TARGETED DUAL FUNCTIONAL NANOPARTICLES FOR THE TREATMENT OF CANCER AND LIVER FIBROSIS

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For decades, a large number of therapeutics have been discovered and developed with high potential for curing various diseases, many of which have been clinically used for years. However, some treatments are greatly limited due to their side effects, which arise from their inability to differentiate between normal and diseased cells. The aim of this dissertation work is to develop nanomaterial-based dual function drug delivery systems to improve overall therapeutic outcomes for the treatment of cancer and liver fibrosis.

The first part of this work focused on the development of FTS-based solid lipid nanoparticles (SLNs) for cancer-targeted delivery of paclitaxel (PTX). Novel SLNs were successfully developed which are capable of solubilizing PTX while simultaneously avoiding unwanted side effects of the clinically used PTX formulation (Taxol). The data from this study demonstrated that the PTX-SLNs system has a significantly improved profile in terms of controlled release kinetics and stability compared to Taxol. Additionally, PTX-SLNs have shown enhanced anticancer activity *in vivo*.

The second part focused on improved delivery of the herbal agent thymoquinone (TQ) to hepatic stellate cells (HSCs) for the treatment of liver fibrosis. Firstly, a study was conducted on the biological effects of TQ on HSCs, which represent the major liver cell type involved in the massive production of extra cellular matrix (ECM) in liver fibrosis. The results revealed that TQ exerts hepatoprotective and anti-fibrotic effects via direct inhibition of the fibrogenic activities of HSCs, which suggests that TQ holds great potential as a new drug candidate for treatment of

liver fibrosis. Secondly, an examination was conducted on the potential of a novel dual functional micellar system PEG_{5k}-Fmoc-FTS_2 to deliver TQ into activated HSCs *in vitro*. TQ-micelles have shown high tolerability in activated HSCs with more efficient anti-fibrotic activity compared to free TQ.

Collectively, this work suggests that an FTS-based SLNs system represents a promising nanocarrier for cancer-targeted delivery which could enhance the therapeutic efficiency of anticancer agents. In addition, TQ holds a great a potential as new therapy for the treatment of liver fibrosis, and formulating TQ into the PEG_{5k}-Fmoc-FTS_2 micelles system could further enhance overall anti-fibrotic activity.

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PREFACE

First and foremost, I wholeheartedly thank Allah for giving me the vision, power, spirit, and endurance to complete my dissertation work.

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ABBREVIATIONS

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BBB	Blood brain barrier
COL1A1	Collagen, Type I, Alpha 1
CBDL	Common bile duct ligation
CCl_4	Carbon tetrachloride
c-FLIPL	Cellular FLICE-inhibitory protein
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethylsulfoxide
DCX	Docetaxel
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
DPBS	Dulbecco's phosphate-buffered saline
EPR	Enhanced permeability and retention
EG-	Ethyleneglycol-
EE	Entrapment efficiency
ECM	Extracellular matrix proteins
ET-1	Endothelin 1
FTS	Farnesyl thiosalicylic acid
FDA	Food and drug administration
FBS	Fetal bovine serum
Fmoc	9-fluorenylmethoxycarbony
GRAS	Generally recognized as safe
GSH	Glutathione
HSCs	Hepatic Stellate Cells
H.E	Hematoxylin and eosin
HR	Hazard ratio
HPR	Horseradish peroxidase
HPLC	High-performance liquid chromatography

HCV	Hepatitis C virus
IL-6	Interleukin 6
i.p.	Intra-peritoneal
i.v.	Intravenous
IR	Tumor growth inhibition rate
LPS	Lipopolysaccharide
LC	Loading capacity
MCP-1	Monocyte chemotactic protein-1
MDR	Multidrug resistance
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide
NF-κB	Nuclear factor kappa-B
NPs	Nanoparticles
NHS	N-hydroxysuccinimide
NASH	Nonalcoholic steatohepatitis
PTX	Paclitaxel
P-gp	P-glycoprotein
PEG	Polyethylenglycol
PLGA	D,L-lactic-co-glycolic acid
PDGF	Platelet-derived growth factor
PPAR	Peroxisomal proliferator activated receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
RES	Reticuloendothelial systems
SLNs	Solid lipid nanoparticles
SEM	Standard error of the mean
SM	Silymarin
SD	Standard deviation
siRNA	Small interfering RNA
SEM	Scanning electron microscope
α-SMA	Actin, aortic smooth muscle
TQ	Thymoquinone
$t_{1/2}$	Half-life
TM	Trimyristin
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor
TNF-α	Tumor necrosis factor-α
TLR4	Toll-like receptor 4
XIAP	X-linked inhibitor of apoptosis protein

1. TARGETED DELIVERY OF ANTICANCER AGENTS

1.1. INTRODUCTION

1.1.1. Current status of cancer treatment

Cancer is the most prominent disease of the 21st century, and represents an increasingly difficult challenge over time. Although science and technology have progressed remarkably over the last decades, they remain incapable of providing a cure for cancer. Cancer is not a single disease, but rather a group of diseases. However, they all begin with abnormal cells that grow uncontrollably.

According to the International Agency for Research on Cancer (IARC) (2015b) , the number of estimated cancer deaths in 2012 was 8.2 million worldwide. This accounts for 58% of the total estimated new cases of the same year. The 2015 United States cancer statistics, revealed by the National Cancer Institute, estimate that 1,658,370 cases are expected to be registered during this year, of which 589,430 are estimated deaths from all cancer types (Siegel et al., 2015). The 5-year survival rate between 2005 and 2011 was 66.5%, which represents an improvement when compared to 49% between 1975 and 1977 (2015a). This is likely due to an improvement in treatment and the constant evolution of tools which assist in the detection of certain types of cancer at their early stages.

Several factors contribute to the incidence of cancer. Tobacco use is the leading cause, accounting for 20-30% of all risk factors. Both active and secondhand smokers are at high risk of developing cancer, because chemicals in tobacco can cause damage to DNA. Another leading cause is linked to diet and obesity, which represents 30% (Doll and Peto, 1981; Anand et al., 2008). In fact, diet is responsible for approximately 70% of colorectal cancer cases. This factor is difficult to control, since many carcinogenic materials such as nitrate, pesticide, and dioxin are used in the food industry (Abnet, 2007; Hogg, 2007). Infectious diseases play a major role in cancer occurrence. Up to 20% of cancer cases are related to infections, of which viral infection being the dominant type. For example, nearly all cases of cervical cancer are caused by human papillomavirus in Western developed countries (Pisani et al., 1997; Parkin, 2006). Although cancer is caused by molecular genetic mutations, these mutations are the result of exposure and interaction with external environmental factors. Only approximately 5-10% of cancers are linked to genetic defects inherited from parents. The remaining percentage includes environmental pollution, alcohol, family history, radiation, and other factors (Loeb and Loeb, 2000; Mucci et al., 2001; Hahn and Weinberg, 2002).

Currently, the most common treatment options include surgery, radiotherapy, chemotherapy, immunotherapy, and targeted therapy. Additionally, a combinational therapy including some of the aforementioned treatments can be considered depending on the type and stage of the disease. However, many limitations and downsides exist for each treatment option. Although surgery is the only treatment option that may provide a realistic chance for curing cancer, it is only applicable when the cancer is localized and hasn’t spread to other tissues; and also if tumor removal will not damage any vital organs. Typically, surgery will be the first option if cancer is detected early. Radiation treatment is characterized by its specific targeting to the

tumor area using special radiation such as x-rays and gamma rays. However, radiation itself may cause skin irritation, damage to nearby tissue, and patients may suffer from fatigue. Patients undergoing chemotherapy normally suffer from side effects caused by anticancer drugs, including hair loss, nausea, vomiting, and loss of appetite. Despite the high efficacy of these chemotherapeutic agents, they lack the specificity in tumor targeting. Consequently, the agents spread into healthy tissues and kill normal cells simultaneously (Sutradhar and Amin, 2014). Therefore, safer alternatives which guarantee antitumor efficacy without the accompanying harmful side effects are highly in demand.

A variety of techniques have been investigated over the past few decades which are sought to discover an optimal remedy for cancer treatments; the most promising technique among them is nanotechnology. The potential therapeutic benefits of nanotechnology are not only limited to cancer treatment, but also in the detection and prevention of the disease. The unique functional properties of nanotechnology, such as the possibility of delivering drugs specifically into tumor cells without harming normal cells, and the potential for overcoming drug resistance and side effects have made it a very attractive field in cancer research. In fact, several nanocarriers such as Doxil, Abraxane and Myocet have successfully made it into the clinic for their significant advantages over conventional treatments (Pillai, 2014).

1.1.2. Nanotechnology and its application in cancer treatment

Nanotechnology is a multidisciplinary field which represents a combination of many basic sciences, such as chemistry, physics, engineering, and biology. Nanotechnology can be defined as materials of nanoscale size between 1-100 nm which possess the ability to deliver drugs or

imaging agents for the purpose of treatment or diagnosis (Peppas, 2004; Ferrari, 2005). A Nanoparticle system is typically composed of a core, corona, payload, and targeting ligand (Figure 1).

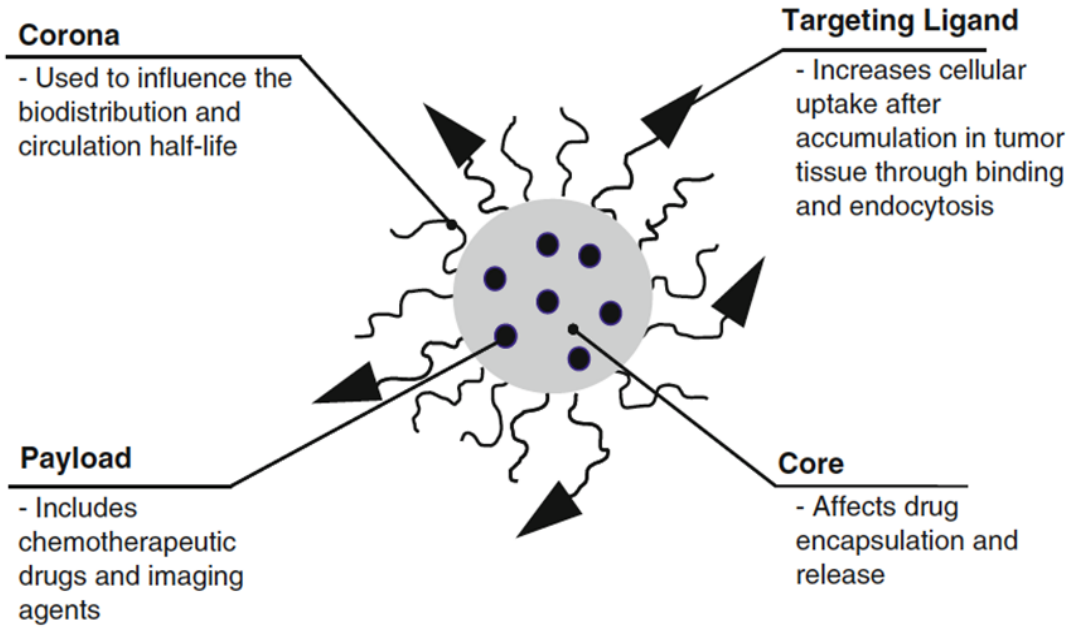

Figure 1. Nanoparticles platform structure, including core, payload, corona, targeting ligand.

Adapted from (Alexis et al., 2010)

The early discovery of nanotechnology was first described in 1960s by British haematologist Alec Bangham. When Bangham and his colleague R.W. Horne were testing a new electron microscope at the Babraham Institute, in Cambridge, UK using dry phospholipids dispersed in water, they noticed that this material was able to form closed-membrane spherical vesicles similar to cell membrane which was known later as liposomes (Bangham et al., 1965). Following this discovery, remarkable progress has been achieved, enabling improved design and fabrication of nanosystems. In 1976, Langer and Folkman introduced the first controlled release

system for macromolecule delivery (Langer and Folkman, 1976), followed by the first application of antibody-decorated liposomes for targeted therapy in 1980 (Heath et al., 1980; Leserman et al., 1980). When PEGylated liposomes were introduced in 1990, they greatly improved nanoparticle stability and prolonged their circulation time in blood, representing another major breakthrough (Klibanov et al., 1990; Gref et al., 1994). This opened new scopes for the development of new strategies to re-evaluate existing effective nanosystems which were abandoned for their short stability in blood.

Many obstacles for conventional chemotherapy have hindered its clinical applications, including poor water solubility, non-specific targeting, and multidrug resistance (Dong and Mumper, 2010; Chidambaram et al., 2011; Sutradhar and Amin, 2014). Most anticancer agents are hydrophobic in nature, which make their oral administration very difficult, and additionally expose them to GI metabolism, leading to degradation or modification to inactive or toxic metabolites. Therefore, the intravenous (i.v.) route represents a better option that increases the bioavailability and benefit of most drugs (Hande et al., 1999). However, hydrophobic drugs must be dissolved in an aqueous solution, which creates additional challenges in suitability for i.v. administration. Increasing drug hydrophilicity by chemical modification of parent compounds is possible, but the probability of a loss in drug activity becomes high (Savjani et al., 2012). Moreover, the use of adjuvant solvents to solve this issue, such as Taxol (Paclitaxel dissolved in Cremophore EL and dehydrated ethanol) could likely cause unwanted side effects such as hypersensitivity reactions (Gelderblom et al., 2001b). Moreover, conventional chemotherapy is distributed throughout the entire body following i.v. administration, and non-specifically targets normal cells, which causes severe side effects. Furthermore, substantial amount of drug are quickly cleared by the reticuloendothelial system (RES) (Alexis et al., 2008; Li and Huang,

2010; Wang et al., 2012), leading to insufficient drug levels becoming present in the tumor, resulting in minimal antitumor activity. Such treatments could even worsen disease condition by triggering tumor cell resistance to anticancer drugs. Tumor cells have the capability to respond to treatment by launching defensive mechanisms via the overexpression of P-glycoprotein (P-gp) efflux membrane transporter, resulting in multidrug resistance (MDR) and the production of more aggressive tumor forms (Gottesman, 1993; Dong and Mumper, 2010). Therefore, solubilizing hydrophobic drugs in a carrier that selectively targets tumors and is capable of escaping clearance by RES would greatly maximize therapeutic benefits while avoiding the potential side effects of conventional therapy.

Nanotechnology offers potentially promising solutions for all of the aforementioned challenges. The physiochemical properties and small particles sizes that characterize nanoparticles (NPs) enable them to improve drug solubility and efficiently decrease their interaction with blood proteins, allowing them to circulate longer in blood stream. Moreover, these properties play a role in the ability to specifically target tumor sites and increase drug bioavailability by taking advantage of the leaky vasculatures and poor lymphatic drainage in tumor tissues. This phenomenon is known as the enhanced permeability and retention (EPR) effect (Maeda et al., 2000; Duan et al., 2010a) (Figure 2).

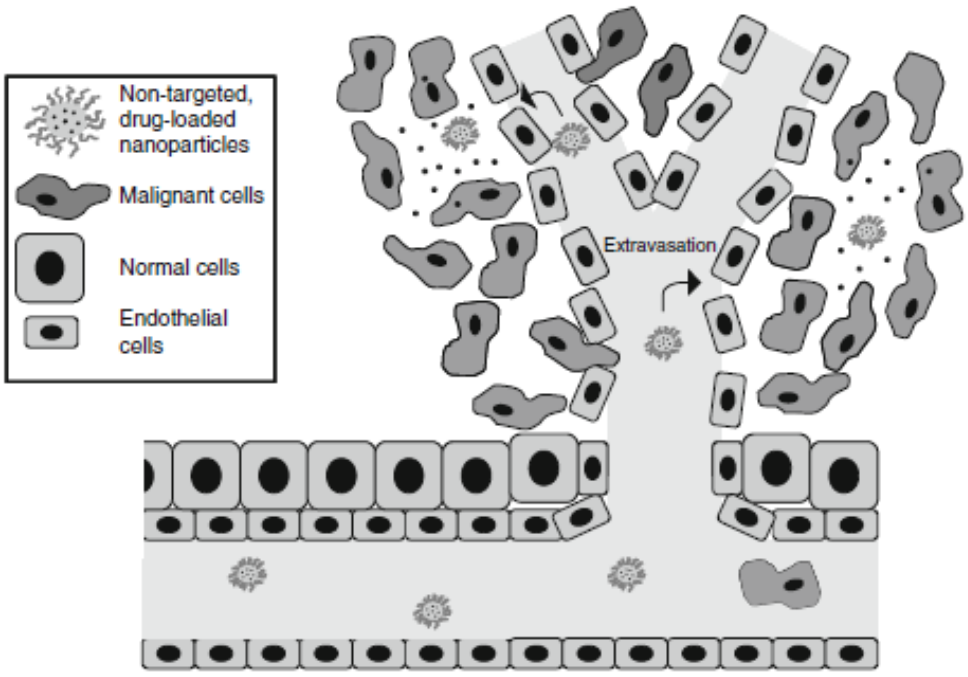

Figure 2. Enhanced permeability and retention (EPR) effect.

Adapted from (Alexis et al., 2010)

A variety of nanocarriers have been developed for treatment and diagnostic purposes. Among these nanocarriers, liposomes are the most extensively studied platform. In fact, several formulations have made it to clinical applications for the treatment of Kaposi's sarcoma, ovarian and breast cancer, with many applications currently in clinical trials (Fan and Zhang, 2013). Liposomes are composed of phospholipids that, in aqueous solution, form spherical vesicles with a hydrophilic core encapsulated by at least one lipid bilayer. Liposomes have the ability to carry both hydrophilic and lipophilic agents. Doxil is one example of a liposomal formulation that is coated with polyethylene glycol (PEG) to deliver doxorubicin for cancer treatment (Senior, 1986; Torchilin, 2005; Immordino et al., 2006).

Another example, polymeric NPs are composed of biodegradable polymers that self-assemble in an aqueous environment, thereby encapsulating hydrophobic agents. Genexol-PM is a poly (D,L-lactic-co-glycolic acid) (PLGA) NP for paclitaxel delivery that has been approved in South Korea and currently under phase III-IV clinical trials in the United States for the patients with recurrent breast cancer (Soppimath et al., 2001; Kim et al., 2007; Ledet and Mandal, 2012; Wang et al., 2012).

The aforementioned platforms are categorized as passive targeting NPs, which essentially rely on the physicochemical properties of NPs and the EPR effect in the tumor area to reach their target. Another technique is active targeting, where the NP's surface can be fabricated with a targeting moiety such as proteins, antibodies, or other ligands that can selectively interact with the antigens or cell receptors overexpressed in tumor cells. Currently, several NPs that are based on passive targeting have been approved for clinical use; however, active targeting NPs are still in clinical trials (Schäfer-Korting, 2010).

1.1.2.1. PEG-decorated nanoparticles

Although nanotechnology has improved the therapeutic outcomes of conventional chemotherapy, one major barrier against the use of NPs is their attack by opsonins in the bloodstream, and the subsequent removal by RES within short time after i.v. injection. The processes known as opsonization and phagocytosis are the first line defense against any foreign bodies or particles entering blood circulation. In order for phagocytes to recognize foreign materials, opsonin proteins must guide them by binding to a particle's surface so it may be identified as an invader. Then, phagocytic cells take up the foreign particles, and destroy them. Opsonins represent any blood materials which are involved in the process of phagocytic recognition; these include

albumin, immunoglobulins, fibronectin, and laminin, among others (Moghimi and Szebeni, 2003; Owens and Peppas, 2006).

One key step in escaping opsonization involves preventing recognition by phagocytes. One of the most successful methods used to achieve this goal is the covering of NPs' surfaces using a hydrophilic polymer which acts as shield against opsonins and eventually bypasses phagocytic recognition (Klibanov et al., 1990; Li and Huang, 2010). Among these polymers, PEG, an FDA-approved hydrophilic polymer, holds the most promise based on its flexibility and excellent safety profile. The development of NPs decorated by PEG molecules (PEGylated-NPs) not only reduces exposure to RES uptake, but also extends the half-lives when compared to non-PEGylated NPs. Early attempts to use PEG in the field of drug delivery dates back to a 1977 study, where methoxy-PEGs (mPEGs) was conjugated to bovine serum albumin and liver catalase (Abuchowski et al., 1977). Additionally, modification of microspheres' surfaces with surface-localized human serum albumin with polyethylene glycol resulted in a two-fold increase in the half-life of the carrier (Arturson et al., 1983). Ten years later, Li and coworker reported that latex particles coated with PEG 5000 kDa remained in rat blood for 13 h, while uncoated particles only lasted for 20 min (Tan et al., 1993). Klibanov and Huang discovered that the fabrication of liposome NPs with dioleoyl N-(monomethoxy polyethyleneglycol succinyl) phosphotidylethanolamine (PEG-PE) increased their $t_{1/2}$ 10-fold without significant leakage of liposome content (Klibanov et al., 1990). This research in PEG applications for drug delivery development led to the first FDA-approved PEGylated NPs in the mid-1990s: Doxil® (liposomal delivery vehicle for doxorubicin) and oncospar (PEG-l-asparaginase). Doxil formulations have increased doxorubicin bioavailability 90-fold when compared to free drug administration.

1.1.2.2. Dual functional carriers

The vast majority of NPs being developed for optimal drug delivery are usually based on inert carriers. These carriers are biologically inactive regardless of their advantageous properties in terms of drug solubility and stability. However, these carries add to the cost and raises potential safety concerns. The recruitment of bioactive compounds which can functionally replace the carrier's inert part and offer potential therapeutic benefits is being investigated as an effective approach for the design of dual functional NPs. Our laboratory has been investigating a number of therapeutic agents for this purpose. Indeed, *in vitro* and *in vivo* data from several promising PEG-conjugate micellar systems have demonstrated significantly improved antitumor activity. These conjugates improved the overall anticancer effect via synergizing with delivered anticancer agents such as paclitaxel and doxorubicin, while enhancing formulation's stability and drug loading capacity (Huang et al., 2012; Lu et al., 2013; Zhang et al., 2013b; Lu et al., 2014b; Zhang et al., 2014d).

For instance, a dual functional carrier based on PEG-derivatized embelin was developed as a novel micellar system for the delivery of paclitaxel. Embelin is a natural compound extracted from *Embelia ribes* BURM. Embelin has been studied for a variety of therapeutic activities, including antidiabetic, anti-inflammatory, and hepatoprotective activities. Moreover, several studies have highlighted its antitumor activities in many types of cancer. Embelin induces apoptosis through the inhibition of X-linked inhibitor of apoptosis protein (XIAP), which is overexpressed in tumor cells, especially in drug-resistant cells, which makes embelin capable of reversing multidrug resistance. Moreover, embelin exhibits significantly less toxicity in normal cells due to the low impact of XIAP in normal cells (Chitra et al., 1994; Danquah et al., 2009). Apart from its therapeutic benefits, the poor water solubility of embelin makes it an excellent

candidate as hydrophobic carrier component that can be conjugated to a PEG hydrophilic polymer to form dual functional micellar systems. *In vitro* cytotoxicity analysis indicated that PEG-embelin micelles not only maintained a similar antitumor activity to free embelin, but also exhibited a synergistic effect with loaded PTX in human breast and prostate cancer lines. Also, *in vivo* antitumor study in murine models of breast and prostate cancers exhibited significantly improved tumor growth inhibition when compared to a Taxol formulation (Huang et al., 2012).

Another dual functional micellar system reported by our group is based on PEG-farnesylthiosalicylic acid (FTS) conjugate. (Zhang et al., 2013b). FTS is a synthetic, non-toxic, and potent Ras inhibitor. Ras mutation occurs in about one-third of cancers, with a highest incidence (nearly 90%) in pancreatic cancer. Mutation of Ras genes leads to permanent activation of the Ras pathway, which subsequently promotes uncontrolled cell growth. One of the major mechanisms by which FTS exerts its antitumor activity is binding to the anchorage domain of Ras protein in the plasma membrane, resulting in Ras protein dislodgement followed by the degradation and inhibition of effector signaling (Marciano et al., 1995; Haklai et al., 1998a). More importantly, FTS (hydrophobic in nature, see Figure 3) conjugated with PEG improved water solubility and self-assembled to very small nanomicellar particles (20-30 nm) suitable for hydrophobic anticancer agents such as PTX. An i*n vitro* release study has indicated slower release kinetics in FTS-micelles when compared to Taxol. Also, PEG-FTS micelles exhibited comparable activity to free FTS, as evidenced by similar cytotoxicity in several cancer cell lines as well as Ras protein downregulation analyzed by Western blot. Moreover, PTX-loaded PEG-FTS micelles have significantly inhibited tumor growth in a syngeneic murine breast cancer model when compared to Taxol formulation, suggesting improved accumulation of

micelle particles at tumor sites and possible synergy between the carrier and PTX (Zhang et al., 2013b).

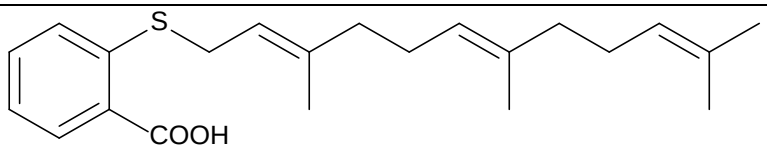

Figure 3. Chemical structure of FTS

1.1.2.3. Solid nanoparticles in cancer targeted therapy

Solid lipid nanoparticles (SLNs) are colloidal systems with a submicron size range. As the name implies, they consist of a hydrophobic lipid core present in a solid state at room and body temperatures. The solid core is surrounded by a monolayer of phospholipid coating, forming spherical particles. The active drug is dissolved or dispersed within the solid matrix (Wissing et al., 2004).

The idea behind SLN development dates back to the early 1990s, when scientists were searching for alternatives to overcome the limitations of NPs through liposome and polymeric NPs. Despite the increased efficacy and reduced side effects of incorporated drugs that liposomes offer, major drawbacks remain, including limited physical stability, leakage of loaded drugs, and large scaling hurdles (Gregoriadis, 1988). Similarly, polymeric NPs provide enhanced tumor targeting and controlled release of loaded drugs, however, there are growing concerns regarding polymer cytotoxicity following their uptake by cells, as well as their scale up difficulty (Fu et al., 2000). SLNs were developed as an alternative carrier for conventional NPs. SLNs combine the advantages and avoid some of the shortcomings of existing traditional carriers (e.g. improved

stability profile, protection of loaded drug from degradation, improved controlled release, enhanced bioavailability, and excellent biocompatibility). Moreover, SLNs are considered less costly in large-scale production when compared to liposomes (Wissing et al., 2004). SLNs also possess a broad range of applications in many diseases, including cancer. However, SLNs also carry some potential disadvantages, including limited drug loading capacity, unpredictable gelation tendency, and particle growth. Researchers have designed SLNs formulations suitable for different routes of administration such as parenteral, oral, dermal, ocular, pulmonary, and rectal routes. Due to the physicochemical properties of SLNs, they have the potential to carry both hydrophilic and hydrophobic drugs. Also, based on their material composition, SLNs are generally recognized as safe (GRAS) (Schwarz, 1999; Muller et al., 2000a; Wissing et al., 2004). A variety of biodegradable lipids can be used to prepare SLNs (e.g. triglycerides and lipid acids); in addition, SLNs work compatibly with many types of surfactants, such as lecithin, Tween 80, and Poloxamer 188. With all of these features, SLNs represent a promising delivery platform for cancer chemotherapy.

Several studies have reported SLNs' ability to protect anticancer drugs from degradation. Researchers have tested all-trans retinoic acid (Rachagani et al.) (Rachagani et al.) stability in SLN formulation (Lim et al., 2004). ATRA is known for its light, heat, and oxidant sensitivity, which upon degradation may yield less active compounds, such as all-trans-4-oxo (Brisaert et al., 2000). Following three months of storing ATRA-loaded SLNs at 4 °C, researchers discovered that over 90% of the ATRA remained active, compared to only 50% of the control (ATRA dissolved in a methanol solution) whithin only one month. A numbers of studies have investigated SLNs' ability to enhance the treatment outcomes of anticancer agents. Naguib et al. have shown an improved antitumor effect of Docetaxel-encapsulated SLN formulation (DCX-

SLN), which is composed of the high melting point lipid trimyristin, compared to free drug being dissolved in a Tween 80/ethanol solution both *in vitro* and *in vivo* (Naguib et al., 2014a). DCX-SLNs' average size was 180 nm. Also, they observed significant accumulation of DCX-SLNs in tumors 12 h after injection. Simultaneously, overall lower uptake by vital organs, especially the liver, has been reported. This study suggests that the SLN system may provide a favorable safety profile for DCX delivery. Another study adopted an active targeting technique, similar to liposomes, in SLNs formulation (Stevens et al., 2004). The researchers developed an SLN formulation equipped with a folate receptor targeting moiety (FR-SLNs) for the delivery of paclitaxel prodrug (paclitaxel-2′-carbonylcholesterol). Folate receptors are highly expressed in many types of cancer. FR-SLNs exhibited much higher cell uptake with a greater cytotoxic effect *in vitro* compared to non-targeted SLNs. Similarly, improved tumor growth inhibition was demonstrated for FR-SLNs compared to both non-targeted and paclitaxel in Cremophore El formulation in a FR (+) M109 tumor model.

SLNs also have been investigated for their potential to deliver genetic materials in cancer treatment. Jin et al. developed a cationic SLNs system for the delivery of PEGylated c-Met siRNA into brain tumors in a mouse model (Jin et al., 2011). The siRNA-PEG/SLNs complex was able to cross the blood brain barrier (BBB) and efficiently downregulate the c-Met gene without the association of systemic toxicity. Although the history of SLNs is relatively short compared to that of many NPs, significant progress toward SLNs development has been achieved. However, no clinical SLNs formulations are available on the market. Considering the features which SLNs may offer to overcome chemotherapy barriers, SLN formulation represents a flexible and promising delivery system which has attracted increasing attention in recent years.

Increased effort should be invested in this formulation to provide a safer and more efficient clinical alternative in the future.

These findings suggest that SLNs can be utilized to improve the targeting ability of NPs and provide a promising tool for the development of targeted therapy.

1.2. NOVEL FARNESYLTHIOSALICYLATE (FTS)-BASED SOLID LIPID NANOPARTICLES FOR IMPROVED TARGETED DELIVERY OF PACLITAXEL

1.2.1. Abstract

Paclitaxel (PTX) is one of the most commonly used chemotherapy agents approved to treat numerous types of cancers, including ovarian cancer, lung cancer, breast cancer, and several others. However, one serious limitation to PTX is its poor-water solubility (< 0.1 μg/mL). Thus, extensive research has been conducted to develop a formulation to deliver this drug effectively. Taxol®, a clinically approved formulation for PTX (Cremophor EL and dehydrated ethanol 1:1), solves the solubility issue of PTX; however, it causes many adverse reactions, such as severe hypersensitivity reactions, nephrotoxicity, neutropenia, and neurotoxicity. In this study, novel solid lipid nanoparticles (SLNs) which are able to solubilize PTX and simultaneously avoid unwanted side effects are introduced. SLNs are composed of a solid lipid core of farnesylthiosalicylate (FTS) conjugated to ethylene glycol. This lipid is solid at room temperature, with a melting point of 52 °C. FTS is a hydrophobic compound that acts as a potent and non-toxic Ras antagonist. The hydrophobic SLN core interacts with PTX, allowing for its encapsulation with a loading efficiency of ~90%. Moreover, PTX-SLN is surface-decorated with PEG_{5k} to enable the nanoparticles to avoid opsonization and circulate in the blood for a longer period of time. The PEGylated SLNs were spherical in shape with sizes ranging between 150 to 200 nm, as determined by Transmission electron microscopy (TEM) imaging and Dynamic Light

Scattering (DLS), respectively. The formulation is stable for 2 months at 4 °C. PTX-SLNs exhibited significantly improved release kinetics when compared to Taxol®. An *in vitro* cytotoxicity study using three cancer cell lines (PC3, DU145, and 4T1.2) showed comparable growth inhibition activity when compared to free PTX. The *in vivo* anticancer activity in the syngeneic murine breast cancer model suggests that PTX-SLNs significantly inhibit tumor growth much more effectively than Taxol formulation. Moreover, histological analysis revealed that PTX-SLNs caused more tumor cell apoptosis when compared to Taxol formulation. PTX-SLNs represent a promising delivery system for PTX, thereby highlighting the potential for improved therapeutic efficacy in cancer treatments while avoiding the hypersensitivity issues of Taxol.

1.2.2. Introduction

Paclitaxel (PTX) is one of the most commonly used chemotherapy agents, and is derived from the bark of *Taxus brevifolia* (northwest Pacific Yew Tree). PTX is an antimitotic drug, which interferes with cancer cell multiplication by stabilizing and preventing microtubules from the disassembly which leads to cell apoptosis (Wani et al., 1971). Paclitaxel has broad-spectrum applications in the treatment of various human malignancies, including breast, lung, and ovarian cancer. However, PTX's clinical use has been hindered by its very poor water solubility. Thus, extensive research has been conducted to develop a formulation for PTX delivery (Goldspiel, 1997; Xie et al., 2007; Duan et al., 2010b; Zhang et al., 2014c; Zhang et al., 2015b). Taxol is an FDA-approved paclitaxel formulation, which involve the use of 50:50 (v/v) mixture of Cremophor EL and dehydrated alcohol. It has solved the solubility issue of PTX. Unfortunately,

a major drawback of this formulation lies in its association with serious hypersensitivity reactions caused by Cremophor EL (Gelderblom et al., 2001a). Therefore, considerable effort is necessary in the development of safer PTX delivery alternatives free from Cremophor EL.

Solid lipid nanoparticles (SLNs) are a new generation of microemulsions in which liquid lipid is replaced by a sold lipid that has a melting point higher than room temperature. SLNs were first introduced in the early 1990s as an alternative delivery system to traditional carriers (Muller et al., 2000b; Mehnert and Mader, 2001). In recent years, increased attention has been directed toward SLNs due to their potential benefits over other carriers. The advantages of SLNs include enhanced stability, protection of carried drugs from chemical degradation, sustained release kinetics, enhanced bioavailability of encapsulated drugs, and scale up feasibility (Mehnert and Mader, 2001; Patel et al., 2014).

Recently, several studies have reported a variety of SLNs formulations composed of physiological lipids for anticancer drug delivery. However, most of these SLN excipients are biologically inactive (Mehnert and Mader, 2001; Radomska-Soukharev, 2007; Naguib et al., 2014b). The replacement of non-functional materials with therapeutically active ones in nanocarriers has been evidenced to provide potential synergy with loaded drugs. For instance, d-α-tocopheryl polyethylene glycol succinate (TPGS), consisting of vitamin E as part of the carrier, has demonstrated a dual function via synergizing with delivered anticancer drugs and enhancing their solubility (Zhang and Feng, 2006). Our group has recently developed additional dual functional micellar systems consisting of polyethylene glycol conjugated with hydrophobic drugs which possess other mechanisms of action (Huang et al., 2012; Zhang et al., 2013b; Lu et al., 2014a).

The aim of this study is to design a PTX-SLN formulation with dual functional ability using Ethylenegylcol-FTS_2 as the solid lipid, decorated with a layer of PEG_{5k}-FTS_2. FTS is a potent inhibitor of Ras-mediated signaling. Mutation in the Ras gene can lead to permanent activation of Ras proteins, resulting in the overactive Ras signaling which ultimately leads to cancer. This phenomenon is evident in over 20% of total human malignancies and 90% of pancreatic cancer. FTS functions via the dislodgement of Ras proteins from the plasma membrane, promoting its degradation (Marom et al., 1995a; Haklai et al., 1998b). We hypothesized that encapsulating PTX in an SLN formulation equipped with FTS, both in the core and inner phase of outer PEG coating, will provide strong stabilization of PTX within the formulation and enhance its loading efficiency. Moreover, this formulation may provide a potential synergy between drug and carrier that could enhance treatment outcomes in cancer therapy. The new PTX-SLNs were characterized with respect to their particle size, loading capacity, and *in vitro* drug release kinetics. *In vitro* and *in vivo* antitumor activity was also investigated.

1.2.3. Materials and methods

PTX (98%) was purchased from AK Scientific Inc. (CA, USA). FTS was synthesized according to the published literature (Marom et al., 1995a). Soy PC (95%) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 4-Dimethylaminopyridine (DMAP) was purchased from Calbiochem-Novabiochem Corporation (CA, USA). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Lonza (MD, USA), and fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Invitrogen (NY, USA). The following chemicals were purchased from Sigma-Aldrich (MO, USA): Ethylene glycol anhydrous, 99.8%, succinate

anhydride, diethanolamine, poly (ethylene glycol) methyl ether (MeO-PEG-OH, Mw = 5000 kDa), tween 80, poloxamer 188, trimyristin (TM), N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and Dulbecco's modified Eagle's medium (DMEM). All solvents used in this study were HPLC grade.

1.2.3.1. Cell lines and animals

4T1.2 (mouse metastatic breast cancer cell line), PC-3, and DU-145 (human prostate cancer cell lines) were cultured in DMEM supplemented with 10% FBS and 1% penicillin−streptomycin at 37 °C and incubated in a humid atmosphere with 5% CO2. Female BALB/c mice, 4−6 weeks in age, were purchased from Charles River (Davis, CA). All animals were housed under pathogen-free conditions according to AAALAC guidelines. All animal-related experiments were performed in full compliance with institutional guidelines and approved by the Animal Use and Care Administrative Advisory Committee at the University of Pittsburgh.

1.2.3.2. Synthesis of EG-FTS_2 lipid

EG-FTS_2 was synthesized via a conjugation reaction of the carboxylic terminal of FTS (2.2 eq) with both hydroxyl terminals of EG (1eq), in the presence of DCC as a coupling reagent and DMAP as a catalyst at 37 °C in DCM for two days. The reaction mixture was filtered through cotton and extracted in a mixture of ethyl acetate and 1N HCL. EG-FTS_2 was then purified by silica gel. EG-FTS_2 purity was confirmed by H^+ NMR, and the melting point was determined by DSC.

1.2.3.3. Synthesis of PEG_{5K}-FTS_2 conjugate

PEG_{5K}-FTS_2 conjugate was synthesized via solution condensation reactions from PEG methyl ether (mPEG-OH) with a molecular weight of 5000, following a previously reported method by Zhang et al. with modification (Zhang et al., 2013b). First, $mPEG_{5k}$-OH (1 eq) was reacted with succinic anhydride (5 eq) and DMAP (5 eq) in DCM at 70°C for 48 h. The PEG product was purified by washing with 10 volume of cold ethanol to remove excess DMAP, followed by precipitation in cold ether. This cycle was repeated three times, (yield = 91%). $mPEG_{5K}$−COOH (1eq) was then reacted with diethanolamine (3 eq) using NHS (3.6 eq) and DCC (3.6 eq) in DCM for 1 day. The derivatized polymer was purified as described in previous step and concentrated under vacuum. FTS (6 eq) was then conjugated with the two hydroxyl terminals in $mPEG_{5k}$-$(OH)_2$ (1 eq), aided by DCC (6 eq) and DMAP (0.2 eq), dissolved in DCM and allowed to react for 1 day at room temperature. The solution was filtered and purified with three cycles of ethanol/ether as mentioned above and concentrated under vacuum.

1.2.3.4. Preparation of SLNs

The PTX-SLNs were prepared according to a modified emulsion/solvent evaporation method (Ye et al., 2008; Naguib et al., 2014b). Briefly, PTX, EG-FTS_2, SPC, and PEG_{5K}-FTS_2 in a weight ratio of 1:10:5:2.5 were dissolved in 100 µL DCM in a glass tube. One mL of Poloxamer 188 aqueous solution was then added and the mixture was sonicated in ice with a probe sonicator at 6 W for 30 min. The emulsion was then stirred at 600 rpm in an oil bath at 70°C for another 30 min to allow for lipid melting, mixing with other ingredients, and evaporation of DCM. SLNs were stirred at room temperature for 30 min, and placed in a vacuum

pump to remove remaining traces of DCM. SLNs were then sonicated for 10 min to be clear of aggregates caused by vacuum pressure, followed by lyophilization with 9.25% (w/v) sucrose as cryoprotectant. The final form of PTX-SLNs was obtained by reconstitution of the resulting lyophilized powder in 1 mL DPBS. The drug-free SLNs were similarly prepared as described above.

1.2.3.5. Characterizations of SLNs

1.2.3.5.1. Particle size analysis

The particle size of SLNs was determined using a Zetasizer (DLS) (Zetasizer Nano ZS instrument, Malvern, Worcestershire, UK). Briefly, 20 μL of SLN suspension was diluted to 1 mL with water, and particle size was measured at room temperature.

1.2.3.5.2. Determination of drug content, entrapment efficiency (EE) and loading capacity (LC)

To determine the drug loading efficiency of PTX-SLNs, 10 μL was dissolved in 0.5 mL methanol, vortexed, and centrifuged at 12000 rpm at 4°C for 10 min. The supernatant was collected and drug content was quantified by high performance liquid chromatography (HPLC) (Alliance 2695− 2998 system). The reverse-phase Lichrospher 100 RP-18 (5 μm) column was used, and the mobile phase consisted of methanol/water (65:35 v/v). The flow rate was set to 0.5 mL/ min and the detection wavelength was 227 nm. Drug entrapment efficiency (EE) and loading capacity (LC) were calculated as follows:

EE (%) = (weight of loaded drug/weight of input drug) × 100%

LC (%) = [(weight of drug loaded/ (weight of SLN + drug used)] 100%

1.2.3.5.3. Differential scanning calorimetry (DSC)

DSC thermal analysis was performed using a Mettler Toledo DSC. Lyophilized samples of PTX, EG-FTS_2, drug-free SLNs, and PTX-SLNs were accurately weighed (3 to 8 mg) and sealed in an aluminum crucible. The crucible was placed in the DSC device and purged with inert nitrogen gas at a rate of 50 mL/min. An empty crucible was used as a reference. Thermograms were obtained by heating samples from 35°C to 230 °C at a heating rate of 5 °C/min.

1.2.3.5.4. Surface morphology by TEM

The morphology of SLN formulation was examined for both drug-free and PTX-loaded forms via TEM imaging. SLNs samples were stained using a copper grid with Formvar immersed in a drop of sample solution and stained with 1% uranyl acetate. Imaging was performed at room temperature on JEOL JEM-1011.

1.2.3.5.5. Stability

Freshly prepared SLNs were stored in parafilm-sealed glass tubes at 4°C. Changes in sizes were monitored by DLS.

1.2.3.6. *In Vitro* release of PTX from SLNs

The kinetics of PTX release from the PTX-SLNs formulation was examined following the method by Zhang et al. (Zhang et al., 2013b). Briefly, 2 mL PTX-SLN emulsion or Taxol solution (1 mg/ mL) was placed into a dialysis bag (MWCO = 12 kDa, Spectrum Laboratories).

The dialysis bag was placed in a beaker with 200 mL DPBS (pH=7.4) containing 1% (w/v) Tween 80 (reported PTX solubility in this medium is 13.8 μg/mL) (Kilfoyle et al., 2012). The beaker was incubated at 37 °C in an oven and agitated using an orbital shaker at 100 rpm. At a predetermined time points, samples were withdrawn from inside the bag and replaced with an equivalent volume to maintain sink condition. PTX concentrations were quantified by HPLC (Alliance 2695–2998 system). The reverse-phase Lichrospher 100 RP-18 (5 μm) column was used and the mobile phase consisted of methanol/water (65:35 v/v). The flow rate was set at 0.5 mL/min and the column effluent was detected at 227 nm with a UV/vis detector. Values were reported as the means from triplicate samples.

1.2.3.7. *In vitro* cytotoxicity study

To investigate the cytotoxicity of the PTX-SLNs formulation, DU145, PC-3, or 4T1.2 (2000 cells/well) were seeded in 96-well plates. Cells were incubated overnight in 10% FBS and 1% streptomycin−penicillin, and media was replaced with new media containing various PTX formulations of indicated concentrations and cells were continuously cultured for 72 h. Then, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) in DPBS was added to each well and cells were further incubated for 3.5 h. The medium was gently removed and DMSO was added to solubilize MTT formazan, resulting in a purple-colored solution. Absorbance was quantified by a microplate reader at a wavelength of 550 nm and reference wavelength at 630 nm. Untreated groups served as controls. Cell viability was calculated as $[(OD_{treat} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%]$.

1.2.3.8. *In vivo* antitumor activity of PTX-SLNs

The *in vivo* tumor growth inhibition effect of PTX-SLN was evaluated using a syngeneic murine breast cancer model (4T1.2). Female BALB/c mice were inoculated subcutaneously at the upper right flank with 200 μL (1×10^5) 4T1.2 cells. The day when tumor volume reached ~50 mm^3 was designated as "day 1". Mice were randomly divided into five groups (n = 4) and administered with i.v. injections of DPBS (control), drug-free SLNs, Taxol, PTX-EG, PTX-TM (FTS-free SLNs formulated with trimyristin lipid and PEG_{5K}-OA_2 instead of EG-FTS_2 lipid), and PEG_{5K}-FTS_2, respectively. The PTX dosage was 10 mg/kg body weight. Injections were repeated on day 3, 5, 8, and 11. Drug-free SLNs were given at an equivalent dosage of carrier in the PTX-EG group. The tumor sizes were monitored three times a week using a digital caliper and the tumor volumes were calculated using the following equation: $(L \times W^2)/2$, where L is the longest and W is the shortest diameter in tumor (mm). On day 18, mice were euthanized and tumor tissues were harvested, weighed, and fixed in a formalin buffer for histological examinations.

1.2.3.9. Histological analysis

Tumors collected from mice were fixed in 10% formalin at 4°C for two days. Each sample was cut and gradually dehydrated in ethanol and xylene and then embedded into paraffin. The paraffin-embedded tumor samples were sectioned into slices at 6 μm using an HM 325 Rotary Microtome, and stained with hematoxylin and eosin (H.E).

1.2.3.10. Statistical Analysis

In all statistical analyses, the significance level was set at a probability of $P < 0.05$. All results were reported as the mean ± standard error (SEM) unless otherwise indicated. Statistical analysis was performed using Student's t test for two groups, and one-way ANOVA for multiple groups, followed by the Newman-Keuls test if $P < 0.05$.

1.2.4. Results

1.2.4.1. Preparation and characterization of SLNs

After successfully synthesizing FTS-based lipid conjugates (EG-FTS_2), the structure was confirmed by ^1H NMR. Additionally, DSC confirmed the lipid melting point at 52.89 °C. SLNs were prepared using an emulsion/solvent evaporation method. The emulsion was composed of PTX, EG-FTS_2, SPC, and PEG_{5K}-FTS_2 dispersed in the aqueous solution Poloxamer 188. The LC%= 5.4%. DLS measurements indicate that drug-free SLNs and PTX-SLNs had an average size of ∼150 nm with PDI = 0.156 and ∼190 nm with PDI = 0.239, respectively. Moreover, TEM observations showed spherical nanoparticles and the sizes on TEM were consistent with those determined by DLS (Figure 4).

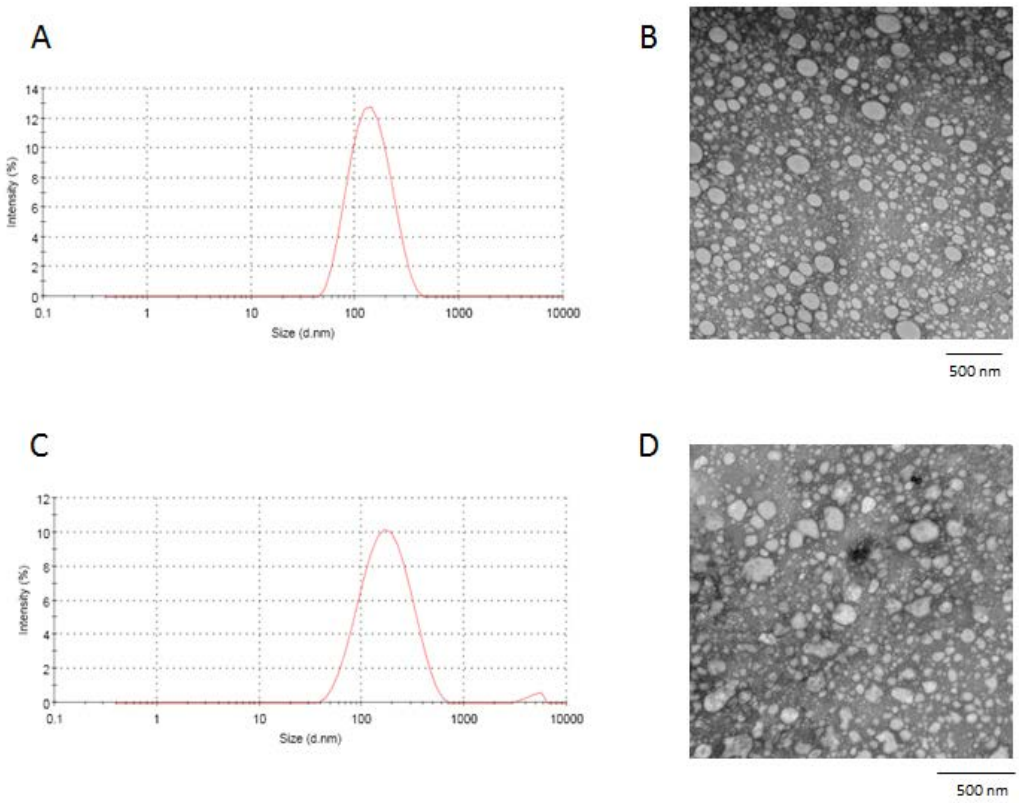

Figure 4. Particle size distribution of SLNs.

Drug-free SLNs (A) and PTX-SLNs (C) as determined by DLS. TEM images of drug-free-SLNs (B) and PTX-SLNs (D).

DSC was used to analyze the physical state of core lipids in SLN formulations (Figure 5). DSC thermograms of PTX revealed a characteristic endothermic melting peak at 217.5°C. This peak completely disappeared in PTX-SLNs formulation, indicating the presence of PTX in the core of SLN formulations. In addition, thermograms indicated sharp endothermic peaks of EG-FTS2, drug-free SLNs, and PTX-SLNs at 52.89°C, 52.28°C, and 52.15°C, respectively. These peaks indicate the presence of lipids in solid crystalline nature, and confirm the solid state of lipids within SLNs.

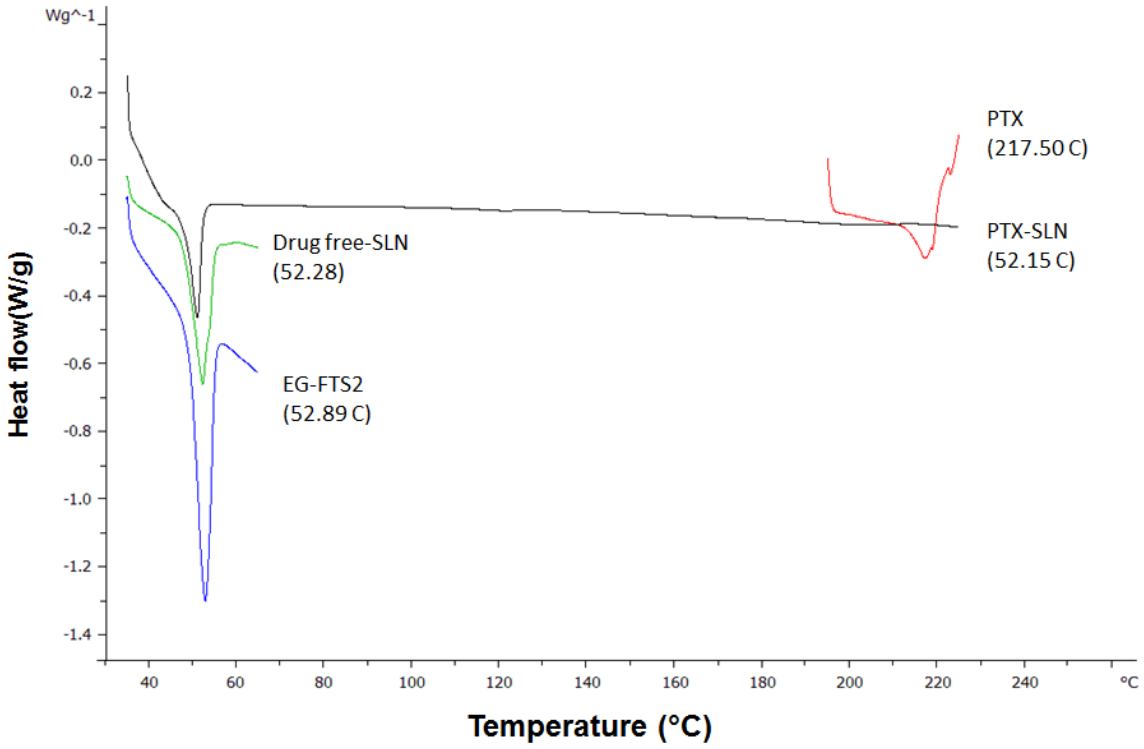

Figure 5. DSC thermograms.

PTX [red], PTX-SLNs [black], Drug free-SLNs [green], and EG-FTS2 bulk lipid [blue].

The SLNs stability was followed for two months. Samples were stored at 4°C and the particle size distribution of both drug-free and PTX-SLNs were measured daily. As shown in Figure 6, no obvious changes in size were found for both formulations, indicating an excellent stability PTX-SLNs at 4°C.

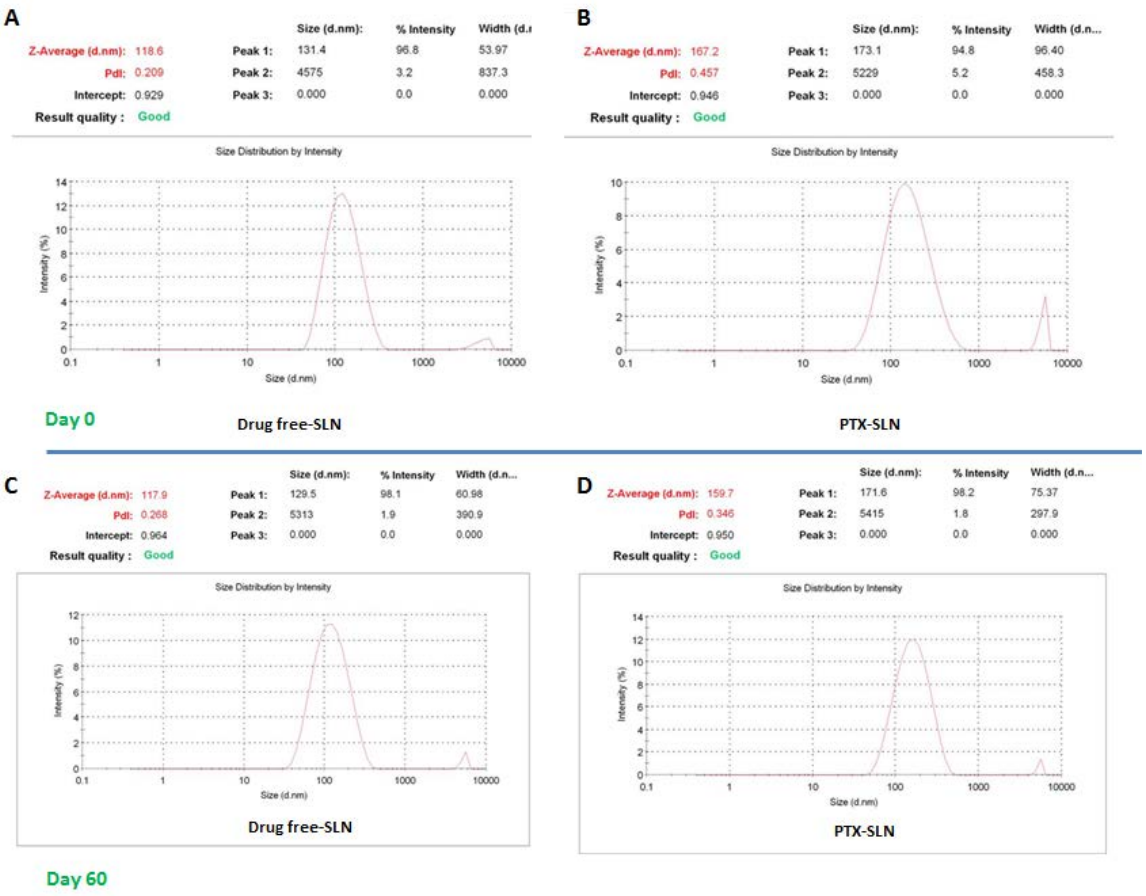

Figure 6. Size distribution of SLNs.

Freshly prepared drug free- SLN (A) and PTX-SLN (B) and their sizes after 60 days (C) and (D), respectively.

1.2.4.2. *In vitro* release kinetics of PTX from SLNs

The release kinetics were examined via a dialysis method and compared to Taxol formulation as a control. As shown in Figure 7, ~5% of PTX was released from the SLN formulation in the first hour, whereas ~35% was released in the Taxol formulation. Twenty-four h later, only ~25% was released from PTX-SLNs compared to 77.5% released from Taxol. This release pattern of PTX-SLNs may suggest strong PTX-EG lipid interaction and the ability of the SLN formulation to avoid burst release of PTX, as seen in Taxol. Moreover, PTX-SLNs demonstrated significantly slower kinetics of PTX release throughout the entire 8 day period. At

the end of the experiment, ~88% was released from the PTX-SLNs formulation. In contrast, a similar percentage was found in Taxol formulation in as early as 2 days.

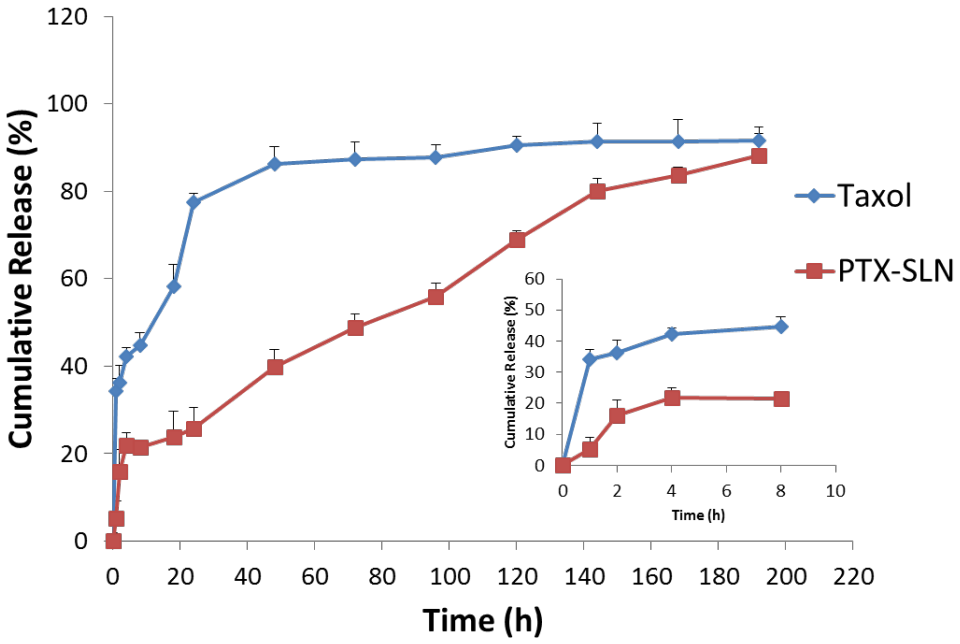

Figure 7. PTX release profile from PTX-SLNs and Taxol.

PTX concentration was 1 mg/mL in both Formulations. Release medium was DPBS (pH 7.4) containing 1% (w/v) Tween 80.

1.2.4.3. *In vitro* cytotoxicity study

The cytotoxicity was investigated in three cancer cell lines: two human prostate cancer cell lines (PC-3 and DU145) and one murine breast cancer cell line (4T1.2). Figure 8 presents the cytotoxicity of free PTX compared to PTX-SLNs in cancer cells. The data reveals that PTX-SLNs exhibited a comparable growth inhibition effect in PC3 cells (Figure 8A), and a slightly improved effect in DU-145 (Figure 8B) and 4T1.2 (Figure 8C). Drug free-SLNs showed minimal cytotoxicity at similar concentrations used in PTX-SLNs (data not shown).

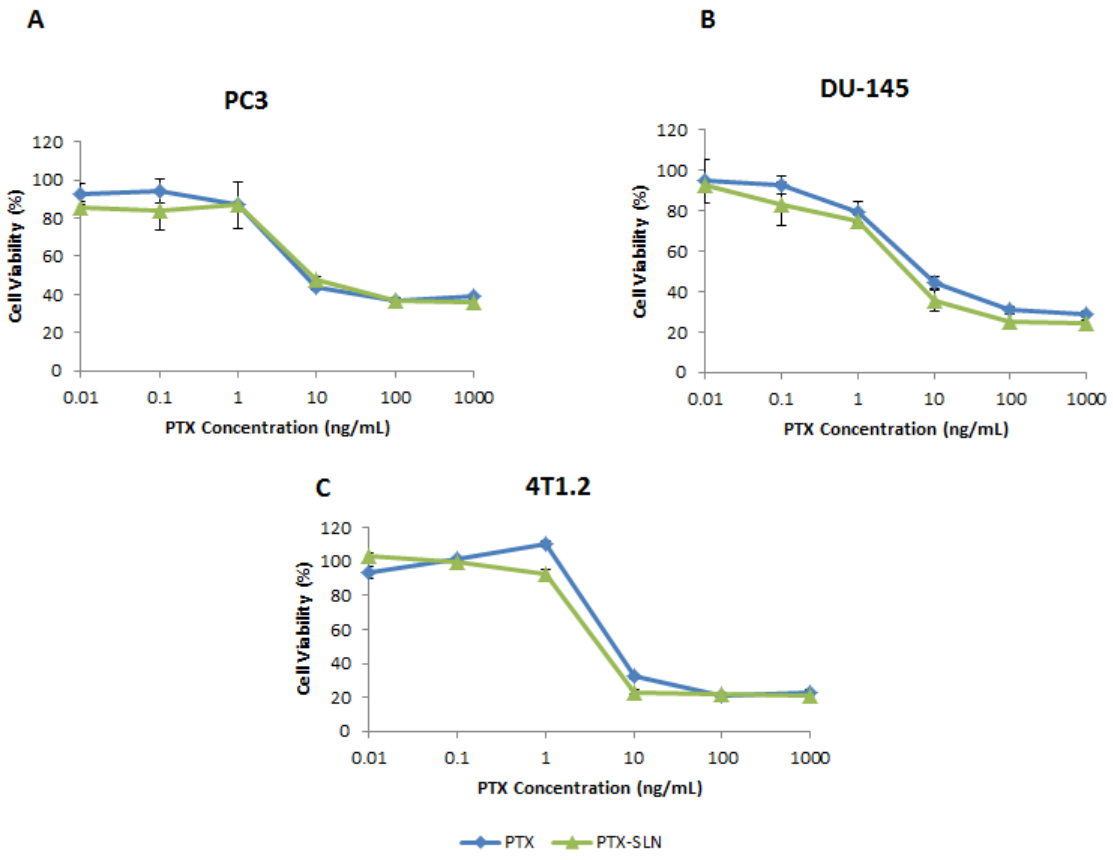

Figure 8. Cytotoxicity of free PTX and PTX-SLNs *in vitro*

Human prostate cancer cell lines, PC-3, IC_{50}= 7 and 9 ng/mL, respectively (A) and DU-145, IC_{50}= 4.9 and 9 ng/mL, respectively (B), and mouse breast cancer cell line 4T1.2, IC_{50}= 4 and 6 ng/mL, respectively (C).

1.2.4.4. *In vivo* antitumor activity in a mouse model

The *in vivo* tumor growth inhibition activity was evaluated in a syngeneic murine breast cancer model (4T1.2). As shown in Figure 9A, at a PTX dosage of 10 mg/kg (Taxol, TM-SLNs, and EG-SLNs) all exhibited significant inhibition of tumor growth when compared to the control group. However, PTX-EG SLNs have shown a clear improvement in antitumor activity when

compared to Taxol and PTX-TM formulations, which showed comparable antitumor effect. Interestingly, drug free-SLNs alone also exhibited moderate antitumor activity although less effectively than PTX-loaded SLNs. This effect may be attributed to the accumulation of FTS already present in the formulation at tumor sites. Figure 9B show the weights of tumors removed at the end of experiments, which were consistent with the data in tumor growth curves (Figure 9A). No changes in body weight were noticed among all treatment groups (Figure 9C).

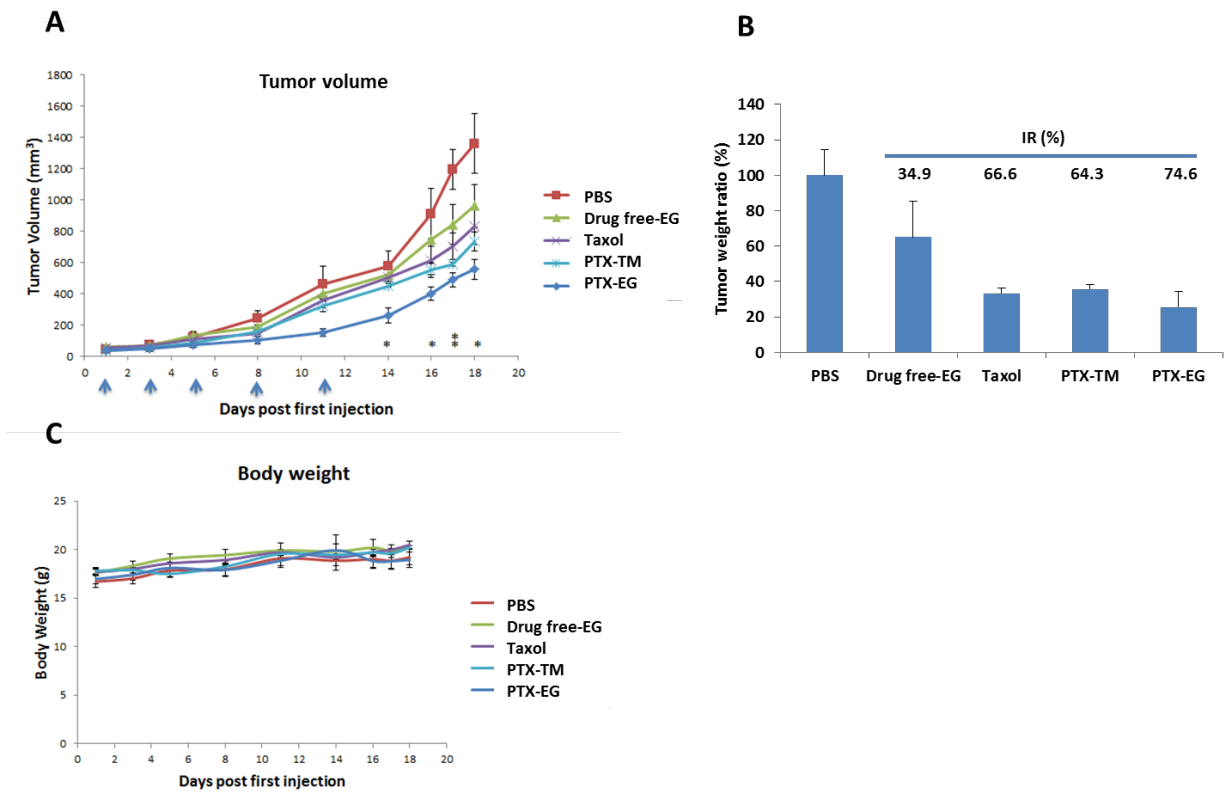

Figure 9. ***In vivo*** **antitumor activity in a mouse model**

(A) *In vivo* antitumor activity of PTX-SLNs. BALB/c mice (n = 4) were inoculated s.c. with 4T1.2 cells (2×10^5 cells/mouse). One week later, mice were treated with various agent indicated above on days 1, 3, 5, 8, and 11, and tumor growth was monitored and plotted tumor volume (mm^3). *P <0.05 (PTX-EG vs. Taxol). **P <0.01 (PTX-EG vs. Taxol). **(B)** body weight changes in all groups. **(C)** Tumors of different groups were excised at the end of experiment. Average weight were measured and plotted, and tumor growth inhibition rate (IR) was calculated as : (1 − (mean tumor weight of drug treated group/mean tumor weight of saline treated group)) * 100%

1.2.4.5. Histological analysis

To further illustrate the therapeutic effect of PTX-SLNs, histological analysis was conducted using H.E staining. Upon completion of the experiment, tumor samples were excised, fixed into paraffin wax, and thin slices were stained and observed under a light microscope. As depicted in Figure 10, tumors in DPBS-treated group consisted of tightly packed tumor cells, indicating uncontrolled tumor proliferation. A decreased tumor cell density with signs of apoptotic cells was observed in tumors treated with drug-free SLNs. In consistent with growth inhibition curves, PTX-SLNs exhibited the highest level of necrotic/apoptotic tumor cells when compared to Taxol and PTX-TM groups.

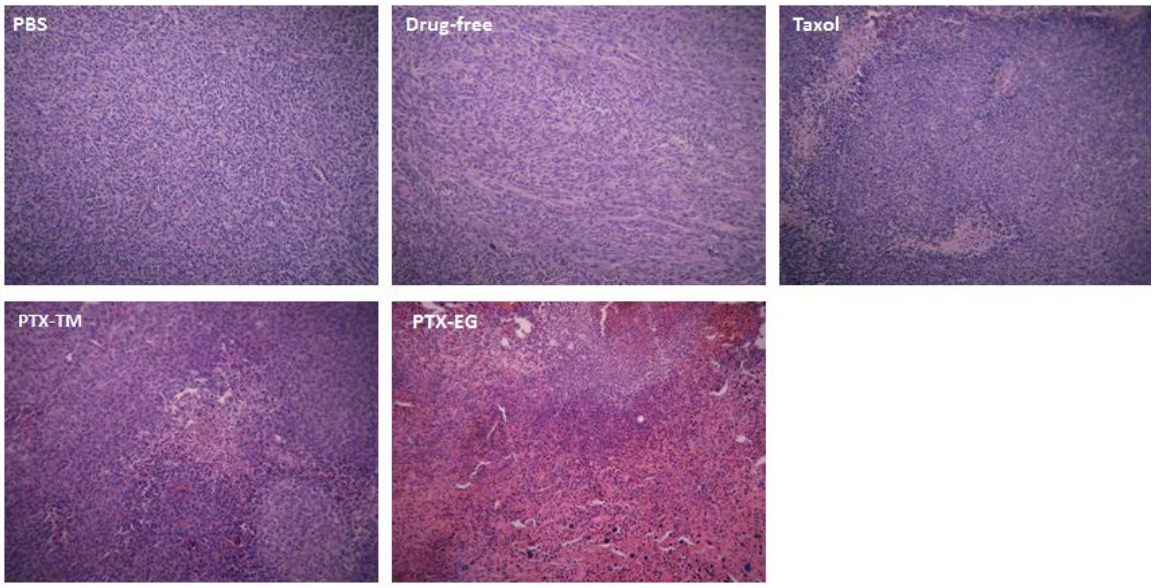

Figure 10. H.E staining of tumor tissues after completion of experiment (18 days)

1.2.5. Discussion

This study involved the development of a novel form of solid lipid nanoparticles which effectively solubilize PTX. EG-SLN formulation is composed of a synthetic EG-FTS_2 lipid as a solid core, and PEG_{5k}-FTS_2 conjugate as shielding molecule. Most importantly, this FTS-based material readily formulated SLNs which can load and deliver PTX.

Most reported SLNs formulations use physiological lipids such as pure glycerides or wax as solid core (Jenning and Gohla, 2000). Although these excipients are considered safe, they do not provide any beneficial therapeutic effect alone. On the other hand, these FTS-based SLNs act as a dual functional carrier that may achieve these goals. FTS is a potent and non-toxic Ras antagonist which is being extensively studied as a new agent for cancer therapy. FTS also exhibits an excellent safety profile among other chemotherapeutic agents (Kloog et al., 1999; Blum and Kloog, 2005; Haklai et al., 2008). In addition, several studies have reported that FTS may provide a synergistic effect for cancer treatment when combined with other anticancer agents (Biran et al., 2011; Mologni et al., 2012; Chen et al., 2014).

A unique advantage of EG-SLNs is their small particle size (150-200 nm). As reported in the literature, this size fits in the optimal size range (100-200 nm) for drug delivery systems, allowing for effective passive targeting via EPR effect (Li and Huang, 2008). Moreover, EG-SLNs presented an excellent stability profile for 2 months at 4°C, which may indicate strong interaction between PTX and FTS hydrophobic moieties present in both EG-FTS_2 and PEG_{5k}-FTS_2. The absence of PTX's characteristic melting peak in PTX-SLNs formulations, this is supported by the data from DSC study, indicates that PTX loses its crystallinity as a result of strong interactions between drug and carrier.

The release kinetics of PTX from SLNs was significantly slower compared to Taxol formulations (Figure 7). The $t_{1/2}$ of Taxol was 12.5 h, while the $t_{1/2}$ of PTX-SLNs was 76 h. The PTX-SLN formulation was also able to avoid early burst release when compared to Taxol. Additionally, PTX-SLNs exhibited a sustained release rate after the 24 h' time-point. The biphasic release behavior observed here (between 0-4 h and 4-200 h) may be attributed to the small fraction of PTX that is present on the surface of SLNs'core which might have relatively weaker interaction with the core. These data support the hypothesis that the incorporation of FTS in the core and outer layer of SLNs should provide strong drug/carrier interaction, leading to enhanced drug loading and stability. This interaction may be attributed to the hydrophobic interaction between PTX and FTS, along with π–π stacking of benzene rings of both compounds, and hydrogen bonding mediated by the acyl groups of FTS. More studies are needed to further elucidate the mechanism of interaction between drug and carrier.

In vitro cytotoxicity in three cell lines (PC3, DU-145, and 4T1.2) indicates that PTX-SLNs retains its anticancer activity by showing similar or slightly higher killing effect when compared to free PTX dissolved in DMSO. This suggests that PTX-SLNs were effectively taken up by cells. Considering the slow kinetics of drug release, perhaps not all of PTX was released from SLNs during the short period of cytotoxicity study. Therefore, the cytotoxic effect of PTX-SLNs might be underestimated.

An aggressive metastatic murine breast cancer model (4T1.2) was used to study the therapeutic effect of PTX-SLNs *in vivo*. PTX-TM SLNs formulation was included as an inert carrier to study the contribution of PTX to overall therapeutic effect of PTX-loaded SLNs in the formulation. PTX-TM SLNs consists of trimyristin, a solid lipid with a melting point of 57 °C,

which is coated with PEG_{5k}-OA_2. PTX-SLNs exhibited improved anticancer activity when compared to Taxol and PTX-TM formulations, although these results are not statistically significant. This is likely due to enhanced antitumor activity caused by FTS incorporation in the system. FTS improves drug stability within SLN formulations, which improves tumor delivery, leading to enhanced bioavailability. Also, FTS may synergize with PTX which may contribute to the overall anticancer activity. Finally, PTX-SLNs were well tolerated in mice as evidenced by a similar body weight when compared to control mice.

1.2.6. Conclusion

We have successfully developed FTS-based SLNs that efficiently solubilize and deliver PTX. PTX-SLNs formed spherically shaped NPs with small particles sizes (< 200 nm) and demonstrated an excellent stability at 4°C. Moreover, this system exhibits a sustained kinetics of drug release and improved *in vivo* antitumor activity when compared to Taxol. PTX-SLNs may represent a promising SLN system that could avoid the hypersensitivity issue of current formulations, and provide a potential synergy with co-delivered anticancer drugs in overall therapeutic activity. Additional studies are required to better understand the mechanism of interaction between drug and carrier for future carrier improvements.

1.3. CONCLUSION AND PERSPECTIVES

The battle against cancer represents one of greatest challenges in modern history. It is expected that cancer may surpass heart diseases as the leading cause of death in the United States within the coming years. However, our strategies to face this fight are developing rapidly, and knowledge regarding cancer is growing due to the ongoing advancement of medical tools. When compared to other classes, chemotherapeutic drugs, especially cytotoxic agents, are the most reactive, toxic, and unstable options due to their diverse structures and physicochemical properties. The application of nanotechnology has greatly overcome many challenges of chemotherapy agents and maximized their potential therapeutic benefits. The development of NP delivery systems has grown over the past decades. This development is expected to have a significant impact on future cancer therapy due to more favorable tumor-specific targeting and controlled release of payload drugs.

Given its unique flexibility, an increased amount of research has been devoted to the study of SLNs system over the past decade. In this chapter, we have experimentally demonstrated that SLNs can be further rendered dual-functional via incorporation of a FTS-based solid lipid. The drug release rate and *in vivo* anticancer activity have been significantly improved when compared to the clinically available PTX formulation (Taxol). Future improvement of SLN systems may result in the development of new generation of anticancer nanocarriers that outperform the ones currently being used. SLN improvement may include the development of dual functional nanocarriers that work synergistically with anticancer agents. Also, decorating NPs with both PEG and a tumor-specific ligand in a more effective manner will achieve higher tumor targeting ability. Moreover, further investigations into the mechanism of

interaction between carrier and drug, along with *in vivo* carrier behavior, will enrich overall understanding and improve the formulation capabilities for clinical applications.

2. THYMOQUINONE TARGETED THERAPY FOR LIVER FIBROSIS

2.1. INTRODUCTION

Liver fibrosis is defined as an excessive accumulation of extracellular matrix proteins (ECM) in response to acute or chronic liver injury. Progression of liver fibrosis leads to a more serious condition called cirrhosis, where most of the liver cells are replaced by scar tissues and ultimately resulting in organ failure. Liver cirrhosis is currently the 12th leading cause of death in the United States, accounting for 32,000 deaths per year (Murphy et al., 2013). In the past, liver fibrosis was considered an irreversible condition due to hepatic parenchyma collapse and the massive accumulation of collagen scarring. Currently, liver fibrosis is recognized as wound healing model response (Benyon and Iredale, 2000a). In acute liver injury, the healing response can completely recover liver damage in a relatively short time, even if a large portion of the liver is destroyed. However, in chronic injury, the liver experiences repetitive damage which causes a loss of regenerative capacity and chronic wound healing response, which includes necrosis and/or apoptosis of parenchymal cells and leads to their replacement by scar tissue. The wound healing process is beneficial in acute injuries, yet, it becomes a pathogenic condition in chronic injures if it continues without treatment (Bataller and Brenner, 2005; Lee et al., 2015). In industrialized countries, the most common causes of liver fibrosis are hepatitis C virus (HCV), heavy alcohol consumption, and nonalcoholic steatohepatitis (Friedman, 2003a). Other diseases,

such as autoimmune diseases, drug-induced liver disease, and trauma can also contribute to the incidence of liver fibrosis (Friedman, 2003a; Rockey and Friedman, 2007). Currently, the only effective treatment is the removal of the underlying cause, such as eradication of viral infection, or immunosuppressive treatment of autoimmune liver disease (Hammel et al., 2001; Kweon et al., 2001; Poynard et al., 2002; Farci et al., 2004). An accumulating amount of evidence shown in animal studies supports the concept that liver fibrosis and cirrhosis are reversible conditions (Dufour et al., 1997a; Iredale et al., 1998; Benyon and Iredale, 2000a). However, the chances of reversibility are much higher in fibrosis, and the possibility of resolution becomes increasingly difficult as the disease progresses toward cirrhosis (Friedman, 2003a). Therefore, focusing on the development of anti-fibrotic therapies holds huge promise toward finding a cure for the disease, considering the long progression time (an estimated 20-30 years) until the development of cirrhosis (Benmanov and DiMartino, 2000; Thein et al., 2008).

Following liver injury, major changes take place in the liver microenvironment, resulting in changes in the phenotype of all liver cells. Among the cells contributing to the production of extracellular matrix proteins (ECM) following liver insult, hepatic stellate cells (HSCs) represent a key fibrogenic effector cell type, whose significant role in the pathology of liver fibrosis has been well clarified. Thus, these are the target cells for anti-fibrotic therapies (Wu and Zern, 2000; Fallowfield, 2011; Mederacke et al., 2013). HSCs, also known as perisinusoidal cells or Ito cells, reside in the space of Disse (a small area between the sinusoids and hepatocytes). Under normal physiological conditions, HSCs (referred as quiescent HSCs), which represent ~15% of the total liver cells population, are considered the principle storage site for vitamin A (Wang, 1999; Bataller and Brenner, 2005; Friedman, 2008). Also, these cells perform a key role in the maintenance of steady-state levels of basement membrane-like matrix, in addition to the

regulation of hepatic blood flow and portal venous pressure (Moreira, 2007b). After chronic injury exposure to the liver, inflammatory lymphocytes infiltrate liver parenchyma, and some injured hepatocytes undergo apoptosis. Apoptotic hepatocytes release cytokines and reactive oxygen species, which in turn activate liver macrophages (Kuppfer cells) to release pro-inflammatory factors such as transforming growth factor-(TGF-β), platelet-derived growth factor (PDGF), and tumor necrosis factor-α (TNF-α) (Wu et al., 1998). These changes promote the HSCs to undergo dramatic phenotypic transformation, termed activation, into myofibroblast-like cells. Activated HSCs lose their vitamin A content and transition into proliferative, fibrogenic, proinflammatory, and contractile cells which determine the overproduction of ECM (mainly collages type I and III) (Iredale et al., 1998; Friedman, 2004). The contractile feature also plays pivotal role in promoting portal hypertension, which facilitates further progression toward cirrhosis. During the resolution of liver fibrosis, HSCs may revert back to the quiescent state or undergo selective clearance by apoptosis (Iredale et al., 1998; Friedman, 2004). Therefore, agents that inhibit or interfere with the activation process or selectively induce apoptosis are of great interest for their significant impact on the treatment of liver fibrosis.

2.2. OVERVIEW OF TQ THERAPEUTIC APPLICATIONS

Due to the limitations of conventional medicine represented in severe and unwanted side effects, increased attention has shifted toward the discovery of therapeutic compounds with natural origins. Over the last decades, the focus on discovering medicinal plants has increased considerably in an effort to find replacements for synthetic drugs, or at least serve as adjunctive therapy to reduce the toxicity of primary drugs. Among these promising plants is *Nigella sativa* (or black seed). This species has traditionally been known to treat a variety of diseases for over 2000 years (Yi et al., 2008). Moreover, it is more commonly used as a condiment and spice in middle and far eastern countries. Thymoquinone (2-isopropyl-5-methylbenzo-1, 4-quinone) (TQ) is the active component of *Nigella sativa* and constitutes ~ 30% of its volatile oil (Al-Ali et al., 2008b)(see TQ structure in Figure 11). Since the extraction of TQ (el-Dakhakhny, 1965), numerous research has been conducted to explore its therapeutic potential for many diseases (Houghton et al., 1995; Badary et al., 1997; Mutabagani and El-Mahdy, 1997; Nagi et al., 1999b; Badary and Gamal, 2000). TQ exhibited a wide spectrum of biological activities in inflammatory diseases and cancer. TQ holds great potential as an antioxidant agent; in addition, it has array of favorable effects, including anti-inflammatory (Mutabagani and El-Mahdy, 1997), antitumor (Majdalawieh et al., 2010; Aikemu et al., 2013), hepatoprotective (Al-Suhaimi, 2012; Talib and Abukhader, 2013), antidiabetic (Fararh et al., 2005; Al Wafai, 2013), and antibacterial activities (Kokoska et al., 2008; Bakathir and Abbas, 2011). TQ mechanisms of action are complex due to multiple activities having been observed in a wide range of diseases. The antioxidant ability of TQ resides in its scavenging effects against reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical, and singlet molecular oxygen, which are elevated in various diseases (Nagi and Mansour, 2000; Mansour et al., 2002). In cancer, TQ shows promising antitumor

activity in multiple cancer types, with the advantage of minimal toxicity against normal cells (Shoieb et al., 2003). Additionally, TQ has demonstrated inhibition of breast cancer *in vitro,* and enhanced the antitumor activity of doxorubicin and 5-florouracil when combined with TQ. These studies have revealed the anticancer activity of TQ through enhanced PPAR-gamma activity and downregulation of several pro-apoptotic genes, including Bcl-2, Bcl-xL, and survivin (Woo et al., 2011). Moreover, a number of studies have reported TQ's hepatoprotective and anti-fibrotic effects *in vitro* and *in vivo* (Badary et al., 1999; Nagi et al., 1999b; Nagi et al., 2010; Bai et al., 2013a). Daba and Abdel-Rahman showed that TQ possessed hepatoprotective properties similar to those seen in Silybin (part of the Silymarin flavonoid complex), a known hepatoprotective agent with antioxidant properties. TQ protected isolated rat hepatocytes against tert-butylhydroperoxide (TBHP)-induced toxicity, as evidenced by the inhibition of intracellular glutathione depletion, protection of cell membrane integrity, and prevention of alanine transaminase (ALT) and aspartic transaminase leakage (Daba and Abdel-Rahman, 1998; Kloosterman and Plasterk, 2006). Bai et al. explored the anti-fibrotic activity of TQ in a thioacetamide-induced liver fibrosis mouse model. TQ substantially attenuated liver fibrosis progression and remarkably decreased ECM accumulation. This study indicated that TQ can negatively regulate the expression of toll-like receptor 4 (TLR4), inhibit phosphatidylinositol 3-kinase (PI3K) phosphorylation, and activate the LKB1-AMPK signaling pathway (Bai et al., 2014). Amein et al. studied lethal dose (LD_{50}) of TQ in rodents. The reported LD_{50} of TQ in experimental animals was as follows: in mice, 104.7 mg/kg, injected intra-peritoneally (i.p.), and 870.9 mg/kg orally; and in rats, 57.5 mg/kg injected i.p., and 794.3 mg/kg orally. These values are 10-15 times greater than the reported i.p. dose for antioxidant, anti-inflammatory, and anti-cancer activity, and 100-150 times greater for oral dose. These data suggest that TQ is relatively

safe in experimental animals, especially when administered orally (Al-Ali et al., 2008a). Moreover, Al-Amri and Bamosa (2009) conducted a phase I study in 21 patients with solid tumors or hematological malignancies for oral TQ. They reported no significant systemic toxicities; with marginal decreases in tumor markers of no more than 25% of baseline levels. This study also discovered that up to 2600 mg/day of TQ was tolerable (Al-Amri and Bamosa, 2009). In general, these studies suggest that TQ is one of promising candidates for liver fibrosis therapy with wide margin of safety in humans.

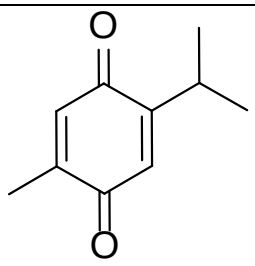

Figure 11. Chemical structure of Thymoquinone (TQ)

2.3. ANTI-FIBROTIC EFFECT OF THYMOQUINONE ON HEPATIC STELLATE CELLS

2.3.1. Abstract

Hepatic stellate cells (HSCs) are the major cells involved in the production of the extracellular matrix in liver. Following liver injury, HSCs undergo a transdifferentiation process from a quiescent state to an activated state, which plays an important role in liver fibrosis. Previous studies have indicated that thymoquinone (TQ) might have a protective effect against liver fibrosis in animal models; however, the underlying mechanism of action is not fully understood. The aim of this study is to examine whether TQ has any direct effect on HSCs. The results of this study indicate that pretreatment of mice with TQ has a protective effect against CCl_4-induced liver injury when compared to the control (untreated) group; this is consistent with previous studies. Moreover, an *in vivo* study indicated that COL1A1 and α-SMA mRNA levels were significantly downregulated by TQ treatment. Similarly, an *in vitro* study confirmed that TQ downregulated COL1A1, COL3A1, and α-SMA mRNA levels in activated rat HSCs and LX2 cells. Pretreatment with TQ also inhibited the LPS-induced proinflammatory response in LX2 cells, as demonstrated by the reduced mRNA expression of IL-6 and MCP-1. Mechanistically, inactivation of the NF-κB pathway is likely to play a role in the TQ-mediated inhibition of proinflammatory response in HSCs. Finally, this study indicates that TQ inhibits the culture-triggered transdifferentiation of freshly isolated rat HSCs, as shown by the significant downregulation of mRNA expression of several fibrosis-related genes. In conclusion, this study suggests that TQ has a direct effect on HSCs, which may contribute to its overall anti-fibrotic effect.

2.3.2. Introduction

Liver fibrosis is one of the leading causes of morbidity and mortality worldwide (Moreira, 2007a). Liver fibrosis results from an excessive accumulation of extracellular matrix proteins, which represents the liver's response to injury. Liver fibrosis is not an independent disease, but rather an outcome of many chronic liver diseases, such as HCV infection, alcohol abuse, and nonalcoholic steatohepatitis (NASH) (Friedman, 2003b; Bataller and Brenner, 2005). Untreated liver fibrosis results in progression to cirrhosis, liver failure, and portal hypertension (Bataller and Brenner, 2005). Importantly, strong evidence now exists to support the concept of liver fibrosis being a reversible condition (Dufour et al., 1997b; Benyon and Iredale, 2000b; Friedman and Bansal, 2006b). Therefore, the chances of reversibility at the fibrosis stage are much higher than if the condition had progressed to cirrhosis (Friedman, 2003b).

Currently, no standard treatment exists for liver fibrosis. Removal of the causative agent is still considered the most effective therapy (Bataller and Brenner, 2001; Davis et al., 2003; Cheng and Mahato, 2007). Within the past decade, a significant amount of progress has been made in comprehending the underlying mechanisms of liver fibrosis. It has been well established that hepatic stellate cells (HSCs) play a central role in liver fibrosis (Bataller and Brenner, 2001). Following liver injury, HSCs undergo an "activation" or transdifferentiation process from quiescent vitamin A storing cells to myofibroblast-like cells. This process leads to notable changes in the phenotypic features of HSCs, including: increased expression of α-SMA (the protein involved in cell motility and contractility), loss of retinoid-storing capacity, enhanced cell migration and adhesion, increased proliferation, production of chemotactic proteins, enrichment of the rough endoplasmic reticulum, and acquisition of fibrogenic capacity (Moreira, 2007a; Atzori et al., 2009).

Thymoquinone (TQ) is the main active constituent of *Nigella sativa* plant oil, also known as black seed or black cumin(Sayed, 1980). TQ is commonly used as a food additive, and has been known for its ability to prevent and cure many diseases for centuries (Al-Ghamdi, 2003). Several studies have shown that TQ has many pharmacological effects, including antioxidation and hepatoprotective effects against hepatotoxins (Nagi et al., 1999a; Mansour, 2000; Ragheb et al., 2009; Woo et al., 2012). For instance, pretreatment of mice with TQ led to significant decreases in CCl_4-induced liver injury, as indicated by histology examination and serum enzyme (ALT, AST, and LDH) tests (Anderson and Smith, 2003). A recent study by Oguz et al. (2012) suggests that TQ inhibits common bile duct ligation (CBDL)-induced liver damage in rats, including fibrotic changes in the liver. So far, the hepatoprotective and anti-fibrotic effects of TQ are largely attributed to its antioxidative activity, which leads to decreased hepatocyte damage and thus decreased transactivation of HSCs. However, the detailed mechanism remains to be completely understood. Particularly, the role of TQ in directly inhibiting the fibrogenic activity of HSCs has not been studied.

In this study, we showed that TQ can protect liver damage induced by CCl_4, which is consistent with the published work. This study also indicates that TQ can act directly on HSCs via inhibiting their transactivation and the expression of fibrosis-related genes. This study unveils a new mechanism which may contribute to the overall anti-fibrotic effect of TQ.

2.3.3. Materials and Methods

2.3.3.1. Animal

Female CD-1 mice weighing 25g were purchased from The Jackson Laboratory (Bar Harbor, ME). Male Sprague-Dawley rats (200-250 g) from Charles River Laboratories (Wilmington, MA) were used for HSC isolation.

2.3.3.2. Chemical

Lipopolysaccharides (LPS), silymarin (SM), and TQ were purchased from Sigma-Aldrich (St. Louis, MO). The purity (GC) of TQ is ≥98.5% as per the manufacturer's specification. Carbon tetrachloride (CCl_4) was purchased from Merck (Whitehouse Station, NJ).

2.3.3.3. Cell line

LX2, an immortalized human hepatic stellate cell line, was kindly provided by Dr. Scott L. Friedman (Mount Sinai School of Medicine, New York, NY)and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics.

2.3.3.4. Rat HSCs Isolation

Retired male Sprague-Dawley rats weighing 200-250 g, obtained from Charles River Laboratories (Wilmington, MA), were used to isolate HSCs. HSCs were isolated via *in situ* proteinase/collagenase perfusion followed by density gradient centrifugation as described (Li et al., 2011). The purity of isolated cells was >90%. Isolated HSCs were cultured in 6-well plate in DMEM with 10% FBS and antibiotics for 7 days to allow the process of activation.

2.3.3.5. Assessment of TQ protective effect in mice

Four groups were used (n = 4) in this study; including group A that received no treatment, group B that was treated with oral TQ (25 mg/kg dissolved in sesame oil), group C that received i.p. injection of CCl_4 (dissolved in sesame oil), and group D that received both TQ and CCl_4 treatment. Mice in groups B and D were treated with TQ daily for 7 days while mice in groups C and D received a single dose of CCl_4 on day 6. All mice were sacrificed 2 days after CCl_4 injection. Samples from liver tissues were directly stored in -80°C freezer for gene expression study or fixed in 4% paraformaldehyde for histology study.

2.3.3.6. Mice liver tissue processing

The liver was sectioned and fixed in phosphate-buffered 10% formaldehyde for histological analysis. Each formaldehyde-fixed sample was embedded in paraffin, cut into 5 micron-thick sections and stained with hematoxylin-eosin (H.E.) and Masson's trichrome according to standard procedures.

2.3.3.7. Cytotoxicity assay

LX2 Cells were seeded in 96-well plate and incubated in DMEM containing 10% FBS overnight. Cells were then treated with various concentrations of TQ (in DMSO) for 24 h. Cell viability was measured by MTT assay (Roche Diagnostics, Indianapolis, IN) as described (Huang et al., 2012).

2.3.3.8. RNA isolation and qRT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen). The total RNA was measured by NanoDrop 2000 (Thermo Scientific). Extracted RNA concentration was adjusted to be 1µg per reverse transcription reaction using SuperScript III reverse transcriptase (Invitrogen). The primers for COL1A1, COL3A1, α-SMA, MCP-1, IL-6 and GAPDH were obtained from MWG Biotech. After synthesis of first strand cDNA, Real-Time PCR was performed using SYBR Green-based assays with the ABI Prism 7300 (Applied Biosystems, Foster City, CA) (Li et al., 2008a).

2.3.3.9. Assessment of TQ inhibitory effect on LPS-induced proinflammatory response in LX2 cells

LX2 cells were seeded in a 6-well plate in DMEM containing 10% FBS overnight. Cells were pretreated with TQ for 24 h, challenged with LPS (100 ng/mL) for 4h, and then were harvested with TRIzol reagent. MCP-1 and IL-6 mRNA expression levels were measured as described above.

2.3.3.10. Assessment of TQ inhibitory effect on culture-triggered transdifferentiation, and fully activated primary rat HSCs

Rat HSCs were isolated as described above. Cells were divided into 7 groups: untreated cells that were harvested on day 1, vehicle treated cells that were treated with DMSO (0.1%) and harvested on day 7, TQ-treated cells that were treated with TQ (4 µM) once every 2 days and harvested on day 7, and cells cultured for 7 days followed by treatment with TQ (1 and 4 µM) for 24 h, and cells cultured for 7 days followed by treatment with silymarin (10 and 40 µM) for 24 h. The mRNA expression levels of COL1A1 and α-SMA genes were assessed by qRT-PCR

as described above. Similarly, LX2 cells were treated with different concentrations of TQ and SM for 24 h, and the mRNA expression levels of COL1A1 and α-SMA genes were assessed by qRT-PCR

2.3.3.11. Western Blot

Cells were harvested after being washed and lysed in lysis buffer (0.2% Triton X-100) for 5 min on ice. Cell lysates were collected and centrifuged 12,000 rpm for 10 min at 4 °C. Equal amounts of proteins were heated to 95 °C for 5 min in loading buffer and then separated on 10% SDS-polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride membranes (Thermo Scientific) that were blocked for 1hand then probed overnight at 4°C with an antibody specific to p-NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. HRP was detected using chemiluminescence detection reagent (*Denville Scientific* Inc).

2.3.3.12. Statistical analysis

Values for all measurements are expressed as mean ± SEM. Each experiment was performed in triplicate. Comparisons between two groups were made using unpaired Student's *t* test. Differences were considered statistically significant if the P value was less than 0.05.

2.3.4. Results

2.3.4.1. TQ protects against CCl_4-induced liver damage

We first investigated TQ protective effect in mice that were pretreated with TQ (25 mg/kg) for 5 days followed by CCl_4 injection. TQ-pretreated group showed significant resistance to CCl_4-induced injury compared to the group that received only CCl_4 (without TQ pretreatment) (Figure 12C and D). Mice treated with TQ alone showed normal liver histology similar to untreated group (Figure 12A and B).

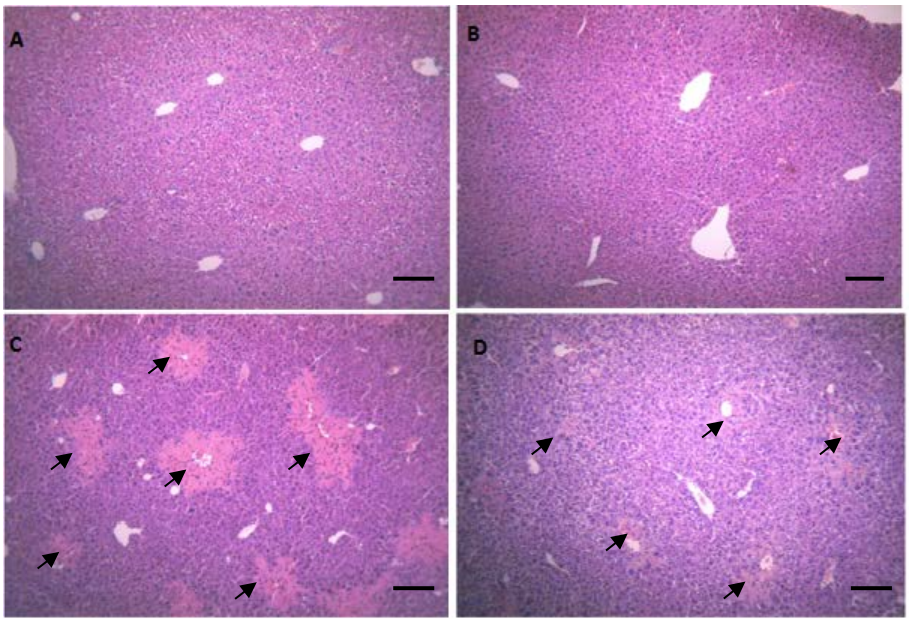

Figure 12. TQ protects mice against CCl_4-induced liver damage.

Mice (n = 4) received daily TQ treatment orally for 5 days followed by a single injection of CCl_4 (i.p.). Liver tissues were collected 2 days after CCl_4 injection and subjected to H.E. staining. A: Control (untreated), B: TQ (25 mg/kg), C: CCl_4, and D: TQ pretreatment followed by CCl_4 injection. N = 4. Bar represent 100µm

2.3.4.2. TQ downregulates the mRNA expression of COL1A1 and α-SMA in mouse liver

Following the demonstration of the hepatoprotective effect of TQ, we then investigated the effect of TQ on the mRNA expression of COL1A1 and α-SMA in the liver of CCl_4-treated mice. COL1A1 is the major collagen in fibrotic liver (Friedman et al., 1985) and its mRNA expression level was significantly upregulated in the liver of CCl_4-treated mice (Figure. 13). TQ pretreatment significantly inhibited the CCl_4-induced upregulation of COL1A1 mRNA expression in mouse liver. TQ similarly inhibited the CCl_4-induced upregulation of α-SMA, a known marker for HSCs activation (Mabuchi et al., 2004) (Figure. 13). These data suggest that TQ exhibits anti-fibrotic activity.

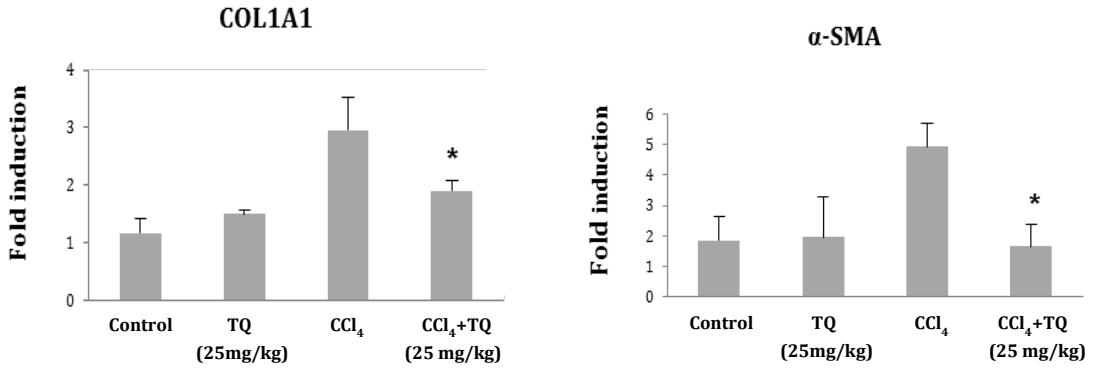

Figure. 13. TQ downregulates the mRNA expression of COL1A1 and α-SMA in mouse liver

TQ significantly downregulates mRNA expression of COL1A1 and α -SMA in the liver of CCl_4-treated mice (n = 4). Mice were treated with TQ followed by CCl_4 as described in Figure 12. and mRNA expression of COL1A1 and α-SMA in mouse liver was determined via qRT-PCR.

2.3.4.3. *In vitro* cytotoxicity of TQ

The hepatoprotective effect of TQ is likely to play a role in its anti-fibrotic activity due to the decreased damage of hepatocytes and subsequent decrease in HSC activation. It was hypothesized that TQ also inhibits fibrogenic activity via a direct effect on HSCs. To test this hypothesis, the effect of TQ on the proliferation of HSC *in vitro* was examined, as the induction of HSC apoptosis is currently being explored as a therapeutic approach for the prevention and/or treatment of liver fibrosis (Hagens et al., 2008). Figure. 14 shows the results of a MTT assay with LX2 cells. LX2 is an immortalized human hepatic stellate cell line, and these cells are fully activated (Xu et al., 2005). Our results indicate that TQ inhibited the proliferation of LX2 cells in a dose-dependent manner. Silymarin showed a minimal effect on the proliferation of HSCs at the concentrations used (Figure. 14).

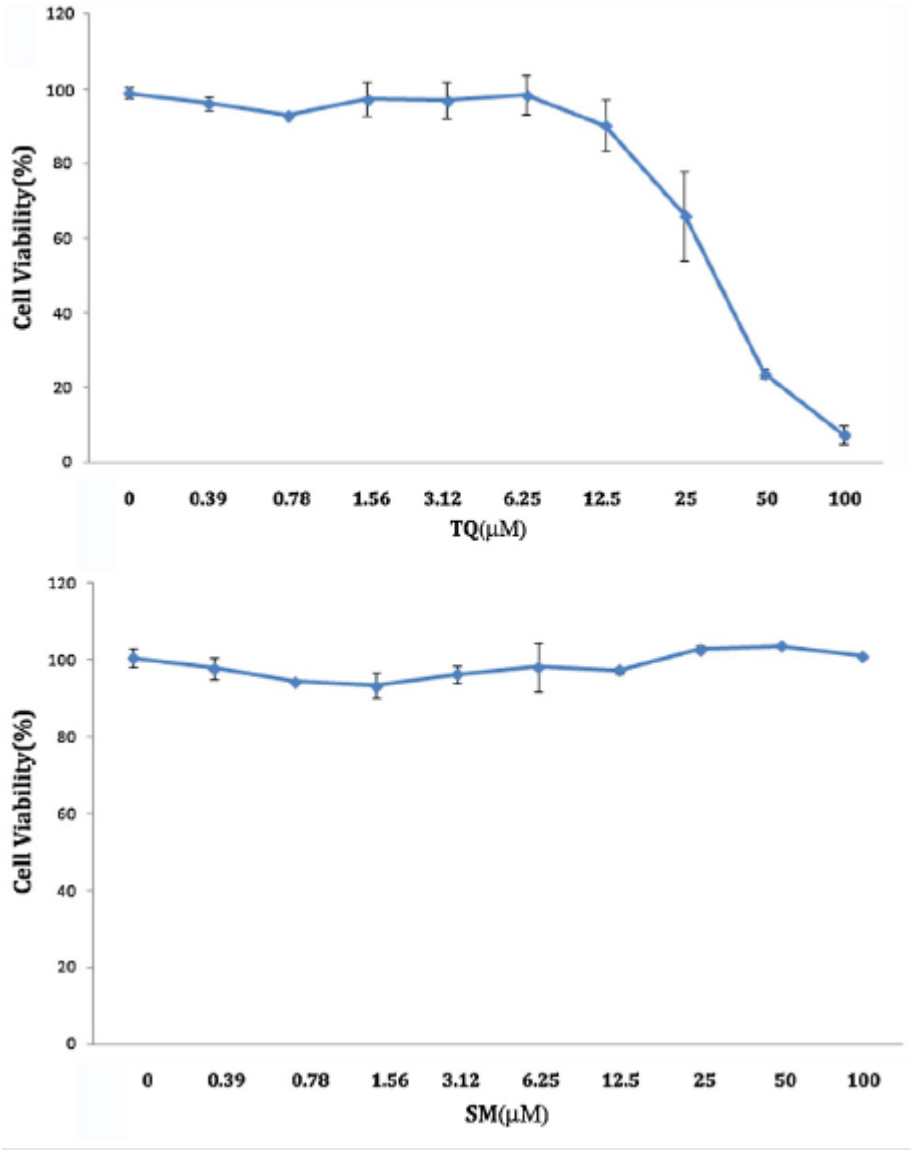

Figure. 14. *In vitro* cytotoxicity of TQ.

Cytotoxic effect of TQ and SM on LX2 cells. Cells were treated with various concentrations of TQ and SM. MTT assay was performed 24 h later.

2.3.4.4. TQ downregulates the expression of several fibrosis-related genes in LX2 cells and culture-activated primary rat HSCs

Following the demonstration of the growth inhibitory effect of TQ on LX2 cells, we went on to further examine the effect of TQ on the mRNA expression of several fibrosis-related genes including COL1A1, COL3A1, and α-SMA in both LX2 and culture-activated primary rat HSCs. TQ was applied to cells at the concentrations that were shown to have minimal effect on cell growth (Figure. 14). As shown in Figure. 15A, TQ inhibited the mRNA expression of all of the three genes in LX2 cells in a concentration dependent manner. Moreover, TQ significantly downregulated COL1A1 and α-SMA mRNA expression in culture-activated primary rat HSCs as shown in Figure. 15B. Similar effects were shown for silymarin at concentrations that were nontoxic to the cells (Figure. 15A and B).

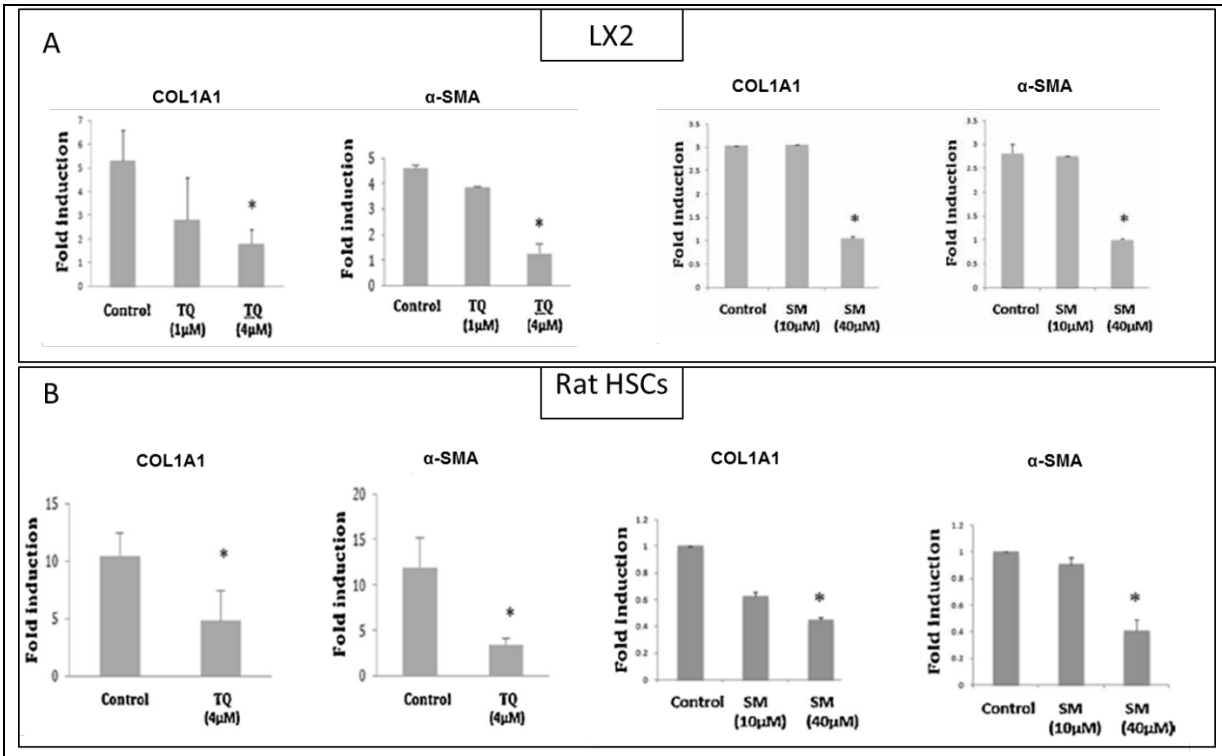

Figure. 15. TQ downregulates the expression of several fibrosis-related genes in LX2 cells and culture activated primary rat HSCs.

TQ and SM significantly downregulates the mRNA expression of COL1A1 and α -SMA genes in LX2 cells (A) and culture-activated primary rat HSCs (B): Cells were treated with TQ and SM in DMEM containing 10% FBS for 24 h. Cells were harvested with TRIzol reagent and the mRNA expression of several fibrosis-related genes was measured by qRT-PCR. Expression folds were normalized against GAPDH gene expression.

2.3.4.5. TQ suppresses the LPS-induced proinflammatory response in LX2 cells

It is known that HSCs can produce certain types of chemokines and cytokines in response to various types of stimuli, such as Lipopolysaccharide (LPS), which could have both paracrine and autocrine effects (Gressner and Weiskirchen, 2006). LPS is an endotoxin from Gram-negative bacteria which interact with TLR4 and induce proinflammatory response through activation of **NF-κB.** To examine whether TQ can inhibit the proinflammatory response of HSCs

towards LPS, LX2 cells were pretreated with TQ followed by challenge with LPS and the mRNA expression of monocyte chemoattractant protein-1(MCP-1) and interlukin-6 (IL-6). As shown in Figure. **16**, LPS induced significant upregulation in the mRNA expression of both MCP-1 and IL-6 genes. Both responses were significantly inhibited when LX2 cells were pretreated with TQ.

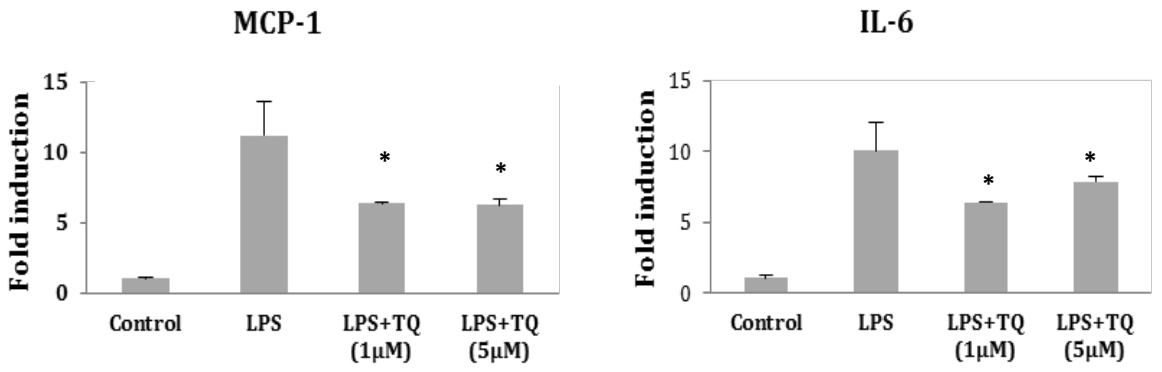

Figure. 16. TQ significantly suppresses pro-inflammatory response after LPS challenge.

Cells were plated in 6-well plates overnight followed by treatment with TQ at 1 and 5μM in DMEM containing 10% FBS for 24h. Cells were then challenged with LPS (0.1μg/mL) for 4h and the mRNA expression levels of MCP-1 and IL-6 were determined by qRT-PCR.

2.3.4.6. TQ inhibits NF-κB activation induced by LPS

It is known that MCP-1 and IL-6 are two target genes of NF-κB signaling (Libermann and Baltimore, 1990; Ueda et al., 1994). Thus, we investigated the effect of TQ on NF-κB signaling to elucidate the potential role of NF-κB inhibition in TQ-mediated inhibition of LPS-induced MCP-1/IL-6 response. Western blot analysis showed that TQ significantly suppressed NF-κB

activation induced by LPS as evidenced by decreased levels of phosphorylated NF-κB p65 protein (p-NF-κB p65) (Figure. 17). In addition, TQ pretreatment inhibited the serum-induced phosphorylation of NF-κB p65 (Figure. 17).

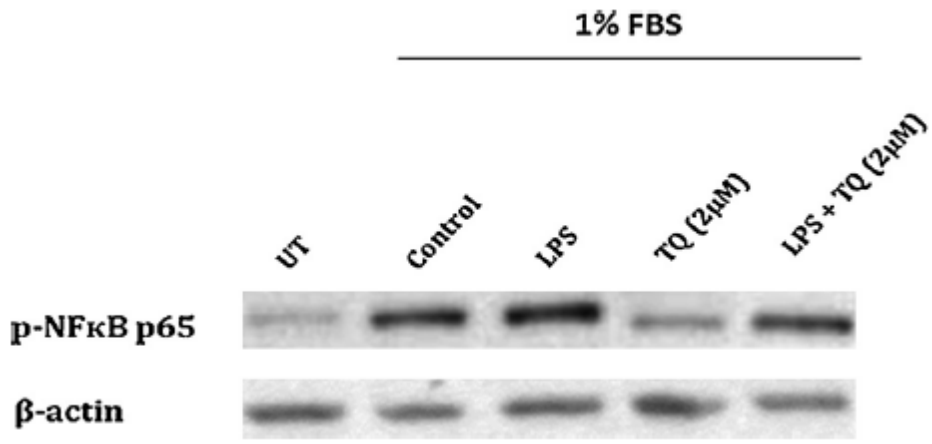

Figure. 17. TQ significantly suppresses LPS–induced activation of NF-κB pathway.

LX2 Cells were plated in 6-well plates and serum starved overnight. Cells were pretreated with TQ (2μM) in serum free medium for 24h and challenged with LPS in DMEM medium containing 1% FBS for 10 min. Cells were harvested with lysis buffer and total protein was measured. Western blots were performed to detect phosphorylated-NF-κB p65 and β-actin protein levels

2.3.4.7. TQ inhibits culture-triggered transdifferentiation of primary rat HSCs

The aforementioned studies have demonstrated the anti-fibrotic and anti-proinflammatory activity of TQ on LX2, a fully transactivated human HSC cell line and fully activated primary rat HSCs. To examine the effect of TQ on the transactivation, freshly isolated rat HSCs were cultured for 7 days in the presence or absence of TQ and the mRNA expression levels of COL1A1, COL3A1 and α-SMA genes were determined via qRT-PCR. As shown in Figure. 18, there were significant increases in the mRNA expression levels of all three genes examined after

7 days in culture, suggesting culture-induced transactivation of rat HSCs. In contrast, TQ treatment significantly inhibited the culture-induced upregulation of the expression of all three genes, suggesting that TQ effectively inhibited the culture-triggered transactivation of primary HSCs.

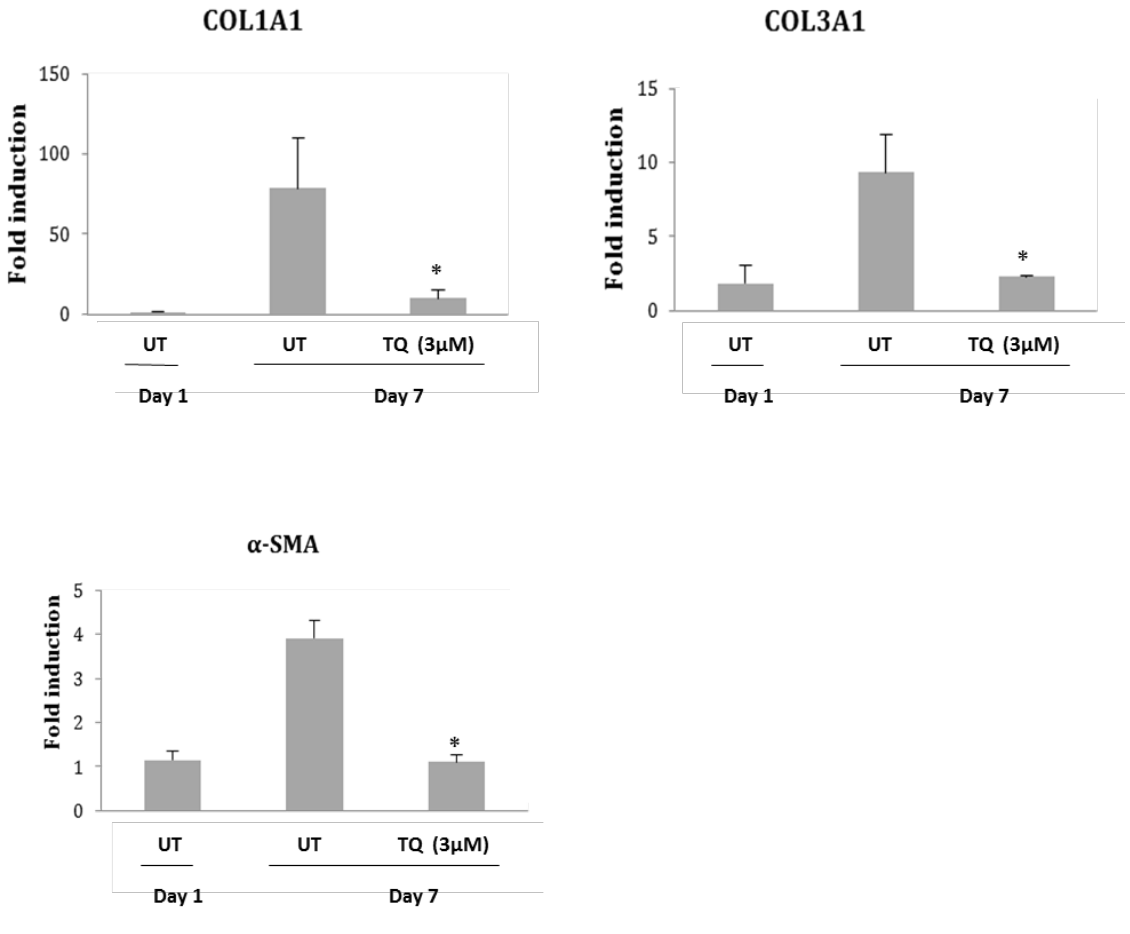

Figure. 18. TQ inhibited the culture-induced transdifferentiation of primary rat HSCs.

Freshly isolated rat HSCs were cultured for one or seven days in the presence or absence of TQ and the mRNA expression levels of COL1A1, COL3A1, and α-SMA genes were measured by qRT-PCR.

2.3.5. Discussion

TQ is a natural product with various biological activities including hepatoprotective effects against hepatotoxins. A recent study has also demonstrated anti-fibrotic effect for TQ in a rat model of CBDL-induced liver injury (Oguz et al., 2012). However, the detailed mechanism for the anti-fibrotic activity of TQ remains largely unknown. It is generally regarded that the hepatoprotective activity of TQ plays an important role as decreased hepatocyte damage shall lead to reduced activation of HSC and the associated fibrogenic events. The results from the present study clearly demonstrated a direct inhibitory effect of TQ on HSCs, unveiling a new mechanism for the anti-fibrotic effect of TQ.

Activation of HSCs represents the key initial step in the pathogenesis of liver fibrosis. Our data showed that TQ treatment significantly inhibited the culture-induced transactivation of primary rat HSCs as demonstrated by drastic inhibition of culture-triggered upregulation of several fibrosis-related genes including COL1A1, COL3A1, and α-SMA. In addition to blocking the transactivation of quiescent HSCs, TQ significantly inhibited the fibrogenic activity in fully activated cells: TQ-treated LX2 cells showed significantly reduced mRNA expression levels of COL1A1 and COL3A1 compared to vehicle-treated cells. A recent study by Bai et al. showed that TQ treatment reduced the LPS-induced upregulation of COL1A1 and α-SMA in T-HSC/Cl-6 cells, an immortalized rat hepatic stellate cell line (Bai et al., 2013b). We have further shown that TQ could inhibit the LPS-induced proinflammatory response as demonstrated by significant inhibition of LPS-induced upregulation in the mRNA expression levels of MCP-1 and IL-6. It is well known the cytokines and chemokines produced by activated HSCs can work on both HSCs and neighboring cells in autocrine and paracrine fashions, which serve to amplify the

proinflammatory and fibrogenic events (Bataller and Brenner, 2001). Thus, TQ can intervene at various steps of the complex fibrogenic processes.

In addition to direct inhibition of fibrogenic activity in HSCs, TQ shows growth inhibitory effect on HSCs. It has been accepted that liver fibrosis is a reversible process, particularly at early stage, and elimination of activated HSCs via apoptosis plays an important role in the resolution of fibrotic changes (Wright et al., 2001). This has led to the development of a number of therapeutic strategies targeted at the induction of apoptosis of activated HSCs in fibrotic liver (Li et al., 2008b). The inhibitory effect of TQ on the proliferation of HSCs likely contributes to the overall anti-fibrotic activity of TQ *in vivo*, which will be further addressed in the future.

The mechanism for the anti-fibrotic activity of TQ is not completely understood; however, its antioxidant activity likely plays an important role. It is known that the LPS/TLR4 signaling is critically involved in transactivation of HSCs during liver injury (Bai et al., 2013b). It has also been known that LPS initially promotes the production of reactive oxygen species (ROS), which elicits a wide spectrum of responses by activating transcription factor NF-κB through MAPKs and PI3K/Akt pathways (Bai et al., 2013b; Shi et al., 2013). Activation of NF-κB leads to the expression of not only various proinflammatory and profibrogenic factors but also antiapoptotic proteins such as XIAP and cellular FLIP (c-FLIPL) that might enhance the survival and proliferation of activated HSCs. Our data showed that activation of NF-κB by LPS in LX2 cells was significantly inhibited by TQ. The study by Bai et al showed that LPS treatment led to activation of PI3K signaling, which was significantly attenuated by TQ. These data are in consistent with the antioxidant activity of TQ. This is further supported by the observation that silymarin, a well-established antioxidant (Bindoli et al., 1977; Trappoliere et al., 2009), similarly

inhibited the fibrogenic activity in both human and rat HSCs. More studies are needed to better understand the mechanism by which TQ exerts its anti-fibrotic and antiproliferation activity in HSCs. In addition to these data, we have done a side experiment to investigate other mechanisms that may be involved. We tested weather TQ has an impact in inhibition of serum-induced contraction of LX2 cells *in vitro*. Our preliminary results showed that TQ significantly inhibited LX2 contraction induced by serum using collagen lattices gel. Moreover, TQ treatment of culture-induced transactivation of primary rat HSCs significantly inhibited vasoactive mediators ET-1 gene expression, which is heavily involved in the contractile properties of activated HSCs (data shown in Appendix A).

In summary, this study supports the notion that TQ has a direct anti-fibrotic and antiproliferation effect on HSCs. Considering its excellent safety profile and various favorable biological properties, TQ may represent a new type of anti-fibrotic therapy that warrants further study

2.4. PEG_{5K}-FMOC-FTS_2 MICELLE SYSTEM AS A DUAL FUNCTIONAL DELIVERY SYSTEM FOR TQ IN THE TREATMENT OF LIVER FIBROSIS

2.4.1. Introduction

Chronic liver diseases represent major health problem worldwide; the major causes include chronic viral hepatitis, alcohol abuse, autoimmune disease, and metabolic disorder. Long-term exposure to liver injury results in an abnormal wound healing response termed fibrosis (Friedman, 2003a; Bataller and Brenner, 2005). Injured liver cells undergo phenotypic changes, leading to the overproduction and deposition of ECM, which results in the replacement of healthy cells with scar tissue that subsequently leads to major complications, such as liver cirrhosis and hepatocellular carcinoma (Bataller and Brenner, 2005; Friedman and Bansal, 2006a). There have been significant advances in the understanding of liver fibrosis pathology and treatment approaches. Although many potent potential anti-fibrotic drugs were discovered and are currently being tested in clinical trials, such as angiotensin II antagonists, interferon gamma, and peroxisomal proliferator activated receptor (PPAR), they often show minimal *in vivo* effect when compared to their in vitro effect (Rockey, 2008). This is likely attributed to lower drug concentrations reaching target sites and non-specific distribution to many organs, resulting in adverse drug effects. This may explain the absence of anti-fibrotic drugs in clinical practice.

The recent emerging nanotherapy has introduced a promising alternative to conventional therapy. Nanotechnology is a growing field attracting more and more scientists over the past decades. Nanocarriers possess the flexibility to be engineered with wide spectrum of biocompatible materials in order to get the most suitable nanocarriers for the treatment purposes.

Numerous *in vitro* and *in vivo* studies have suggested that TQ carries significant therapeutic applications for many diseases, with a relatively low toxicity profile (Badary et al., 1997; Al-Shabanah et al., 1998; Mansour et al., 2001; Zafeer et al., 2012). We have recently shown that TQ shows promise as a hepatoprotective and anti-fibrotic agent, particularly in activated HSCs, major cells for producing the ECM (Ghazwani et al., 2014). Previously, our lab has developed a FTS-based micellar system , a promising dual functional carrier for delivery of anticancer agents such as PTX (Zhang et al., 2013b; Chen et al., 2014; Zhang et al., 2014d; Zhang et al., 2014e; Zhang et al., 2014f). FTS is a potent Ras antagonist, currently being extensively studied as new agent for cancer treatment, with the advantage of having an excellent safety profile (Marom et al., 1995b; Haklai et al., 1998a; Gana-Weisz et al., 2002). Also, several studies have reported its therapeutic activity in animal models of liver diseases. It was discovered that FTS exerts an anti-fibrotic effect in a rat model via inhibition of hepatic fibrogenesis associated with a decrease in Ras expression and reduction of HSCs proliferation and migration (Reif et al., 2004). Moreover, FTS accelerates the regression of experimentally induced hepatic cirrhosis in rats via increased collagenolytic activity, as evidenced by increased expression of MMP-2, MMP-9, and TIMP-2 (Reif et al., 1999).

This study developed a PEG_{5k}-Fmoc-FTS_2 micellar system for TQ delivery. 9-fluorenylmethoxycarbony (Fmoc) moiety is a functional amino acid protecting group typically used in chemical reactions. Our lab has shown that Fmoc group is the most potent drug-interactive moiety among numerous screened compounds (Gao et al., 2012). Fmoc can improve both drug-loading capacity and formulation stability (Zhang et al., 2014a; Zhang et al., 2014b; Zhang et al., 2014g; Zhang et al., 2015a). Here, we examine the potential of this formulation for the delivery of TQ *in vitro* using LX2, an immortalized human hepatic stellate cell line.

2.4.2. Materials and methods

TQ was purchased from Sigma–Aldrich (St. Louis, MO). The purity (GC) of TQ was ≥98.5%, as per the manufacturer's specification. FTS was synthesized according to published literature (Zhang et al., 2013a). 4-Dimethylaminopyridine (DMAP) was purchased from Calbiochem-Novabiochem Corporation (CA, USA). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Lonza (MD, USA). Fetal bovine serum (FBS) and penicillin-streptomycin solution were from Invitrogen (NY, USA). The following chemicals were purchased from Sigma-Aldrich (MO, USA): succinate anhydride, diethanolamine, poly (ethylene glycol) methyl ether (MeO-PEG-OH, Mw = 5000 kDa), N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and Dulbecco's modified Eagle's medium (DMEM).

2.4.2.1 Synthesis of PEG_{5k}-Fmoc-FTS_2 conjugate

The solution condensation method was utilized to synthesize PEG_{5k}-Fmoc-FTS_2 conjugate. MeO-PEG-OH (1 equiv) was reacted with succinate anhydride (5 equiv) in CH_2Cl_2 overnight in the presence of DMAP (5 equiv) as catalyst. The resultant PEG derivative was purified by precipitation in cold ether solution (twice), and extra DMAP was washed away by cold ethanol (twice). The resultant product ($mPEG_{5K}$–COOH) was then reacted with tris(hydroxymethyl)aminomethane (tris), NHS (3 equiv) and DCC (3 equiv) in CH_2Cl_2 for 1 day. The product was similarly purified as previously above. P-toluenesulfonic acid (TsOH) was used

as a catalyst in acetone to block the two hydroxyl groups of PEG-derivatized Tris by forming acetonide. Then, the remaining hydroxyl group of Tris was coupled with Fmoc group through reaction with 9-fluorenylmethoxycarbonyl chloride (Fmoc–Cl) (2 equiv) and triethylamine (3 equiv) in CH_2Cl_2 overnight. The product was purified as mentioned above, and the acetonide group was removed by the addition of 1% TsOH in CH_2Cl_2. Finally, FTS was conjugated with the two hydroxyl groups via reaction with DCC (4 equiv) and DMAP (0.4 equiv) as the coupling reagents. The reaction solution was filtered through cotton and the final product was purified by precipitation / washing as mentioned above.

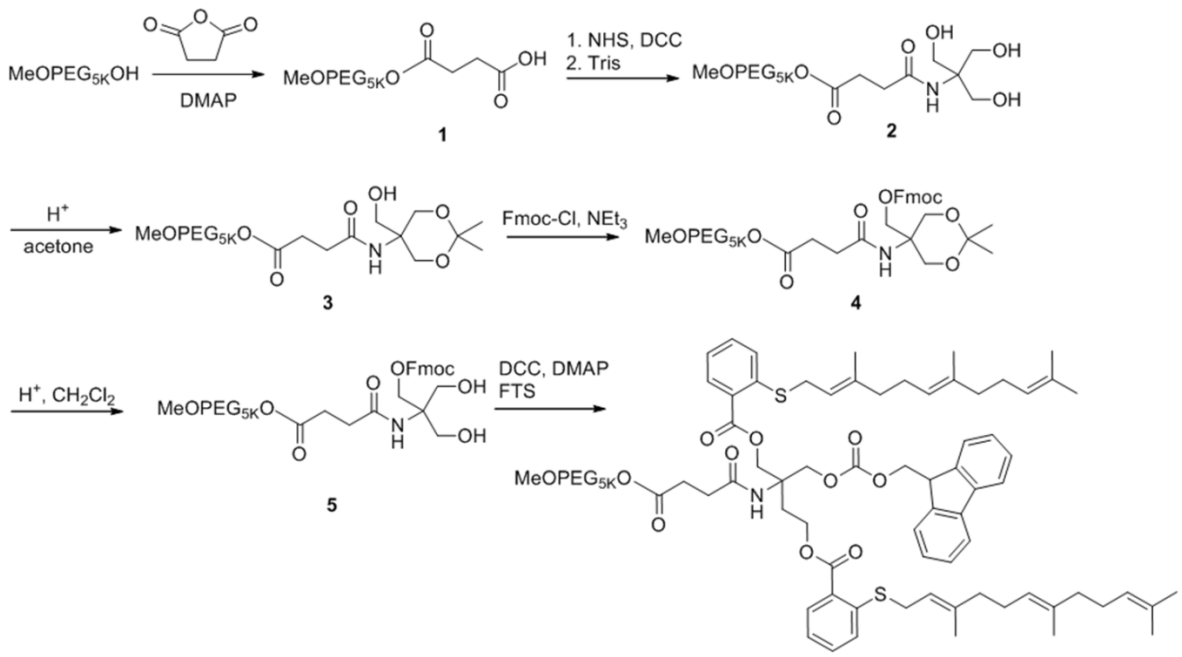

Figure. 19. Synthesis scheme of PEG_{5K}-Fmoc-FTS_2 conjugate

2.4.2.2 Preparation of PEG_{5k}-Fmoc-FTS_2 loaded TQ

TQ (10 mM dissolved in CH_2Cl_2) and PEG_{5k}-Fmoc-FTS_2 (10 mM dissolved in CH_2Cl_2) were mixed with different carrier /drug ratios in glass tube as shown in table 1. The solvent was removed by nitrogen flow to form thin film of the mixture. Traces of solvent were removed by glass vacuum desiccator. Micelles were formed by hydration of film with DPBS, followed by gentle vortexing.

2.4.2.3 Particles size and surface morphology measurement

TQ-micelles concentration was kept at (Bakathir and Abbas). Particles size was measured via dynamic light scattering (DLS) (Zetasizer Nano ZS instrument, Malvern, Worcestershire, UK). Briefly, 20 μL of micelle solution was diluted with 1 mL of distilled water, and particle size was measured at room temperature. Size distribution and morphology was examined for both drug-free and TQ-loaded micelles via TEM imaging. Sample from micelles were stained using a copper grid with Formvar immersed in a drop of sample solution and stained with 1% uranyl acetate. Imaging was performed at room temperature on JEOL JEM-1011.

2.4.2.4 Determination of drug content, entrapment efficiency (EE) and loading capacity (LC)

To determine the drug loading efficiency of TQ-micelle, 10 μL was dissolved in 0.5 mL methanol, vortexed, and centrifuged at 12000 rpm at 4°C for 10 min. The supernatant was collected and drug content was quantified by high performance liquid chromatography (HPLC) (Alliance 2695− 2998 system). The reverse-phase Lichrospher 100 RP-18 (5 μm) column was used, and the mobile phase consisted of methanol/water (5:95 v/v). The flow rate was set to 0.8

mL/ min and the detection wavelength was 242 nm. Drug entrapment efficiency (EE) and loading capacity (LC) were calculated as follows:

EE (%) = (weight of loaded drug/weight of input drug) × 100%

LC (%) = [(weight of drug loaded/ (weight of SLN + drug used)] 100%

2.4.2.5 Stability

For stability study, freshly prepared micelle samples were stored in parafilm-sealed glass tubes at room temperature. Size measurements were performed daily until changes in size is observed.

2.4.2.6 *In vitro* cytotoxicity study

LX2 (2000 cells/well) were seeded in 96-well plates. Cells were incubated overnight in 10% FBS and 1% streptomycin−penicillin. Fresh media containing TQ with indicated concentrations for 72 h was added to replace old media. Following, 20 µL of MTT (5 mg/mL) in DPBS was added to each well and further incubated for 3.5 h. Well contents were gently removed and DMSO was added to solubilize MTT formazan, resulting in a purple-colored solution. A microplate reader quantified absorbance with a wavelength of 550 nm and reference wavelength of 630 nm. Untreated groups were used as controls. Cell viability was calculated as $[(OD_{treat} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%]$. Drug-free micelles and free TQ in DMSO were examined using a similar method.

2.4.2.7 RNA extraction and qRT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen). The total RNA was measured by NanoDrop 2000 (Thermo Scientific). Extracted RNA concentration was adjusted to be 1 µg per reverse transcription reaction using SuperScript III reverse transcriptase (Invitrogen). The primers for COL1A1, α-SMA and GAPDH were obtained from MWG Biotech. Following synthesis of first strand cDNA, real-time PCR was performed using SYBR Green based assays using an ABI Prism 7300 (Applied Biosystems, Foster City, CA).

2.4.2.8 Statistical analysis

In all statistical analyses, the significance level was set at a probability of $P < 0.05$. All results were reported as the mean ± standard error (SEM) unless otherwise indicated. Statistical analysis was performed by Student's *t* test for two groups.

2.4.3 Results

2.4.3.1 Characterizations of micelles

Following the successful synthesis of PEG_{5k}-Fmoc-FTS_2, determined by NMR, micelles were formed by film hydration as previously described. DLS size distribution indicates that drug-free micelles and TQ-micelles had an average size of ~30 nm each. TEM observations confirmed spherically-shaped micelles with a size distribution that is consistent with that seen in DLS (Figure 19). Moreover, an entrapment efficiency of greater than 80 % was observed for TQ-micelles of carrier/drug ratios of (0.2:1) and (0.5:1), respectively, and the drug-loaded micelles were stable for more than two weeks (**Table 1**).

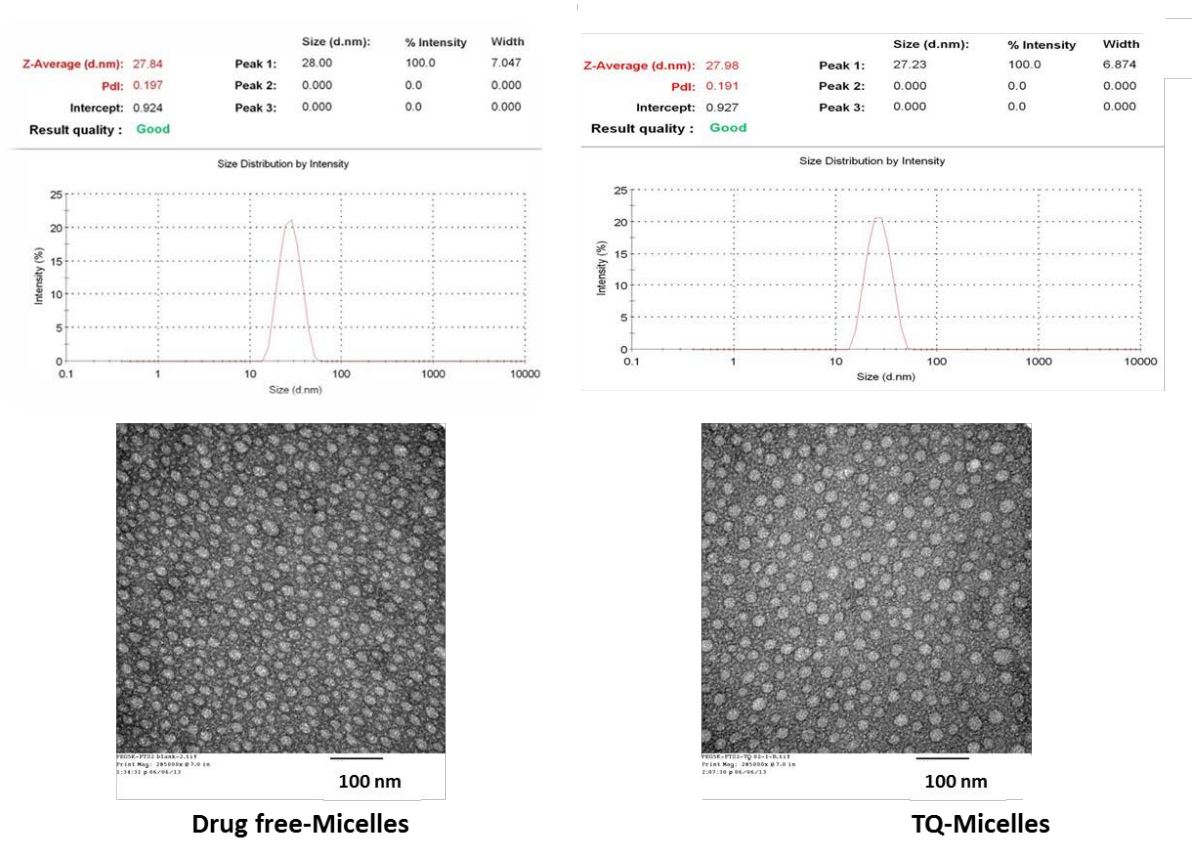

Figure 19. Particles size distribution of micelles.

Drug-free micelles (A) and TQ-micelles (B) as determined by DLS. TEM images of drug-free-micelles (C) and TQ-micelles (D).

2.4.3.2 MTT assay analysis

Free TQ in DMSO exhibited a cytotoxic effect against LX2 cells in a dose-dependent manner. However, TQ-loaded micelles only exhibited a minimal killing effect of about 20% at high concentrations (100 µM) (Figure 20). No toxic effect was observed in drug-free micelles (data not shown).

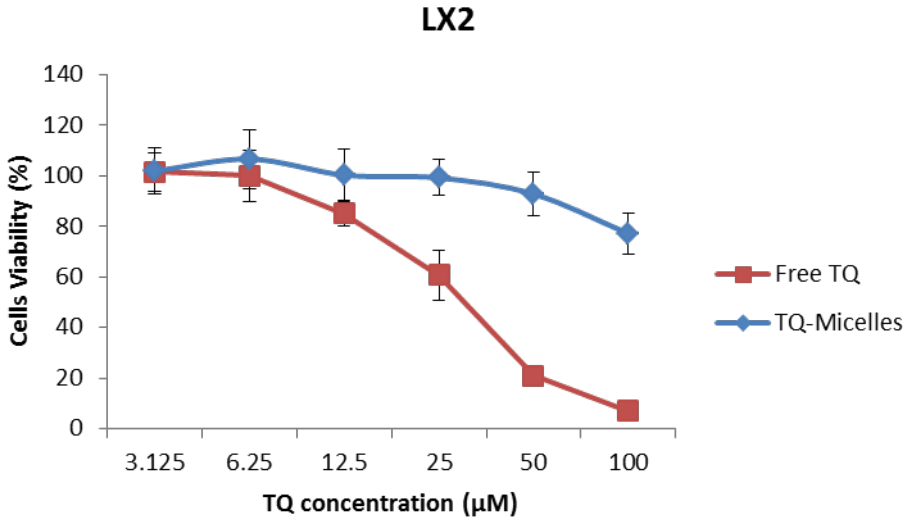

Figure 20. Cytotoxicity assay of free TQ and TQ-micelles in immortalized activated human HSCs line (LX2).

2.4.3.3 mRNA expression analysis of fibrogenic markers

Cells treated with free TQ exhibited significant downregulation of both fibrogenic markers COL1A1, a major component of ECM proteins, and α-SMA – a marker for HSCs activation. In contrast, TQ-micelles of similar concentrations had no effect on both genes. However, at a higher concentration of 20 µM, micellar TQ exhibited significant inhibition of both COL1A1 and α-SMA genes (Figure 21).

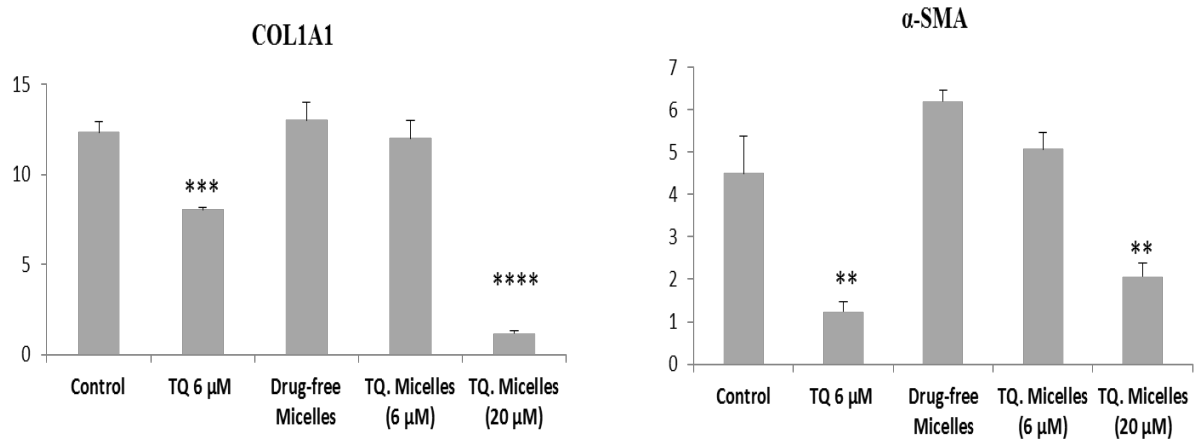

Figure 21. qRT-PCR analysis of fibrotic genes expression after treatment with TQ-Micelles.

LX2 cells were plated overnight, followed by treatment of free TQ in DMSO, drug-free micelles, and two concentrations of TQ-Micelles for 24 hrs. Expression of COL1A1 and α-SMA were determined by qRT-PCR.

Table 1. Stability of TQ-micelles in different molar ratios

PEG_{5K}-Fmoc- FTS_2	EE (%)	Appearance	Stability	LC (%)
0.1:1	37	Clear	3 days	26
0.2:1	82	Clear	2 weeks	13
0.5:1	87	Clear	2 weeks	5

2.4.4 Discussion

This study explores the delivery of TQ into activated human HSCs (LX2) *in vitro*, using an innovative micellar system, PEG_{5k}-Fmoc-FTS_2. The system is based on a PEG conjugate with

two molecules of FTS together with a Fmoc group at the interfacial region. The preparation method of this micelle system can be easily handled and achieved in short time period. Previously, our lab has developed micellar delivery systems equipped with Fmoc moieties acting as drug interactive motifs, which has been shown to efficiently improve carrier/drug compatibility. The findings demonstrated that the Fmoc group can provide strong carrier/drug interactions, when compared to conventional micelles, by forming π-π stacking with other compounds carrying aromatic rings. Thus, the π-π stacking can add to the hydrophobic interaction between drug and carrier, allowing for increased compatibility and stability. This formulation readily solubilized TQ and formed spherical micelles in an aqueous solution with a very small size (<30 nm). TQ could be loaded into PEG_{5k}-Fmoc-FTS_2 micelles at a carrier/drug molar ratio as low as 0.2:1. The drug loading capacity for TQ at this ratio is 13%, with a drug loading efficiency of 82%. The formulation remained stable for 2 weeks at room temperature (**Table 1**). It is possible that the interaction between TQ and hydrophobic core of micelles may not be very high, probably because the water solubility of TQ in water is 0.87 mg/mL at 25 °C, which may suggest that small amount of TQ may partially dissolve in aqueous phase. This may explain why the loading efficiency didn't reach 100%.

The cytotoxicity study showed that free TQ inhibited the proliferation of LX2 cells in a concentration-dependent manner, while TQ formulated in PEG5k-Fmoc-FTS2 micelles did not show toxicity at parallel concentrations, except at a high concentration (100 μM), resulting in ~20% cell killing effect. Although apoptosis induction in activated HSCs is considered to be one of the resolution pathways in liver fibrosis therapy, the reversion of activated HSCs to a quiescent state is also of equivalent importance (Friedman, 2008). Interestingly, dramatic inhibition of COL1A1 expression was observed in the group treated with TQ-micelles. This may

be explained by the efficient uptake of TQ-micelles by LX2 cells; and once TQ and FTS are released inside the cells, they work synergistically to inhibit collagen expression. These data are consistent with the reported anti-fibrotic effects of TQ and the effects of FTS against collagen expression (Reif et al., 1999; Reif et al., 2004). Also, the significant downregulation of α-SMA expression following TQ-micelles treatment (20 μM) suggests that TQ-micelles have a slow release kinetics which also may explain the minimal toxicity effect at a high dose (100 μM), as seen in the cytotoxicity assay. These data suggest that LX2 cells show high tolerability to TQ formulated in micelles, as evidenced by their low toxicity compared to the free drug group. Nevertheless, the TQ formulated in micelles demonstrated significant therapeutic effect as an anti-fibrotic agent.

Despite the demonstrated potential of this formulation, further studies are necessary to make it a better candidate for *in vivo* studies. Necessary studies include: the characterization of release kinetics, an uptake study, stability, mechanisms of action pathways, and incorporation of HSCs-specific ligands (e.g. mannose-6-phosphate/insulin-like growth factor receptor (M6P/IGFII receptor) for improved targeting.

2.5. CONCLUSION AND PERSPECTIVE

Chronic liver diseases (CLD) have become an increasingly important problem worldwide. The prevalence of liver disease is increasing rather than decreasing, particularly when compared to other major causes of mortality. Current knowledge and understanding of liver disease pathology has advanced greatly. However, current treatment options are relatively ineffective – especially in advanced liver fibrosis. Therefore, there is a high demand for identifying novel approaches to curing liver fibrosis. Currently, herbal medicine is a popular area of research due to its promising therapeutic potential for liver disease. Moreover, the low cost, high availability, safety, and efficacy of medicinal plants make them very attractive to scientists. In addition, many herbal plants are widely used by various cultures or religions worldwide. The active component of *Nigella sativa* or black seed (Thymoquinone), is an example of a phytochemical with diverse therapeutic applications and an excellent safety profile. This study investigates TQ's potential impact for the treatment of liver fibrosis. Consistent with previous reports, this study has indicated that TQ plays an important role in the prevention and treatment of liver fibrosis. This study has also shown that TQ can directly affect HSCs, the major collagen producing cells in liver fibrosis, by inhibition of their fibrogenic activities. Additionally, TQ significantly reduced the mRNA expression of the fibrosis-related genes COL1A1, COL3A1, and α-SMA. Moreover, TQ blocked the transactivation of primary rat HSCs *in vitro*. This study has also indicated that TQ acts via inhibiting the activity of NF- κ B induced by LPS, which may attributed to TQ's antioxidant activity. However, further studies are needed to elucidate the mechanism of action for TQ in liver fibrosis.

Following an investigation of TQ's therapeutic impact on HSCs, this study further explored the delivery aspect of TQ into HSCs. This study developed a dual functional micellar

system PEG_{5k}-Fmoc-FTS_2. FTS carries therapeutic potential as an anti-fibrotic drug with an excellent safety profile. Also, FTS may potentially synergize with the codelivered anti-fibrotic drugs. TQ-micelles have very small size (<30 nm) with good stability up to two weeks at room temperature. This formulation didn't exhibit cytotoxicity in LX2 cells compared to free TQ, and, more importantly, was able to deliver higher doses of TQ to achieve anti-fibrotic effect. However, further characterization studies are needed to better understand the system's capabilities and limitations. Also, incorporation of HSC-specific ligands will further improve the potential of *in vivo* therapeutic outcomes.

3 CONCLUSION AND FUTURE DIRECTIONS

3.4 MAJOR FINDINGS AND IMPLICATIONS

Nanomaterial-based drug delivery systems hold promising expectations in the development of novel therapeutic products. The application of nanotechnology in medicine has achieved a great deal in solving many obstacles of conventional therapy. These obstacles include poor water solubility, a low therapeutic window, off-target effects, and multidrug resistance issues. The objective of this dissertation is to explore and develop novel nanocarriers with dual functional properties, rendering them capable of delivering therapeutic agents and contribute to overall therapy outcomes.

In the first chapter, the development of an FTS-based SLNs formulation to deliver PTX is described. PTX is a potent chemotherapeutic agent that has been used for years to treat various human malignancies, including breast, lung, and ovarian cancer. However, its clinical application is greatly limited by its very low water solubility. Currently, PTX is formulated with the use of surfactants including Cremophore EL, which improves the drug solubility. Unfortunately, Cremophore EL is associated with serious side effects, such as hypersensitivity reactions. This study introduced a SLN formulation consisting of a solid lipid core of ethyleneglycol-di-FTS stabilized with PEG5k-FTS2 polymers as a dual functional delivery system for PTX. This study recruited FTS as part of delivery system, with potential bioactivity as an anticancer agent. FTS is non-toxic and a potent Ras antagonist that has been extensively studied as new anticancer agent

– especially in cancers with Ras mutations. Additionally, a phase-I clinical trial has demonstrated the safety of FTS. The SLN system in this study is characterized by small particle size (<200 nm), which is within the size range that can penetrate through tumor leaky vasculatures. Also, this system exhibited an excellent stability profile. In addition, this study suggests that the PTX delivered by this formulation has significantly slower release kinetics with the advantage of avoiding burst release, when compared to Taxol formulation. Most importantly, the *in vivo* data revealed that PTX-loaded SLNs exhibit enhanced antitumor activity, as evidenced by improved tumor growth inhibition in mice treated with PTX-loaded SLNs when compared to both Taxol and control SLNs without FTS. Our results suggest that FTS-based SLNs may represent a better alternative to the currently used Taxol formulation for the following reasons: solving the solubility issue of PTX, providing potential synergy with PTX *in vivo*, stable formulation with controlled release kinetics, and targeted delivery to tumor sites. Moreover, the FTS-based SLNs could serve as a platform for the delivery of other antineoplastic agents with hydrophobic nature.

In the second chapter, this study explores the possibility of delivering TQ to activated HSCs for treatment of liver fibrosis. The first section of the chapter is dedicated to studying the biological activity of TQ in activated HSCs. The second section aims to explore the delivery aspect of TQ to activated HSCs.

TQ is the bioactive constituent of the *Nigella sativa* plant, also known as black seed. A large number of studies have reported TQ's therapeutic potential for the treatment of various inflammatory diseases, including liver fibrosis. This study aimed to determine whether TQ could exert its anti-fibrotic activity directly through inhibiting the fibrogenic activity of HSCs, since HSCs are critical in the initiation and progression of liver fibrosis following liver injury. The preliminary data of this study suggest that TQ could protect against acute liver injury induced by

CCl4 in mice. Additionally, the gene expression of COL1A1 and α-SMA was significantly inhibited. *In vitro* analysis using human activated HSCs (LX2) cells suggest that TQ acts by suppressing the NF-κB pathway, leading to a reduction in expression of LPS-induced pro-inflammatory cytokines. Moreover, TQ treatment significantly blocked the culture-induced transactivation of primary rat HSCs, as demonstrated by the drastic inhibition of culture-triggered upregulation of several fibrosis-related genes. These data suggest that TQ holds great potential as a new drug candidate with many favorable therapeutic effects and an excellent safety profile for the treatment of liver fibrosis. However, future studies are still needed to fully understand its mechanism of action.

The second section of chapter two outlines the development of a simple dual functional micellar system that is based on PEG conjugate with two FTS molecules together with a Fmoc at interfacial region. Aside from the antitumor activity of FTS, several studies have demonstrated its anti-fibrotic effect in animal models. The purpose of study is to develop a nanocarrier system that both efficiently delivers TQ and enhances the overall anti-fibrotic effect. Here, the novel micellar system was able to solubilize TQ in an aqueous solution with very small particles sizes (< 30 nm). The *in vitro* analysis, using LX2 cells, has shown that this formulation is safe and highly efficient in the suppression of the fibrotic genes COL1A1 and α-SMA. The incorporation of FTS in the micelle system may provide a potential synergistic effect with TQ, which could further enhance the overall anti-fibrotic activity.

3.5 LIMITATION AND FUTURE STUDIES

In first chapter, although PTX-SLNs formulation has demonstrated improved performance over clinical formulation Taxol in terms of stability, controlled release and efficacy in vivo, there are several limitations that should be considered for future improvement. First, the biphasic release could be due to insufficient interaction between small amount of PTX and SLN's core, which results in the release of ~ 25% in the first 24 h. The addition of another FTS group to solid lipid to become EG-FTS3 might increase the interaction strength with PTX and allow for slower release especially in the first few hours. Keeping in mind that these modifications will not significantly alter the formulation stability and efficacy. Additionally, we assumed that the decent antitumor activity of EG-FTS2 SLNs *in vivo* is due to FTS, since it possess anticancer activity by itself. However, in order to roll out any toxic contribution coming from EG itself, inclusion of another group of mice receiving only EG would strongly support our explanation if EG didn't show any toxic effect by itself.

Future studies are necessary to further enhance and strengthen FTS-based SLNs for cancer delivery. This may include further analysis of carrier/drug interactions to improve understanding of how to enhance formulation stability and drug release rate. Also, incorporation of a tumor-sensitive cleavable linker within the ethyleneglycol-di-FTS lipid may improve the controlled release of anticancer agents in tumor sites. For example, the disulfide bond is glutathione (GSH) responsive linker that has been extensively studied in various nanocarriers. Disulfide bonds showed strong stability under normal physiological conditions, with low GSH concentrations, and can be highly reduced in tumor environments with high concentrations of GSH. Consequently, this would enhance SLNs' bioavailability in tumor tissues and avoid off-target effects.

In the second chapter, the anti-fibrotic effect of TQ was studied in mice pretreated with TQ followed by liver injury using CCl4. The effect was further confirmed *in vitro* using LX2 cell line. However, it would be more reliable to examine TQ's anti-fibrotic effect in an established liver fibrosis model. Using such a model will reflect the extent by which TQ could inhibit or reverse liver fibrosis. Moreover, although PEG5K-Fmoc-FTS2 shows a great potential as delivery system for TQ, the entrapment efficiency remains limited (~ 80%). This may be attributed to insufficient hydrophobic interaction between TQ and the carrier. Considering the solubility of TQ in water, liposomes might be another delivery system candidate for TQ because of its capability to load both hydrophilic and hydrophobic agents, assuming that TQ might have partial water solubility.

Additional studies are needed to further improve the micellar delivery system for the treatment of liver fibrosis in the future. One direction could include the incorporation of HSC-specific ligands in the micelle surface to allow for active targeting of activated HSCs, and enhance their cellular uptake while minimizing unwanted off-target effects. Moreover, studying the mechanism of interaction between drug and carrier is of great importance to elucidating new perspectives on the improvement of delivery system stability and compatibility with delivered drugs.

APPENDIX A

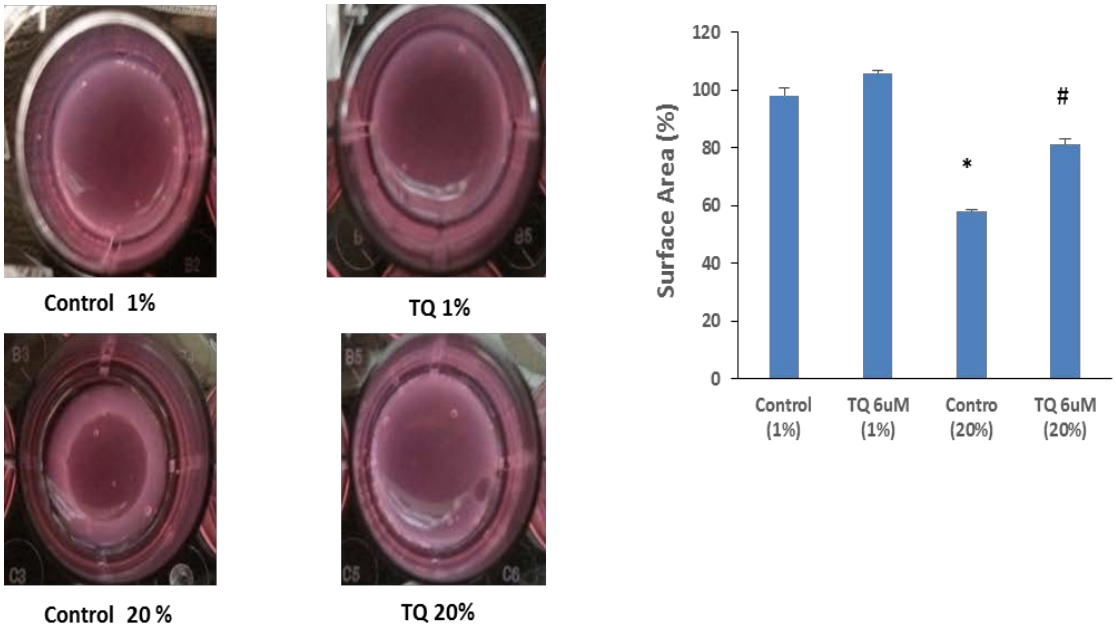

Figure 22. TQ significantly inhibited LX2 serum-induced contraction.

Collagen gel lattices were prepared, and then LX2 were plated over the lattices for overnight. Cells were pretreated with TQ in 1% FBS for 24 hrs, followed by addition of 20% FBS and collagen lattices edges were released from wells. 24 hrs later, pictures were taken and quantified.

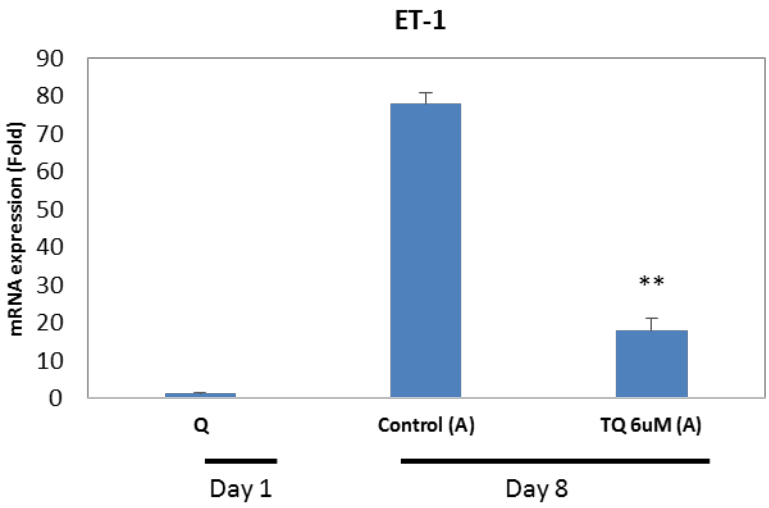

Figure 23. TQ treatment during transactivation process of cultured-activated rat HSCs significantly inhibited ET-1 expression.

APPENDIX B

Table 2. Sequences of real-time PCR primers

Gene	Forward primer	Reverse primer
Homo sapiens COL 1A1	AAGAGGAAGGCCAAGTCGAG	CACACGTCTCGGTCATGGTA
Homo sapiens COL 3A1	CTGGTCCAAAGGGTGACAAG	GCCAGGAGGACCAATAGGA
Homo sapiens α-SMA	AGCCAAGCACTGTCAGGAAT	CCAGAGCCATTGTCACACAC
Homo sapiens GAPDH	ACCTTCCAGCAGATGTGGAT	AGTCATAGTCCGCCTAGAAGC
Homo sapiens MCP-1	TAGCAGCCACCTTCATTCCC	CTGCACTGAGATCTTCCTATTGG
Homo sapiens IL-6	CCAGAGCTGTGCAGATGAGT	GCATTTGTGGTTGGGTCAGG
Homo sapiens ET-1	TCTCTCTGCTGTTTGTGGCT	GGACTGGGAGTGGGTTTCTC
Mus musculus COL1A1	GTTCAGCTTTGTGGACCTCC	TTTCCACGTCTCACCATTGG
Mus musculus α-SMA	GACACCACCCACCCAGAG	CAGAGCCATTGTCGCACAC

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