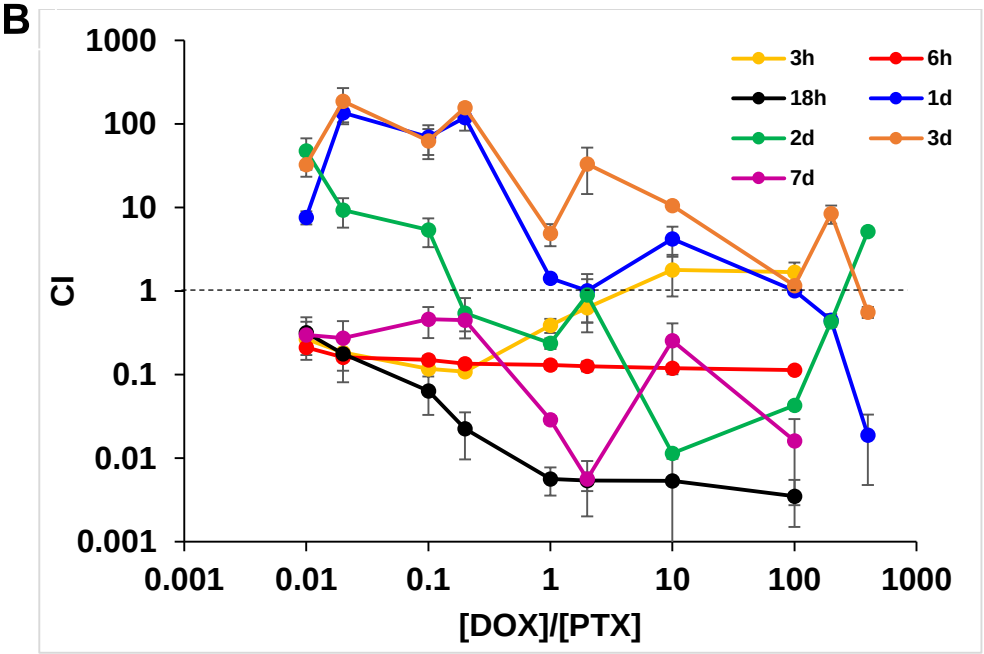
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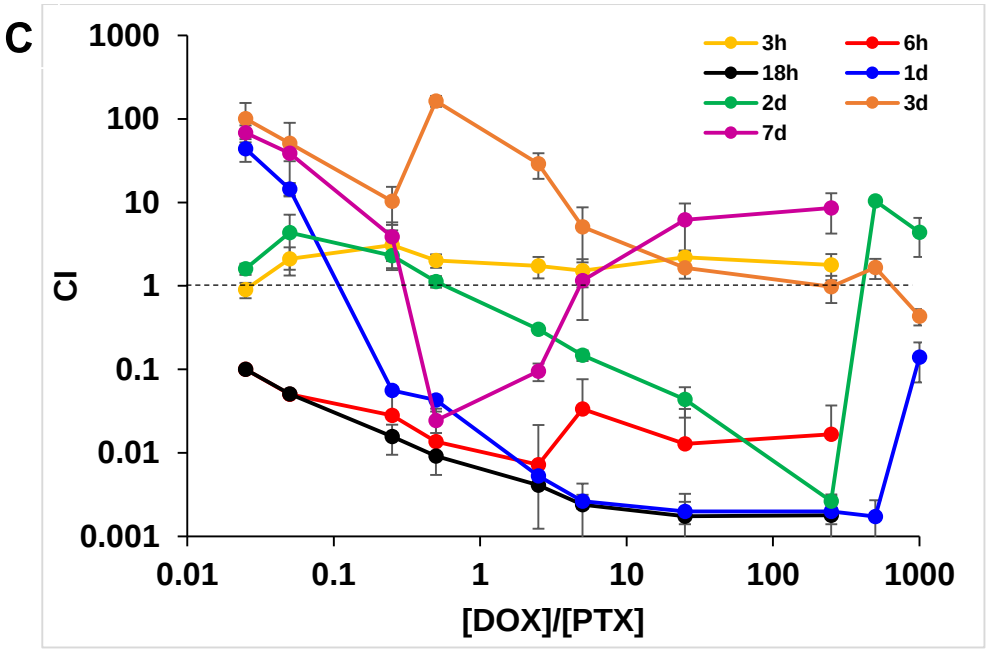

Figure 25. The combination index (CI) of DOX and PTX at pre-incubation for 3h, 6h, 18h, 1d, 2d, 3d and 7d. The concentration of DOX as 0.05 μg/ml (A), 0.1 μg/ml (B) and 0.25 μg/ml (C).

Effect of pre-incubated combined DOX and PTX depots on HepG2 cells

According to the results of DOX/PTX synergism, the suitable condition for developing drug release from depots was controlled by drug ratio in a range of 1-100:1 (DOX:PTX) and pre-incubation for 6 hours to 2 days. *In vitro* study, we investigated the efficacy of combined drug depot at effective ratio of DOX:PTX. The cytotoxic study of combined drug depot demonstrated that there was non-cytotoxic effect on HepG2 cell of empty and PTX depots, but presented from single DOX depot and combined DOX-PTX depot at 1-day pre-incubation by cell viability as shown 19.81 ± 7.44 and 10.03 ± 0.78%, respectively. For 3-day pre-incubation, there was exhibited more cytotoxicity effect of DOX depot and DOX-PTX depot as 5.79 ± 0.96 and 2.14 ± 1.43%, respectively (**Table 10** and **Figure 26**). From this point specified that PTX could be released at significantly low amount from polymeric depot after 1 and 3 days incubation which could be effect of hydrophobic nature of the depot.

The amount of DOX:PTX release at 1 and 3 days pre-incubation was controllable at 18.55 ± 0.30 and 17.55 ± 0.39 mg, respectively. The synergistic effect of DOX and PTX were distinctly revealed after pre-incubation for 1 and 3 days with combination index (CI) less than 1.0 (0.0050 ± 0.0002 and 0.0032 ± 0.0002) (**Table 10**). Cell morphology of drug polymeric depot-treated HepG2 cell was demonstrated in Figure 27. Consequently, the combination of DOX and PTX established the synergistic effect on HepG2 cell that can help support in future research in liver cancer therapy.

Table 10. Combination index (CI) of combined DOX/PTX depots

Pre-incubation times	Condition	Amount of drug (mg)	% Cell viability	Release of DOX/PTX	CI
1 day	Empty depot	-	122.87 ± 0.24	-	NA
	DOX depot	162.9	19.81 ± 4.59	-	NA
	PTX depot	8.5	107.02 ± 0.05	-	NA
	DOX-PTX depot	171.4	10.03 ± 0.51	18.55 ± 0.30	0.0050 ± 0.0002
	DOX depot	171.4	10.71 ± 0.85	-	NA
	PTX depot	171.4	115.32 ± 2.98	-	NA
3 days	Empty depot	-	93.02 ± 1.13	-	NA
	DOX depot	162.9	5.79 ± 0.52	-	NA
	PTX depot	8.5	96.78 ± 4.48	-	NA
	DOX-PTX depot	171.4	2.14 ± 1.01	17.55 ± 0.39	0.0032 ± 0.0002
	DOX depot	171.4	4.72 ± 0.81	-	NA
	PTX depot	171.4	113.04 ± 9.56	-	NA

NA; Not analysis, values are expressed as mean ± S.D.

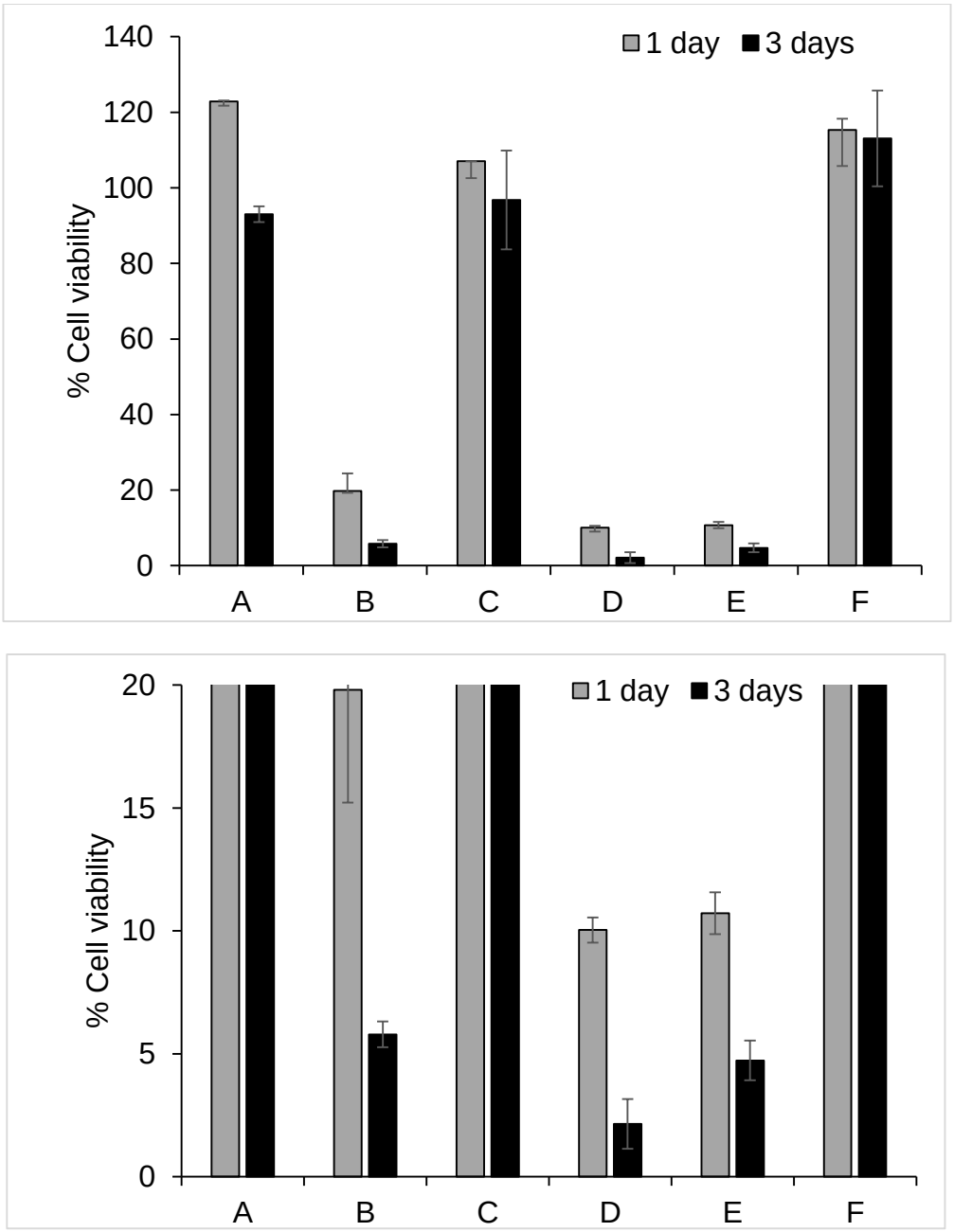

Figure 26. Cytotoxicity of DOX and PTX at pre-incubation for 1d and 3d. A; Empty depot, B; DOX depot (162.9 mg), C; PTX depot (8.5 mg), D; DOX-PTX depot (DOX 162.9 mg and PTX 8.5 mg), E; DOX depot (171.4 mg), F; PTX depot (171.4 mg).

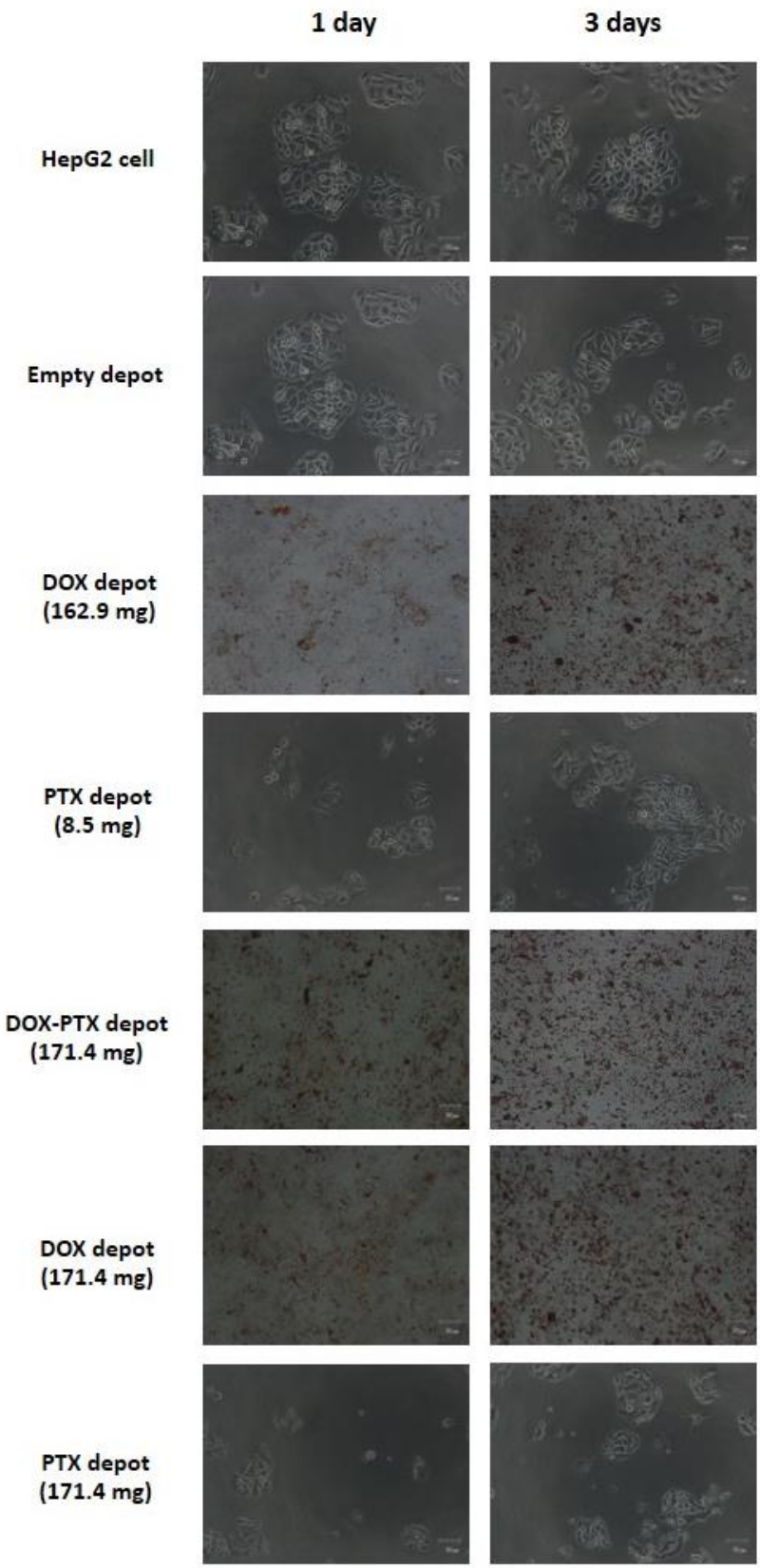

Figure 27. Cell morphology of DOX/PTX-PLGA depot-treated HepG2 cells

Conclusion

The pre-incubation of DOX or PTX could have a concentration-dependent cytotoxic effect on HepG2 cell. However, the capability of drugs were not related to period of pre-incubation by decreasing drug efficiency after 3 to 18-hours pre-incubation. Thus, the anticancer properties of DOX and PTX were depended on drug concentration and pre-incubation times. Otherwise, the combination of DOX and PTX was able to generate the synergistic effect, obviously at prolonged pre-incubation. This might be suggested that the activity of combined drug should have an appropriate time interval to take effect. In order to develop the combined DOX and PTX efficacy, the broad range of drug concentrations and pre-incubation times were investigated at 1, 2, 3 and 7 days. The synergism of DOX/PTX was diverged and performed as time-independent for these time points. Conspicuously at 2 days pre-incubation, there was noticeable synergism at the range of DOX:PTX ratio (5-50:1). These data predicted that the pre-incubation for 6 hours, 18 hours and 2 days of DOX/PTX at 10:1 were the appropriate conditions for developing drug depots with synergistic effect.

In vitro release study, there was cytotoxic response from release of DOX depot and combined DOX-PTX depot at 1-day pre-incubation (cell viability were 19.81 ± 7.44 and 10.03 ± 0.78%, respectively) and 3-day pre-incubation (cell viability were 5.79 ± 0.96 and 2.14 ± 1.43%, respectively). Interestingly, there was a synergistic effect of DOX and PTX after pre-incubation for 1 and 3 days. Accordingly, these investigation can be advantage for further liver cancer treatment.

การเผยแพร่/ประชาสัมพันธ์ *(กรุณาให้รายละเอียด พร้อมแนบหลักฐาน)*

1. สิ่งพิมพ์ หรือสื่อทั่วไป

   ☐ หนังสือพิมพ์ ☐ วารสาร ☐ โทรทัศน์ ☐ วิทยุ ☐ เว็บไซต์ ☐ คู่มือ/แผ่นพับ ☐ จัดประชุม/อบรม ☐ อื่น ๆ

2. สิ่งพิมพ์ทางวิชาการ *(วารสาร, การประชุม ให้ระบุรายละเอียดแบบการเขียนเอกสารอ้างอิง เพื่อการค้นหาซึ่งควรประกอบด้วยชื่อผู้แต่ง ชื่อเรื่อง แหล่งพิมพ์ ปี พ.ศ. (ค.ศ.) ฉบับที่ หน้า)*

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   • Pinunta Nittayacharn, Varakhan Theekhamongkhon, Preawpan Pongklang, Sintana Thepintha, and Norased Nasongkla*, Modification of Burst Release of Doxorubicin from Polymeric Depot via Cyclodextrin Inclusion Complex, IUPAC World Polymer Congress Macro2014, 6 - 11 July 2014, Chaing Mai, Thailand, Accepted as poster presentation and proceeding

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