Thymoquinone loaded in nanostructured lipid carrier showed enhanced anticancer activity in 4T1 tumor-bearing mice

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Aim: To investigate the enhancement of anticancer activity of thymoquinone (TQ) by the use of nanostructured lipid carrier (NLC) in 4T1 tumor-bearing female BALB/c mice. **Material & methods:** TQ was incorporated into NLC (TQNLC) by using high pressure homogenization. TQNLC and TQ were orally administered to the mice. **Results & conclusion:** TQNLC and TQ are potential chemotherapeutic drugs as they exhibited anticancer activity. The use of NLC as a carrier has enhanced the therapeutic property of TQ by increasing the survival rate of mice. The antimetastasis effect of TQNLC and TQ to the lungs was evidence by downregulation of MMP-2. TQNLC and TQ induced apoptosis via modulation of Bcl-2 and caspase-8 in the intrinsic apoptotic pathway.

First draft submitted: 16 October 2017; Accepted for publication: 11 April 2018; Published online: 20 July 2018

Keywords: 4T1 tumor-bearing female BALB/c mice • anticancer • nanoparticle • nanostructured lipid carrier • thymoquinone

Breast cancer is the most common cause of cancer death among women with 1.7 million new cases diagnosed and 522,000 death cases reported in 2012 [1]. Despite the recent remarkable advances in cancer therapy, breast cancer still ranks as the second leading cause of cancer death even in developed countries mainly due to the adverse effects of treatment and metastasis [2]. Hence, more effective strategies in treating the cancer are critically needed. One of the promising approaches is the use of novel pharmacologically active compound(s) from natural products especially medicinal plants [3]. Historically, medicinal plants have been the invaluable source for various currently available cancer chemotherapeutic agents such as vincristine, paclitaxel (Taxol[®], Bristol-Myers Squibb Company, NY, USA), docetaxel and topotecan [4]. Another plant-derived compound, thymoquinone (TQ), has raised the interest of researchers for its wide spectrum of biological activities such as anti-oxidant, anti-inflammatory [5] and anticancer properties [6].

TQ (2-methyl-5-isopropyl-1,4-benzoquinone) is the bioactive compound isolated from *Nigella sativa* (black seed) oil [7]. Almost one-third of the publications on TQ in the last 5 years described its anticancer properties [8]. TQ was cytotoxic against various cancer cell lines such as A549 adenocarcinoma alveolar basal epithelial cells [9], SH-SY5Y human neuroblastoma cells [10], SW 626 human colon cancer cells [11], ES-2 human ovarian cancer cells [12], HeLa human cervical carcinoma cells [13] and SiHa human cervical squamous carcinoma cells [14]. Its anticancer property was also demonstrated in several *in vivo* animal models such as MDA-MB-231 breast cancer nude mice, WEHI-3 leukemic mice [15], C57BL/6 ovarian cancer mice [16] and HCT116 colorectal cancer mice [17]. Mechanistically *in vivo*, TQ has shown to inhibit various hallmarks of cancer such as inhibition of carcinogen activation, inflammation, tumor cell proliferation, activation of anti-oxidant, induction of cancer cell death, suppression of tumor angiogenesis, invasion and metastasis [18]. The attractive feature of TQ is that it is not

only selectively targeting tumor cells by several molecular pathways, it can also protect the normal tissues from toxicity [8].

Unfortunately, just like 40% of the currently available drugs, TQ is found not suitable for oral delivery due to its hydrophobic nature. This leads to poor oral bioavailability and subsequently reduced pharmacological action due to insufficient concentration of the drug reaching the targeted site [19]. Although TQ can be administered by other routes such as intraperitoneal and intravenous, oral administration route has always been of preference due to several advantages such as patient compliance, ease of administration and cost-effectiveness [20].

Therefore, researchers have attempted several approaches to enhance the bioavailability of therapeutic agents such as nano-sizing the drug molecules, salt formation, prodrug synthesis and encapsulation of drug in nano-sized carriers such as polymeric micelles, liposomes and emulsions [20–22]. In early 2000, Muller *et al.* have worked extensively on the development and modification of lipid nanoparticle known as nanostructured lipid carrier (NLC) [23]. The use of NLC to deliver anticancer agents via ‘passive targeting’ whereby the particles are accumulated in the tumor area based on the leaky vasculature-enhanced permeability and retention effect has been extensively studied [24]. Of advantage, NLC, which is composed of solid and liquid lipids, creates an imperfect matrix with a larger cavity. This offers a firmer inclusion of drug molecules and increases the drug encapsulation efficiency [25]. Since NLC is in nano-size, it has a larger surface area-to-volume ratio which increases the drug reactivity. Moreover, NLC is composed of biodegradable and biocompatible excipients that can be tolerated by human [26].

Our research group has successfully incorporated TQ into NLC (hereinafter referred to as TQNLC). TQNLC has excellent physicochemical properties such as high encapsulation efficiency, particle diameter less than 50 nm and good stability up to 2 years. In our previous work, TQNLC has shown remarkable antiproliferative activity toward breast cancer cell lines (MDA-MB-231 and MCF-7) and cervical cancer cell lines (HeLa and SiHa) [27]. Our recent report indicated that TQNLC was safe for either short- or long-term oral consumption [28]. In this study, we further examined the anticancer activity of TQNLC in 4T1 tumor-bearing female BALB/c mice in comparison to TQ and the commercial drug, doxorubicin.

Materials & methods

Preparation of NLC & TQNLC

The NLC and TQNLC were prepared by hot high-pressure homogenization technique in accordance to the previous study [27]. Briefly, the lipid phase of NLC was made up of hydrogenated palm oil (Wilfarin™ hydrogenated refined palm oil), lecithin (Phospholipon® 90G) and olive oil in a ratio of 7:3:3. Meanwhile, the aqueous phase was made up of sorbitol, polysorbate 80 (Tween® 80), thimerosal and deionized water. Both the lipid and aqueous phases were heated up to 70°C separately. Before dispersion of the aqueous phase into the lipid phase, TQ was added and dissolved in the hot lipid phase. The mixture was then homogenized using a high shear disperser Ultra-Turrax® (IKA-Werke GmbH & Co., Staufen im Breisgau, Germany), at 13,000 rpm for 10 min at 70°C. The pre-emulsion was further homogenized at 1000 bars for 20 cycles using a high-pressure homogenizer EmulsiFlex®-C50 (Avestin, Mannheim, Germany). The hot nanoemulsion was rapidly cooled down to room temperature (25°C) to form TQNLC. Blank NLC was prepared in a similar way, but without addition of TQ into the lipid phase. The physicochemical properties of TQNLC such as mean diameter, polydispersity index, ζ potential, encapsulation efficiency and drug loading capacity were determined by laser Doppler electrophoresis using the Zetasizer Nano ZS (Malvern Instruments GmbH, Malvern, UK).

Cell culture

The mouse mammary breast cancer cell line 4T1 (Catalog Number: CRL-2539) was obtained from the American Type and Culture Collection (MD, USA) and maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin and streptomycin.

Development of mouse mammary tumor model & treatment

The Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia has approved the conduct of this study (UPM/IACUC/AUP-R027/2014). Briefly, female BALB/c mice of 15–25 g in weight at the age of 6–8 weeks were used. They were housed individually in cages under standard laboratory conditions with a period of 12/12-h light/dark cycle, at 20–24°C with 40–50% relative humidity. The animals were acclimatized for one week before the actual experiment. They were fed with a standard chow pellet (Specialty Food, Australia) and allowed to drink water *ad libitum*. After acclimatization, the mice were injected subcutaneously in the right mammary fat

pad with 5×10^4 of 4T1 cells suspended in 100 μ l of ice cold PBS. Tumor growth was assessed morphometrically using an electrical caliper [29]. Tumor volume was calculated according to the following formula [30]:

$$\text{Volume (mm}^3\text{)} = \text{Length (major axis)} \times \text{Width}^2 (\text{minor axis}) \times 0.523$$

When the tumor has reached a volume of 50–100 mm³, the 4T1 tumor-bearing female BALB/c mice were randomly assigned into ten groups (n = 12), which were: negative control (distilled water, daily), positive control (2 mg/kg of doxorubicin, injected intraperitoneally twice weekly), treatment groups with TQNLC and TQ dissolved in olive oil (25, 50 and 100 mg/kg, oral administration daily) and two vehicle groups (blank NLC and olive oil, oral administration daily). All the treatments were given for 28 days. During the treatment period, body weight of each mouse was measured twice a week. Survival of the mice was recorded daily.

Tissue sampling & gross evaluation

After euthanization, the major organs such as tumor, liver, kidneys, heart, lungs and spleen were harvested and weighed. Any changes on the organs were observed grossly before being fixed in 10% formalin. The organs were then embedded with paraffin and sectioned into slides.

Evaluation of lung metastases

The use of 15% of India ink to stain the lobes of lungs to visualize the tumor was adopted [31]. Briefly, ribs of the mice were cut to expose the lungs and trachea. Next, 15% of India ink was slowly injected into the lungs via trachea (intratracheal injection) until they inflated. The lungs were then washed in normal saline and fixed in Fekete's solution (100 ml of 70% ethanol, 10 ml of 10% buffered formalin and 5 ml of glacial acetic acid) overnight [31]. The tumor nodules that did not absorb the India ink appeared as white color, were counted using an image analysis software (ImageJ 1.50i, NIH, MD, USA).

Evaluation of apoptosis

For *in situ* detection of apoptosis in paraffin-embedded tissue sections, the terminal transferase mediated dUTP nick end-labeling kit (TUNEL) assay was carried out in accordance with manufacturer's instructions (*in situ* cell death detection kit, POD, cat no.: 11684817910, Roche Applied Science, Basel, Switzerland). Briefly, 4 μ m paraffin-embedded tissue sections were fished to poly-L-lysine-coated slides and allowed to dry at room temperature. Next, the slides were dewaxed and rehydrated according to the standard protocols [32]. Subsequently, the tissue sections were incubated with 20 μ g/ml of proteinase K working solution for 30 min at 37°C to permeabilise the samples. After washing with PBS, the samples were added with TUNEL reaction mixture and incubated in dark for 1 h at 37°C in a humidified incubator. Negative control was incubated with label solution without terminal transferase while positive control was treated with DNase 1 to induce DNA strands break prior to labelling procedure. With a drop of PBS, the samples were analyzed under a fluorescence microscope. The excitation wavelength was in the range of 450–500 nm and detection wavelength was in the range of 515–565 nm. The TUNEL-positive cells were then counted using image analysis software (ImageJ 1.50i, NIH).

Determination of the expression of apoptotic-related pathway proteins

The tumor was weighed and dissected on ice to prevent protein degradation by protease. The tissue was then placed in a round-bottom microcentrifuge tube and immersed into liquid nitrogen to snap freeze. The samples were stored at -80°C for further use. To a 20 mg tumor lysate, 300 μ l of ice cold RIPA lysis buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 0.1% SDS (w/v), 0.5% sodium deoxycholate (w/v), 1% Triton X-100 (v/v), 1 mM phenylmethylsulfonyl fluoride and 10 μ l/ml of protease inhibitor cocktail) was added. The sample was homogenized using an electrical homogeniser (Qiagen Tissue Ruptor®, Qiagen, Tokyo, Japan) and the blades were rinsed with 200 μ l of RIPA lysis buffer twice. The sample was lysed by constant agitation for two hours at 4°C. The tumor lysate was then centrifuged at 20,000 $\times$ g for 20 min at 4°C. The supernatant was collected and stored at -80°C until further use.

Protein concentration of the tumor lysate was determined by using Bradford assay (BioRad, CA, USA). Equal amount of protein sample (10–20 μ g) was mixed with 2 $\times$ Laemmli buffer (10% β -mercaptoethanol) and heated at 95°C for 5 min. The sample was loaded into SDS-PAGE gel. Next, electrophoresis was run at 80 V in 4%

stacking gel and at 130 V in 12% of resolving gel. The protein from the gel was transferred to a PVDF membrane (Milipore, MA, USA) using a semi-dry method under 12 V constant voltage. The PVDF membrane was blocked with 3% bovine serum albumin (Nacalai Tesque, Kyoto, Japan) in Tris buffer saline containing 0.1% Tween 20 for 1 h at room temperature. Subsequently, the PVDF membrane was incubated overnight with constant agitation at 4°C with respective primary antibodies as follows: anti-BAX (1:500, Santa Cruz Biotechnology, CA, USA), anti-BCL-2 (1:1000, Santa Cruz Biotechnology), anti-MMP-2 (1:1000, Santa Cruz Biotechnology), anti-VEGF (1:500, Santa Cruz Biotechnology), anti-TNF- α (1:1000, ABCAM, Cambridge, MA, USA), anti-caspase 8 (1:1000, ABCAM, Cambridge) and anti- β -actin (1:1000, Santa Cruz Biotechnology). After the washing steps, the membrane was incubated with an appropriate horseradish-conjugated secondary antibody (goat anti-rabbit IgG F(AB')₂, 1:10,000) in TBS-T for one hour at room temperature. Detection of antibody reactivity was performed with a chemiluminescence detection kit, Chemi-Lumi One (Nacalai Tesque) and ChemiDot™ MP System (Biorad) in dark. Equal sample loading was verified by immunodetection of β -actin.

Statistical analysis

All values were expressed as mean $\pm$ standard error of mean (SEM). Comparisons between groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests using SPSS Software. Kaplan–Meier survival curve was analyzed using log-rank test. Probability value less than 0.05 was considered significant.

Results

Physicochemical characteristics of TQNLC

The mean diameter of TQNLC was 46.18 ± 0.17 nm with polydispersity index of 0.163 (Figure 1A) at room temperature (25°C) as determined by Zetasizer Nano ZS. The ζ potential ranged from -4.38 to -6.03 mV (Figure 1B).

Splenomegaly in the 4T1-induced female BALB/c mice

After 7–10 days of inoculation of the 4T1 cancer cells into the mammary fat pad of the female BALB/c mice, the tumor reached 50–100 mm³. Based on the gross observation (Figure 2A) and weight (Figure 2B), the spleens of all mice inoculated with the 4T1 tumor cells (ii) were doubled in size and heavier ($p < 0.05$) as compared with the one of normal mice (i). The histological analysis (Figure 2C) revealed an expanded granulocyte-rich red pulp with reduction of white pulp area in the spleen of 4T1 tumor-bearing female BALB/c mice.

Effect of TQNLC on the tumor volume & weight

The tumor volume is illustrated in Figure 3A. On Day 4, there was a significant decrease in the tumor volume ($p < 0.05$) in the mice treated with doxorubicin (84 ± 13.18 mm³), 50 mg/kg of TQNLC (76 ± 13.95 mm³), 100 mg/kg of TQNLC (102 ± 44.59 mm³) and 100 mg/kg of TQ (80.97 ± 18.45 mm³) as compared with the negative control group (201 ± 27.78 mm³). The same trend was noted until Day 25 ($p < 0.05$). There was no significant difference in the tumor volume between groups treated with all the doses of TQ and TQNLC (25, 50 and 100 mg/kg) and doxorubicin ($p > 0.05$).

There was a significant difference in the tumor weight of the mice treated with doxorubicin (0.94 ± 0.150 g) as compared with the negative control group (2.08 ± 0.426 g). Treatment with the highest dose of TQNLC and TQ (100 mg/kg) did not show a significant difference in the tumor weight as compared with both the negative control and doxorubicin-treated groups (Figure 3B).

Effect of TQNLC on the survival rate

As shown in Figure 4, there was no significant difference ($p > 0.05$) in the survival time between all the treatment groups with the negative and positive control groups. The mice treated with doxorubicin and 100 mg/kg of TQNLC have the longest mean of survival time, which were 27.3 and 27.6 days, respectively. The survival day for the negative control group was 23.9.

As presented in Table 1, at the end of the treatment (Day 28), the survival rate of the mice treated with 100 mg/kg of TQNLC was the highest (81.2%). The mice treated with doxorubicin have a survival rate of 75%. The lowest percentage (53.8%) was noted in the group treated with 100 mg/kg of TQ.

Figure 1. Physicochemical characteristics of TQNLC. (A) Mean diameter and polydispersity index of TQNLC as determined by Zetasizer Nano ZS at room temperature (25°C). **(B)** ζ -potential distribution of TQNLC as determined by Zetasizer Nano ZS at room temperature (25°C). Values are expressed as mean $\pm$ standard error of the mean and measured in triplicate.

TQNLC: Thymoquinone-loaded nanostructured lipid carrier.

Figure 2. Spleen of the 4T1-induced female BALB/c mice. (A) Enlarged spleen of a female BALB/c mouse inoculated with 4T1 tumor cells (ii) compared to the normal one (i). **(B)** Spleen weight of the 4T1 tumor-bearing female BALB/c mice on Day 28 after treatment with TQNLC. *Indicates significantly different as compared to the normal group ($p < 0.05$). Values are expressed as mean $\pm$ standard error of the mean ($n = 6$). Normal refers to uninoculated mice; Negative refers to untreated inoculated mice and Positive refers to doxorubicin-treated inoculated mice. **(C)** Spleen section of the 4T1 tumor-bearing female BALB/c mice after treatment with TQNLC stained with hematoxylin and eosin as observed under a light microscope. Expanded area of RP with reduction of WP was observed in all the 4T1 tumor-bearing mice. Each scale bar represents $10\ \mu\text{m}$ ($200\times$ magnification). Normal refers to uninoculated mice; Negative refers to untreated inoculated mice and Positive refers to doxorubicin-treated inoculated mice. RP: Red pulp; TQNLC: Thymoquinone-loaded nanostructured lipid carrier; WP: White pulp.

Table 1. The survival rate and mean survival time of 4T1 tumor-bearing female BALB/c mice.		
Group	Survival percentage	Mean survival time (days)
NLC	56.2	25.0 $\pm$ 1.30
TQNLC 25 mg kg ⁻¹	58.8	24.9 $\pm$ 1.05
TQNLC 50 mg kg ⁻¹	62.5	24.8 $\pm$ 1.48
TQNLC 100 mg kg ⁻¹	81.2	27.6 $\pm$ 0.52
Olive oil	60.0	26.7 $\pm$ 0.52
TQ 25 mg kg ⁻¹	64.7	23.9 $\pm$ 1.71
TQ 50 mg kg ⁻¹	73.3	26.5 $\pm$ 0.74
TQ 100 mg kg ⁻¹	53.8	25.7 $\pm$ 0.71
Negative	60.0	23.9 $\pm$ 1.55
Positive	75.0	27.3 $\pm$ 0.41
Normal	100	N/A

N/A: Not available; NLC: Nanostructured lipid carrier; TQ: Thymoquinone; TQNLC: Thymoquinone-loaded nanostructured lipid carrier.

Effect of TQNLC on the metastasis to lungs

The tumor metastases to the lungs are shown in Figure 5. The number of tumor nodules in the lungs in the groups treated with 50 mg/kg of TQNLC (11.5 nodules) and 25 mg/kg of TQ (10.3 nodules) was lesser ($p < 0.05$) than

Figure 3. Effect of TQNLC on the tumor volume and weight. (A) Tumor volume of the 4T1 tumor-bearing female BALB/c mice after treatment with TQNLC. Values are expressed as mean $\pm$ standard error of the mean (SEM; $n = 6$). **(B)** Tumor weight of the 4T1 tumor-bearing female BALB/c mice after treatment with TQNLC. *Indicates significantly different as compared to the negative control group ($p < 0.05$). Values are expressed as mean $\pm$ SEM ($n = 6$). Negative refers to untreated inoculated mice and Positive refers to doxorubicin-treated inoculated mice.

NLC: Nanostructured lipid carrier; TQ: Thymoquinone; TQNLC: Thymoquinone-loaded nanostructured lipid carrier.

the one of the negative control group (24.5 nodules) but was not significantly different ($p > 0.05$) compared with the one of doxorubicin. The expression of MMP-2 was significantly downregulated ($p < 0.05$) in the mice treated with doxorubicin, 25 and 50 mg/kg of TQNLC and TQ as compared with the negative control group (Figure 6B).

Effect of TQNLC on the angiogenesis

Gross observation revealed that angiogenesis happened on Day 10 in the 4T1 tumor-bearing female BALB/c mice (Figure 6A). Abnormal proliferation of blood vessels has been observed around and within the tumor in the negative

Figure 4. Survival of the 4T1 tumor-bearing female BALB/c mice after treatment with TQNLc for 28 days. Normal refers to un-inoculated mice; Negative refers to untreated inoculated mice and Positive refers to doxorubicin-treated inoculated mice. NLC: Nanostructured lipid carrier; TQ: Thymoquinone; TQNLc: Thymoquinone-loaded nanostructured lipid carrier.

control group. As depicted in Figure 6B, the expression of VEGF in all the treatment groups as compared with the negative and positive control groups was indifferent ($p > 0.05$)

Effect of TQNLc on the expression of apoptotic-related proteins

The number of apoptotic cells in the tumor of the groups treated with TQ and TQNLc was comparable ($p > 0.05$) to the one of doxorubicin. A significant increase in the number of apoptotic cells was noted in the doxorubicin-treated group ($p < 0.05$) compared with the negative group (Figure 7A & B).

Figure 8A indicates that the expression of Bax was upregulated ($p < 0.05$) in the mice treated with doxorubicin and 50 mg/kg of TQNLc, and the expression of Bcl-2 was upregulated in the mice treated with doxorubicin and 25 mg/kg of TQNLc, as compared with the negative control group. The groups treated with doxorubicin, 25 mg/kg of TQNLc and 50 mg/kg of TQ have significant upregulated expression of caspase 8 compared with the negative control group ($p < 0.05$). The expression of TNF- α was not upregulated in all the treatment groups. Figure 8B shows that the ratio of Bax to Bcl-2 in the mice treated with 50 mg/kg of TQNLc was significantly higher compared with the negative control group ($p < 0.05$).

Discussion

Chemotherapy, the most common strategy to treat cancer, has limitations such as drug nonspecificity, drug insolubility and adverse side effects. In order to overcome these drawbacks, various drug delivery systems have been developed. Lipid-based nanocarriers and liposome have been proven to enhance the efficacy of hydrophobic drugs and reduce the toxicity in cancer therapy [33]. In our previous study, TQNLc was produced with ideal characteristics such as small mean diameter (less than 50 nm), low polydispersity index, high ζ potential, excellent encapsulation efficiency and reduced toxicity [28]. In order to determine the efficacy of the formulation as an anticancer agent, new batch of TQNLc was prepared with the same procedure followed by the physicochemical characterization as previously described. Photon correlation spectroscopy analysis showed that TQNLc is formulated with average size of 50 nm. The polydispersity index value of TQNLc was less than 0.2, indicating that the formulation was homogenous with narrow size distribution. The ζ potential of TQNLc that ranged from -4.38 to -6.03 mV indicates that TQNLc was stabilized with negative surface charge. The mentioned characteristics were found reproducible

Normal	Negative control	Positive control	TQNLC 25 mg/kg	TQ 25 mg/kg

0	24.5 ± 6.77	14.3 ± 1.71*	12.8 ± 2.85	10.3 ± 3.45*

TQNLC 50 mg/kg	TQ 50 mg/kg	TQNLC 100 mg/kg	TQ 100 mg/kg

11.5 ± 3.21*	16.33 ± 2.82	24.5 ± 4.54	21.33 ± 1.88

Figure 5. Lungs of the 4T1 tumor-bearing female BALB/c mice stained with India ink. White nodules on the lungs are the metastasized 4T1 tumor cells. *Indicates significantly different as compared to the negative control group ($p < 0.05$). Values are expressed as mean $\pm$ standard error of the mean ($n = 6$). Normal refers to uninoculated mice; Negative refers to untreated inoculated mice and Positive refers to doxorubicin-treated inoculated mice. RP: Red pulp; TQ: Thymoquinone; TQNLC: Thymoquinone-loaded nanostructured lipid carrier; WP: White pulp.

and comparable with the previous batch. It is crucial that every batch of drugs possesses the same characteristics to ensure reproducible therapeutic effects as batch-to-batch variation may lead to untoward consequences to the patients.

In this study, a highly tumorigenic and invasive mouse 4T1 mammary carcinoma model was employed to determine and compare the anticancer activity of all the tested materials (TQ, TQNLC and doxorubicin). Splenomegaly has been observed in all the 4T1 tumor-bearing female BALB/c mice to indicate that the model worked. The spleens experienced extramedullary hematopoiesis whereby a variety of hematopoietic precursor cells such as megakaryoblasts was present in the expanded area of red pulp. It has been previously reported that the formation of tumor has induced leukemoid reaction and led to spleen enlargement [34]. However, there was no significant different in the spleen weight of all the treatment groups as compared with the normal mice, suggesting that the treatments do not suppress the immune and defense systems [35].

In order to benchmark the anticancer activity of TQ and TQNLC, doxorubicin was selected as a reference drug (positive control). Doxorubicin, an anthracycline chemotherapeutic drug, is commonly used for breast cancer treatment [36]. However, its clinical utility has been limited due to low therapeutic index and adverse side effects [37]. In addition, doxorubicin is administered via intraperitoneal route, which causes patient discomfort and

Figure 6. Effect of TQNLC on the lung metastasis and angiogenesis. (A) Blood vessel formation in the solid tumor of the 4T1 tumor-bearing female BALB/c mice (from the negative control group) on Day 10. **(B)** Expression level of the MMP-2 and VEGF in the tumor of the 4T1 tumor-bearing female BALB/c mice after treatment with TQNLC as analyzed by Western blot. Fold change was normalized against beta actin. *Indicates significantly different as compared to the negative control group ($p < 0.05$). Values are expressed as mean of three independent experiments. Negative refers to untreated inoculated mice and Positive refers to doxorubicin-treated inoculated mice. NLC: Nanostructured lipid carrier; TQ: Thymoquinone; TQNLC: Thymoquinone-loaded nanostructured lipid carrier.

incompliance. TQNLC has an advantage as it is to be administered via oral route. Oral route is the most preferred one because it is noninvasive, convenient with the least sterility issues and of lowest cost.

Our hypothesis was rejected whereby TQNLC did not exhibit enhanced anticancer activity compared with TQ, despite NLC has been reported to enhance bioavailability and facilitate the controlled release of a drug [38]. Treatment with TQNLC did not reduce the tumor volume when compared with TQ. Similar results were previously reported [39,40] but the reason remains unclear. Even though TQNLC didn't outperform TQ, the anticancer activity (based on the reduction of tumor volume) of both at 50 and 100 mg/kg was comparable to doxorubicin.

Efficacy of a medicine is defined by the therapeutic index, which is depending on both the drug therapeutic ability (anticancer activity in this case) and toxicity [41]. Majority of the currently available anticancer drugs are not selectively killing cancer cells, and their toxic adverse effects to the normal cells bring about compromised immune system that affects the patient survivability [42]. Encapsulation of drugs with suitable carriers may reduce their toxicity, and that has been proven by our previous report whereby TQNLC was less toxic than TQ [28]. An ideal delivery system will prolong the controlled drug release, which maintains the drug concentration within the therapeutic window, hence, minimizes the episode of toxicity. It was then evidenced by the 4T1 tumor-bearing female BALB/c mice treated with TQNLC having higher survival time and rate as compared with the one treated with TQ at the same dose and doxorubicin.

Figure 7. TUNEL staining. (A) Tumor section of the 4T1 tumor-bearing female BALB/c mice after treatment with TQNLC after staining with TUNEL as observed under a fluorescent microscope. The apoptotic nuclei are stained green. Each scale bar represents 100 μm (200 $\times$ magnification). (B) Apoptotic cells of the 4T1 tumor-bearing female BALB/c mice after treatment with TQNLC stained with TUNEL as analyzed by ImageJ software. *Indicates significantly different as compared to the negative control group ($p < 0.05$). Values are expressed as mean $\pm$ standard error of the mean ($n = 3$). Negative refers to untreated inoculated mice and Positive refers to doxorubicin-treated inoculated mice. NLC: Nanostructured lipid carrier; TQ: Thymoquinone; TQNLC: Thymoquinone-loaded nanostructured lipid carrier; TUNEL: terminal transferase mediated dUTP nick end-labeling kit.

Figure 8. Effect of TQNLC on the apoptosis. (A) Expression level of the apoptotic-related proteins of the 4T1 tumor-bearing female BALB/c mice after treatment with TQNLC as analyzed by Western blot. Fold change was normalized against beta actin. *Indicates significantly different as compared to the negative control group ($p < 0.05$). Values are expressed as mean of three independent experiments. (B) Bax to Bcl-2 ratio of the 4T1 tumor-bearing female BALB/c mice after treatment with TQNLC as analyzed by Western blot. *Indicates significantly different as compared to the negative control group ($p < 0.05$). Values are expressed as mean $\pm$ standard error of the mean ($n = 3$). Negative refers to untreated inoculated mice and Positive refers to doxorubicin-treated inoculated mice. NLC: Nanostructured lipid carrier; TQ: Thymoquinone; TQNLC: Thymoquinone-loaded nanostructured lipid carrier.

Interestingly, treatment at lower doses of TQ and TQNLC, 25 and 50 mg/kg, respectively, inhibited tumor metastasis to the lung comparable to the one of doxorubicin. It is suggested that both TQ and TQNLC require specific dose or effective dose to exhibit the effect [43], whereby their use at higher dose shall then be avoided to reduce toxicity. The lung metastasis is believed to be inhibited via downregulation of the expression of matrix

metalloproteinases (MMPs). MMPs are a family of structurally related zinc-dependent endopeptidases, which play an important role in the degradation of extracellular matrix [44]. One of the members of the MMP family, MMP-2, can degrade type IV collagen of basement membrane, which is the first barrier for cancer invasion and metastasis. The upregulated expression of MMP-2 also correlated with poor prognosis and aggressive behaviour in breast cancer [45]. Previous studies have indicated that tumor metastasis was associated with the overexpression of MMP-2 [46]. As the expression of VEGF was not affected, it is suggested that TQNLC does not possess anti-angiogenic activity.

Induction of apoptosis in cancer cells has been the main strategy in treating cancer owing to the advantages of self-destructive and non-inflammatory responses [47,48]. TQ and doxorubicin have been previously reported to induce apoptosis in cancer cells in *in vitro* and *in vivo* models. Based on the TUNEL assay data of this study, treatment with TQNLC, TQ and doxorubicin induced apoptosis in the solid tumor of the mice. The induction of apoptosis by TQNLC indicates that the apoptotic inducing properties of TQ are reserved and not affected by the encapsulation by NLC. Ideally, the use of any delivery systems should not change the nature of the drug. TQ (2-isopropyl-5-methyl-1,4-benzoquinone) has a similar structure with benzene metabolites that damage DNA by interrupting the function of type II topoisomerase [49].

Treatment at 50 and 100 mg/kg of TQNLC and TQ was found to induce the intrinsic apoptotic pathway by downregulating the expression of Bcl-2. Nevertheless, the expression of Bax was upregulated in the treatment with 50 mg/kg of TQNLC and doxorubicin. This drives to a hypothesis that the treatment with TQNLC and TQ can trigger mitochondrial action and induce the intrinsic apoptotic pathway in the absence or presence of Bax [50]. Based on the upregulated expression of caspase 8, it is also suggested that the intrinsic apoptotic pathway induced by TQNLC and TQ may be activated by other apoptotic signals or proteins including caspase 8. Caspase 8 is an upstream mediator that predominantly activated in the extrinsic apoptotic pathway by signals from death receptors such as TNF- α [51]. However, the expression of TNF- α in this study was not upregulated, suggesting that the activation of caspase 8 is not modulated by TNF- α via the extrinsic apoptotic signalling pathway. Several studies have shown that the activation of caspase 8 did not only occur through the extrinsic apoptotic pathway, but also via the intrinsic apoptotic pathway [52,53].

Conclusion

Encapsulation of TQ by NLC improved the survival rate of the 4T1 tumor-bearing female BALB/c mice better than doxorubicin. Both TQNLC and TQ inhibited the lung metastasis by modulating the expression of MMP-2, and induced the intrinsic apoptotic pathway in the tumor cells with the involvement of Bcl-2, Bax and caspase 8. TQNLC and TQ may be potential candidates as chemotherapeutic drugs for breast cancer treatment since the anticancer activity was comparable to the one of doxorubicin.

Future perspective

The incidence of cancer is always associated with high rates of mortality and morbidity. The need for new anticancer agents, especially those with antimetastatic properties, is crucial. Based on the data of this study, TQNLC holds a promise to be developed as an anticancer agent. The use of NLC has been shown to retain the similar efficacy (anticancer activity) and improve the survival rates of the tumor-bearing animals. Indeed, a lot of studies need to be conducted especially on the absorption, distribution metabolism and excretion and before it is being translated to human.

Financial & competing interesting disclosure

This research was financially supported by Exploratory Research Grant Scheme (ERGS) with vote number 5527119 funded by Ministry of Higher Education, Malaysia. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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Summary points

- Thymoquinone (TQ) incorporated into nanostructured lipid carriers (NLCs) or TQNLC was produced by using hot high pressure homogenization with good physicochemical properties.
- Anticancer study was carried out by using 4T1 tumor-bearing female BALB/c mouse model.
- TQNLC and TQ were administered into the 4T1 tumor-bearing female BALB/c mice by oral gavage.
- NLC enhanced the therapeutic effect of TQ by improving the survival rate of the mice.
- TQNLC did not outperform the anticancer activity of TQ.
- Both TQNLC and TQ exhibited anticancer activity with reduced tumor volume and tumor weight.
- TQNLC and TQ induced apoptosis via modulation of Bcl-2, Bax and caspase-8.
- TQNLC and TQ exhibited antimetastasis effect to the lungs via downregulation of MMP-2.

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