



Thymoquinone poly (lactide-co-glycolide) nanoparticles exhibit enhanced anti-proliferative, anti-inflammatory, and chemosensitization potential

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ABSTRACT

Thymoquinone (TQ), derived from the medicinal spice *Nigella sativa* (also called black cumin), has been shown to exhibit anti-inflammatory and anti-cancer activities. In this report we employed polymer-based nanoparticle approach to improve upon its effectiveness and bioavailability. TQ was encapsulated with 97.5% efficiency in biodegradable nanoparticulate formulation based on poly (lactide-co-glycolide) (PLGA) and the stabilizer polyethylene glycol (PEG)-5000. Dynamic laser light scattering and transmission electron microscopy confirmed particle diameter between 150 and 200 nm. Electrophoretic gel shift mobility assay showed that TQ nanoparticles (NP) were more active than TQ in inhibiting NF-κB activation and in suppressing the expression of cyclin D1, matrix metalloproteinase (MMP)-9, vascular endothelial growth factor (VEGF), those are markers of cell proliferation, metastasis and angiogenesis, respectively. TQ-NP were also more potent than TQ in suppressing proliferation of colon cancer, breast cancer, prostate cancer, and multiple myeloma cells. Esterase staining for plasma membrane integrity revealed that TQ-NP were more potent than TQ in sensitizing leukemic cells to TNF- and paclitaxel-induced apoptosis. Overall our results demonstrate that encapsulation of TQ into nanoparticles enhances its anti-proliferative, anti-inflammatory, and chemosensitizing effects.

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1. Introduction

Most drugs, especially those with hydrophobic nature, when examined in human clinical trials fail because of either lack of safety or due to poor efficacy which could be in part due to poor bioavailability [1]. Although natural products have served as leads for the majority of drugs [2], poor oral bioavailability has hindered their development. Thymoquinone (TQ), derived from the medicinal spice, black cumin (*Nigella sativa*), is a natural product that exhibits anti-inflammatory and anti-cancer activities but has problems with bioavailability [3]. Several components of black cumin have been identified, including thymoquinone, thymol, thymohydroquinone, and dithymoquinone [4]. The most abundant component of black seed oil, TQ has been reported to exhibit antioxidant [5–7], anti-inflammatory, chemosensitization and chemopreventive potential [8–10] effects. It has been shown to suppress the proliferation of various tumor cells, including

colorectal carcinoma, breast adenocarcinoma, osteosarcoma, ovarian carcinoma, myeloblastic leukemia, and pancreatic carcinoma [11–16], although it is minimally toxic to normal cells [17]. Results from our laboratory have indicated that TQ is a potent inhibitor of the NF-κB pathway [18] and can suppress tumor angiogenesis [19]. This molecule can also inhibit the viability of androgen-sensitive and -insensitive prostate cancer cells both *in vitro* and *in vivo* via suppression of the expression of androgen receptor and E2F-1 [20]. Others have shown that this agent can abrogate the stress response pathway sensor CHEK1 and contributes to apoptosis in colorectal cancer cells [12]. It sensitizes pancreatic cancer cells to chemotherapeutic agents [21].

We hypothesized that encapsulation would enhance cytotoxicity based on developments in drug delivery and nanotechnology. Biocompatible nanoparticles have been developed as inert systemic carriers for therapeutic compounds deliver to target cells and tissues [22–24]. To deliver therapeutic agents to tumor cells *in vivo*, one must overcome the following problems: (i) drug resistance at the tumor level due to physiological barriers (non-cellular based mechanisms), (ii) drug resistance at the cellular level (cellular mechanisms), and (iii) distribution, biotransformation and clearance of anti-cancer drugs in the body [25]. Approach involving polymer-based nanoparticles for oral delivery of the drug is being actively investigated for treatment of cancer and various

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other diseases [26]. It has been used to deliver natural products such as coenzyme Q10 [27], estradiol [28], ellagic acid [29], curcumin [30], and chemotherapeutic agents as paclitaxel [31] and doxorubicin [32]. In fact, a nanoparticle formulation of paclitaxel in which serum albumin is used as a carrier (Abraxane) has been approved for the treatment of breast cancer [33].

As a possible solution to these problems, we encapsulated TQ in nanoparticles (referred herein as TQ-NP) and measured its anti-cancer effects in a cell culture system. In the present report, we describe the design of biodegradable TQ-NP formulation based on poly (lactide-co-glycolide) (PLGA) and a stabilizer polyethylene glycol (PEG)-5000 and characterize its effects on cellular response to various agents. We found that TQ-NP had superior anti-inflammatory, anti-proliferative, apoptotic, and chemosensitizing effects to those of free TQ.

2. Materials and methods

2.1. Reagents

Thymoquinone with the chemical structure as shown in (Fig. 1(A)), was obtained from Sigma–Aldrich (St. Louis, MO). Poly

(lactide-co-glycolide) (PLGA)–poly(ethyleneglycol) (PEG) were custom synthesized (Lactal Absorbable polymers, AL). Ammonium persulphate, ferrous ammonium sulphate, TEMED and N,N'-methylene bis acrylamide were obtained from Sigma–Aldrich. Fetal bovine serum (FBS) was supplied by Atlanta Biologicals (Lawrenceville, GA). Antibodies against cyclin D1 and matrix metalloproteinase (MMP)-9 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Bacteria-derived human recombinant tumor necrosis factor (TNF), purified to homogeneity with a specific activity of 5×10^7 U/mg, was provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, and Iscove's modified Dulbecco's medium (IMDM), Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium were obtained from Invitrogen (Grand Island, NY). An anti-vascular endothelial growth factor (VEGF) antibody was purchased from Thermo Scientific (Fremont, CA).

2.2. Cell lines

Human leukemic cell line KBM-5, human prostate cancer PC-3 cells, human multiple myeloma U266, human colon cancer HCT 116, and human breast cancer MCF-7 cell lines were obtained from

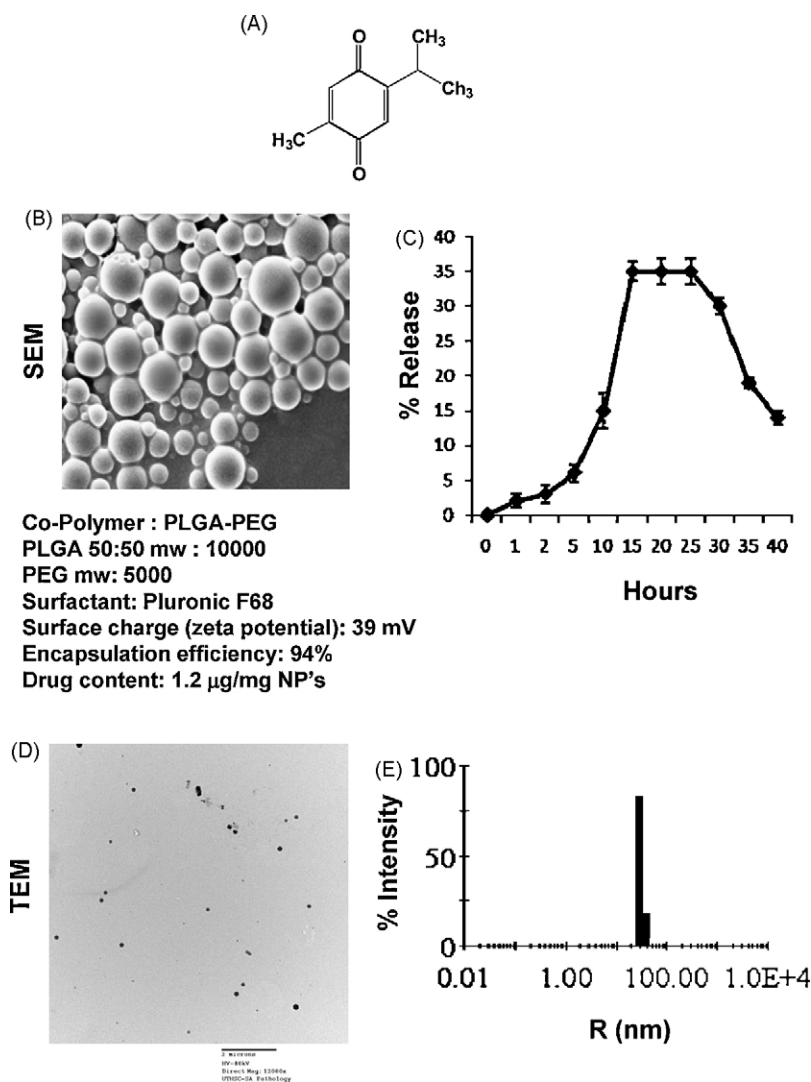


Fig. 1. Design and characterization of thymoquinone nanoparticles. (A) Structure of thymoquinone. (B) Thymoquinone nanoparticles: Morphology by scanning electron microscopy (SEM) and details of the polymer used for the preparation of thymoquinone nanoparticles. (C) Drug release curve for thymoquinone nanoparticles. (D) Nanoparticle size was determined by transmission electron microscopy (TEM) after mechanical extrusion using membrane extruder. The particle size were ranged between 150 and 200 nm. (E) Distribution of the nanoparticles was measured by using micro sampler attached photon correlation spectroscopy. R, radius.

the American Type Culture Collection (ATCC; Manassas, VA). KBM-5 cells were cultured in IMDM with 15% FBS. MCF-7 and HCT 116 cells were cultured in DMEM with 10% FBS. PC-3 and U266 were cultured in RPMI 1640 with 10% FBS. Culture media were also supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin.

2.3. Preparation of polymeric nanoparticles

Nanoprecipitation technique was used to prepare the thymoquinone encapsulated nanoparticles, PLGA–PEG (100 mg) and drug (5 mg) were mixed in acetonitrile (10 mL) added drop wise to an aqueous solution rotating at 5000 rpm containing 0.1% pluronic F-68 as surfactant. The resulting dispersion of nanoparticles was vacuum evaporated to eliminate the organic solvent. The resulting nanoparticles were centrifuged at 15000 rpm for 15 min and washed with deionized water for 3 times and freeze dried with 5% sucrose as a cryoprotectant. The yield of the polymeric nanoparticles was 95% with this protocol. Thymoquinone is directly loaded into the hydrophobic core of nanoparticles by physical entrapment. The drug-loaded nanoparticles are then lyophilized to dry powder for subsequent use.

2.4. Entrapment efficiency (*E*, %)

The entrapment efficiency (*E*, %) of thymoquinone loaded in PLGA–PEG nanoparticles was determined as follows: the nanoparticles were separated from the untrapped free drug using Nanosep (100 kDa cut off) membrane filter and the amount of free drug in the filtrate was measured by spectrophotometer (Spectronic Genesys 5, Thermo Electronic Corp., Waltham, MA) at 450 nm. The *E* (%) was calculated by $E\ (\%) = ([Drug]_{tot} - [Drug]_{free}) / ([Drug]_{tot}) \times 100$.

2.5. Dynamic light scattering (DLS) measurements

DLS measurements of average size and size distribution of the polymeric micelles were performed using a Nanosizer 90ZS (Malvern Instruments, Southborough, MA). The intensity of scattered light was detected at 90° to an incident beam. The freeze-dried powder was dispersed in aqueous buffer, and measurements were taken, after the aqueous micellar solution was filtered with a microfilter having an average pore size of 0.2 µm (Lipex, Vancouver, Canada). All the data analyses were performed in automatic mode. Measured size was presented as the average value of 20 runs, with triplicate measurements within each run.

2.6. Transmission electron microscopy (TEM)

TEM pictures of polymeric nanoparticles were taken in a Joel 1230 (Japan). TEM instrument operating at magnification of 80 kV with 1k × 1k digital images captured using an AMT CCD camera (Danvers, MA). Briefly, a drop of aqueous solution of lyophilized powder (5 mg/mL) was placed on a membrane coated grid surface with a filter paper (Whatman no. 1). A drop of 1% Reynold's lead acetate stain containing sodium citrate, triple distilled water and 1N NaOH solution as immediately added to the surface of the carbon coated grid. After 1 min excess fluid was removed and the grid surface was air dried at room temperature before loaded in the microscope.

2.7. Scanning electron microscopy (SEM)

The surface morphology of the formulated nanoparticle was measured by scanning electron microscopy (SEM) (EM-LEO 435VP, Carl Zeiss SMT Inc., NY) equipped with 15 kV, SE detector with a collector bias of 300 V. The lyophilized samples were spread over

the double-sided conductive tape (12 mm) fixed onto metallic stud.

2.8. Anti-proliferative assay

The anti-proliferative effects of TQ-NP and free TQ on the various cell lines were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) uptake method as described previously [18].

2.9. Apoptosis assay

An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface of membrane to the extracellular surface. This loss of membrane asymmetry can be detected by using the binding properties of annexin V. Annexin V assay was done as described previously [18], using an annexin V antibody, which was conjugated with the FITC fluorescence dye. Briefly, 1×10^6 cells were pretreated with TQ or TQ-NP, treated with TNF for 16 h at 37 °C, and subjected to annexin V staining. The cells were washed in PBS, resuspended in 100 µL of binding buffer containing an FITC-conjugated anti-annexin V antibody, and then analyzed with a flow cytometer (FACS Calibur, BD Biosciences).

2.10. Plasma membrane integrity assay

To evaluate the effect of TQ-NP on plasma membrane integrity, another indicator of apoptosis, we used a Live/Dead assay kit (Invitrogen) that determines intracellular esterase activity and plasma membrane integrity. This assay uses calcein, a polyanionic, green fluorescent dye that is retained within live cells, and a red fluorescent ethidium homodimer dye that can enter cells through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membranes of live cells. In brief, cells (5000 per well) were incubated with TQ or TQ-NP for 24 h. Cells were then stained with assay reagents for 30 min at ambient temperature. Cell viability was determined under a fluorescence microscope by counting live (green) and dead (red) cells.

2.11. Electrophoretic mobility shift assay

To assess NF-κB activation, we isolated nuclear extracts from cells treated with either TQ-NP or TQ, and carried out electrophoretic mobility shift assays (EMSA). In brief, nuclear extracts prepared from cancer cells (1×10^6 /mL) were incubated with ³²P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (4 µg of protein with 16 fmol of DNA) from the HIV long terminal repeat (5'-TTGTTACAAGGGACTTTCCGCTGGGCACTTCCAGGGAGGCGTGG-3'; boldface indicates NF-κB binding sites) for 15 min at 37 °C. The resulting DNA–protein complex was separated from free oligonucleotides on 6.6% native polyacrylamide gels. The dried gels were visualized, and radioactive bands were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

2.12. Western blot analysis

Cancer cells were incubated on ice for 30 min in 0.5 mL of ice-cold whole-cell lysate buffer (10% NP-40, 5 M NaCl, 1 M HEPES, 0.1 M EGTA, 0.5 M EDTA, 0.1 M phenylmethylsulfonyl fluoride, 0.2 M sodium orthovanadate, 1 M NaF, aprotinin [2 µg/mL], and leupeptin [2 µg/mL]). Proteins were then fractionated by SDS-polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).

2.13. Statistical analysis

All experiments were done thrice and the values were compared using one-way ANOVA to with the level of significance set at $P < 0.05$.

3. Results

The goal of this study was first to design thymoquinone nanoparticles (TQ-NP) and then to characterize them for their anti-inflammatory, anti-proliferative, and chemosensitizing effects against different types of cancer cells. Whether TQ-NP are as potent as thymoquinone, were investigated.

3.1. Design and characterization of nanoparticles

The morphology of PLGA–PEG nanoparticles of thymoquinone as prepared is shown in Fig. 1(B). The encapsulation efficiency was

around 94%, and each milligram of the nanoparticle had 1.2 μg of the TQ. When examined for the ability to release the drug, maximum release was observed 15–30 h later and then declined (Fig. 1(C)). Nanoparticle size was determined by transmission electron microscopy after mechanical extrusion using membrane extruder. The particle size was ranged between 150 and 200 nm (Fig. 1(D)). Distribution of the nanoparticles was measured by using micro-sampler attached photon correlation spectroscopy (Fig. 1(E)).

3.2. TQ-NP exhibit enhanced anti-proliferative and apoptosis-inducing ability

We compared the ability of TQ and TQ-NP to inhibit the proliferation of human chronic myeloid leukemia (KBM-5) cells. TQ-NP inhibited the proliferation of these cells in a dose-dependent manner with an IC_{50} value of 1.9 μM as compared to IC_{50} of 3.85 μM for TQ (Fig. 2(A)), thus suggesting that TQ-NP are more potent than TQ.

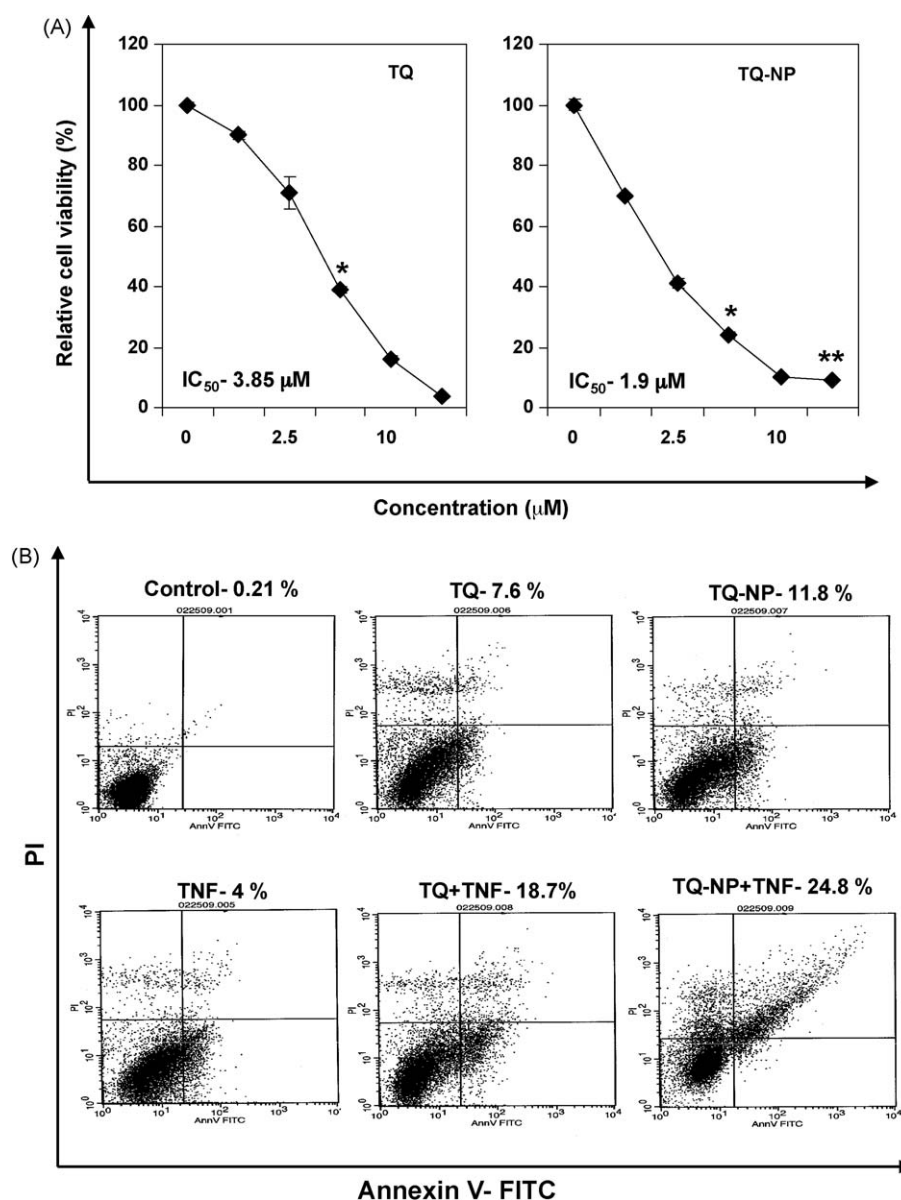


Fig. 2. Effect of thymoquinone and thymoquinone nanoparticles on cell proliferation and apoptosis in myeloid leukemia (KBM-5) cells. (A) Five thousand cells, per well were seeded in triplicate onto 96-well plates; treated with different concentrations of TQ and TQ-NP for 72 h; measured cell viability by the MTT method and presented as percent cell viability. Data are the representative of three independent experiments. Points, mean ($n = 3$); bars, S.E. * $P < 0.05$; ** $P < 0.01$ vs. untreated cells. (B) Cells were treated with 10 μM TQ or TQ-NP for 8 h and the cells were then treated with TNF (1 nM) for 24 h. Cells were stained with PI/annexin V and analyzed by flow cytometry.

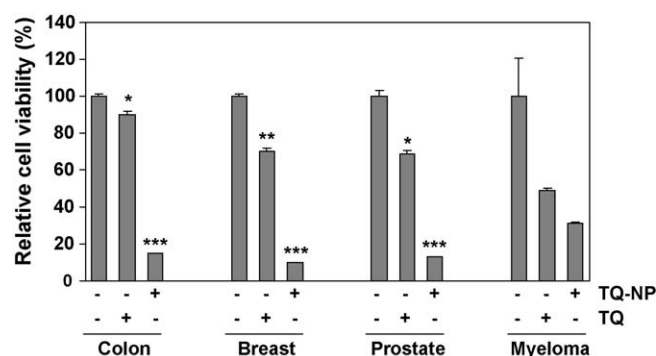


Fig. 3. Effect of thymoquinone and thymoquinone nanoparticles on cell proliferation in different tumor cells. Five thousand human colon cancer (HCT-116), breast (MCF-7), prostate (PC-3) and multiple myeloma (U-266) cells, per well were seeded in triplicate onto 96-well plates; treated with the each compound at 10 μ M for 72 h; measured cell viability by the MTT method and presented as percent cell viability. Data are the representative of three independent experiments. Columns, mean ($n = 3$); bars, S.E. Data are the representative of three independent experiments. Points, mean ($n = 3$); bars, S.E. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ vs. untreated cells.

Previously we have shown that TQ can enhance TNF-induced apoptosis [18]. Next, annexin V assay was used to compare apoptosis induced by TQ and TQ-NP alone and in combination with TNF. TQ-NP alone were more efficient in inducing apoptosis than TQ alone (11.8% vs. 7.7%). When TNF was used to induce apoptosis, TQ-NP enhanced the TNF-induced apoptosis rate to 24.8% vs. 18.7% for TQ (Fig. 2(B)).

3.3. TQ-NP exhibit enhanced anti-proliferative effects against different cell types

Whether TQ-NP can suppress the proliferation of human cancer cells other than chronic myeloid leukemia (KBM-5) was investigated next. The results in Fig. 3 show that TQ-NP had enhanced anti-proliferative effects against colon (HCT-116), breast (MCF-7), and prostate cancer cells (PC-3) and against multiple myeloma (U-266) cells (Fig. 3). The relative rate of growth suppression of TQ and TQ-NP for colon cancer cells was 15% vs. 85%; for breast cancer cells 30% vs. 88%; for prostate cancer cells, 30% vs. 85% and for multiple myeloma 55% and 70%, respectively.

3.4. TQ-NP exhibit enhanced ability to suppress NF- κ B activation

TNF is one of the most potent activators of NF- κ B, which has been linked to inflammation and to proliferation of tumor cells. To determine whether TQ-NP enhance the ability of TQ to suppress TNF-induced NF- κ B, we incubated the cells with different concentrations of TQ or TQ-NP for 4 h and then exposed the cells to 0.1 nmol/L TNF for 30 min. Neither TQ nor TQ-NP alone at 25 μ M activated NF- κ B, but they abolished TNF-induced NF- κ B activation in a concentration-dependent manner (Fig. 4(A)). TQ inhibited NF- κ B activation at 10 μ M by less than 20%, whereas TQ-NP at the same dose inhibited it by 85% (Fig. 4(B)).

3.5. TQ-NP exhibit enhanced ability to suppress NF- κ B-dependent gene products involved in cell proliferation, invasion, and angiogenesis

Numerous gene products that mediate cellular proliferation, invasion, and angiogenesis such as cyclin D1, MMP-9 and VEGF,

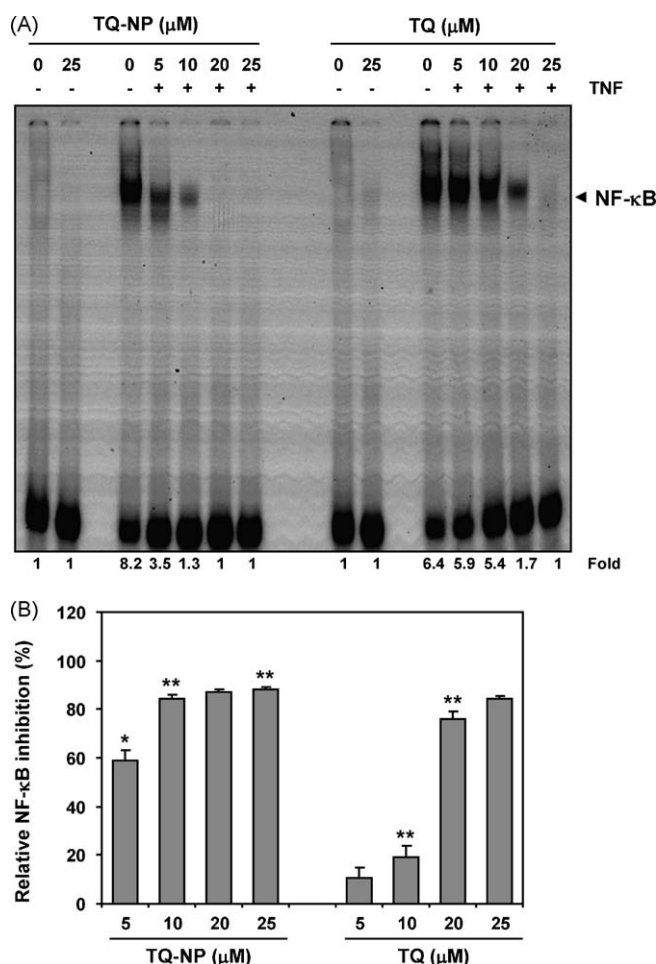


Fig. 4. Effect of thymoquinone nanoparticles on TNF-induced NF- κ B activation. (A) KBM-5 (1×10^6 cells/mL) cells were treated with indicated concentrations of thymoquinone or thymoquinone nanoparticles for 4 h then co-incubated with TNF (0.1 nM) for 30 min. Nuclear extracts were prepared and the NF- κ B activity was examined by EMSA (panel A). (B) Relative NF- κ B activation by TNF and its inhibition by TQ and TQ-NP. Data are the representative of three independent experiments. Points, mean ($n = 3$); bars, S.E. * $P < 0.05$; ** $P < 0.01$ vs. untreated cells.

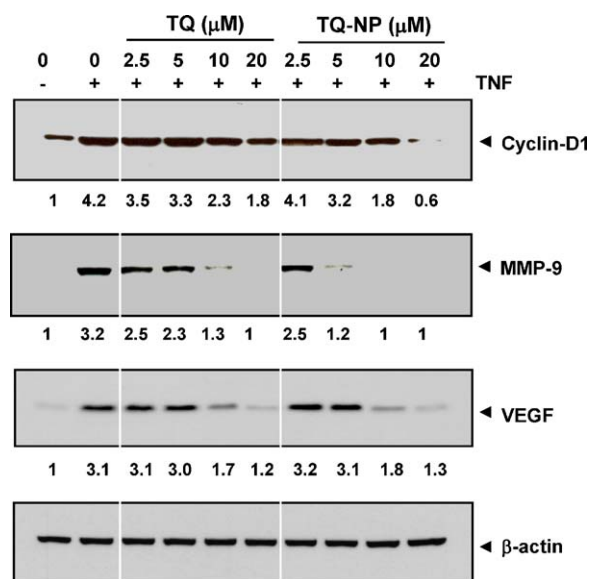


Fig. 5. Effect of thymoquinone nanoparticles on the expression of TNF-induced NF- κ B-regulated gene products. KBM-5 (1×10^6 cells/mL) cells were co-incubated with indicated concentration of TQ or TQ-NP and TNF (1 nM) for 16 h. The cells were harvested and analyzed for the expression of cyclin D1, MMP-9, and VEGF by Western blot. β -actin was used as a loading control. Densitometric values of bands were corrected based on β -actin and expressed relative to that of untreated cells, which was set at 1.0.

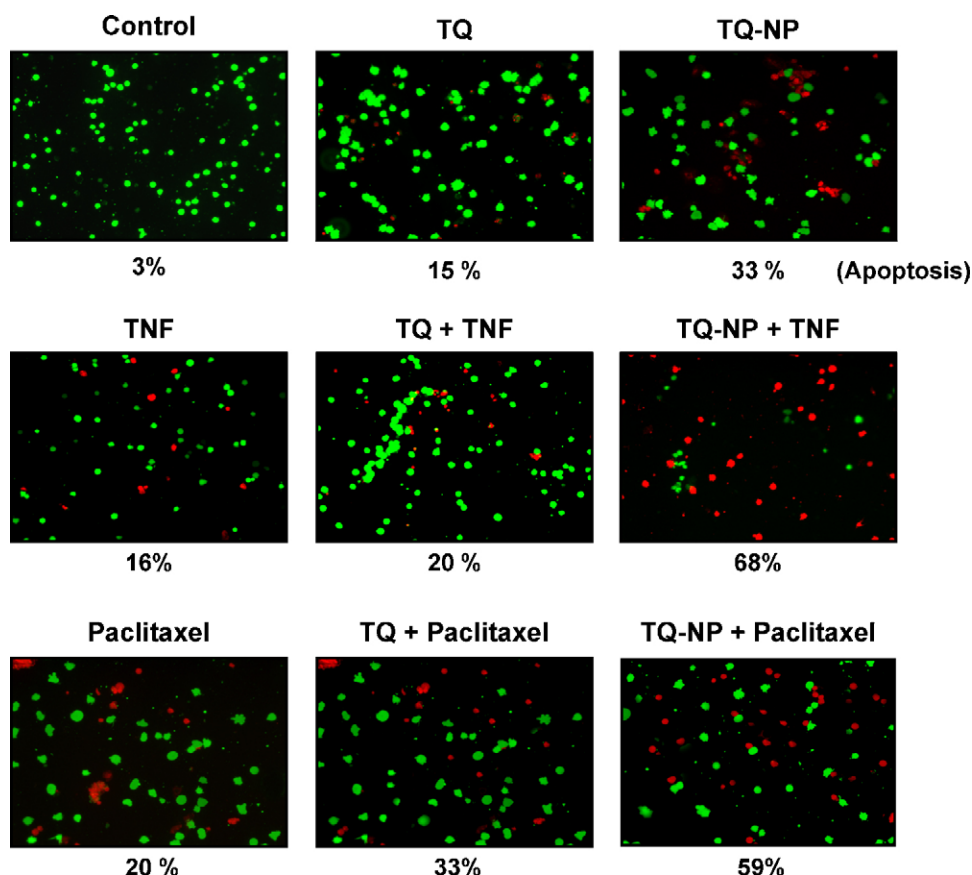


Fig. 6. Effect of thymoquinone nanoparticles on sensitization of tumor cells to TNF and paclitaxel. KBM-5 (1×10^6 cells/mL) cells were incubated with $10 \mu\text{M}$ of TQ and TQ-NP for 4 h. The cells were treated with TNF (1 nM) or paclitaxel (5 nM) for 16 h and were harvested and stained with Live/Dead assay reagent as per the manufacturer's protocol as described in Section 2.

have NF- κ B-binding sites in their promoters [18,34]. The ability of TQ-NP and TQ to modulate expression of these gene products in response to TNF was compared (Fig. 5). While TNF-induced cyclin D1 expression was partially inhibited by TQ at $20 \mu\text{M}$, complete inhibition was observed with TQ-NP at that dose. TNF-induced MMP-9 expression was partially inhibited by $10 \mu\text{M}$ TQ, but TQ-NP completely inhibited it. Interestingly, TQ and TQ-NP showed similar inhibitory effect on TNF-induced VEGF expression.

3.6. TQ-NP are more potent than TQ for sensitization of cells to TNF and chemotherapeutic agents

NF- κ B activation has been shown to suppress apoptosis induced by TNF and chemotherapeutic agents through the expression of gene products regulated by NF- κ B [35]. So we next compared the changes in TNF- and paclitaxel-induced cytotoxicity mediated by TQ-NP and TQ. We found that TQ-NP significantly enhanced the cytotoxic effects of TNF, from 33% to 68% vs. 15% to 20% with TQ (Fig. 6). We also found that TQ-NP significantly enhanced the cytotoxic effects of paclitaxel from 33% to 59% vs. 15% to 33% with TQ (Fig. 6).

4. Discussion

Bioavailability of a drug to the cells, whether *in vitro* or *in vivo*, is critical for its optimal efficacy. Agents which affect the metabolism of drugs, liposomal encapsulation or nanocapsulation methods are employed to enhance the bioavailability. To enhance the solubility of drugs in aqueous solvents, increase their bioavailability, enhance serum half-life, for tumor cell targeting and bioimaging

[36–39], nanotechnology has recently emerged as a new technology of choice. Conjugations of drugs with lipids, carbohydrates, and proteins have been used to make nanoparticles. Recent studies from our laboratory have shown that enhanced uptake of curcumin nanoparticles correlates with enhanced anti-tumor effects [40].

The current studies were designed to prepare TQ-NP and investigate their ability to inhibit proliferation of cancer cells, induce apoptosis, suppress NF- κ B and NF- κ B regulated gene products, and sensitizes cancer cells to cytokines and chemotherapeutic agents. These nanoparticles had high encapsulation efficiency (94%) and sustained release capacity. This is first report on the encapsulation of TQ in nanoparticles.

When examined for its ability to suppress the growth of leukemia cells, we found that TQ-NP were at least twice as potent as free TQ, possibly due to enhanced uptake. These effects were not specific to just leukemia but were also observed with colon, breast, prostate cancer cells and multiple myeloma cells.

Among various mechanisms, apoptosis is one of the mechanisms by which various agents kill tumor cells. As monitored for plasma membrane integrity by annexin V staining or Live/Dead assay, TQ-NP were again found to be more potent than TQ. Activation of NF- κ B pathway has emerged as a major pathway for proliferation of tumor cells, for chemoresistance, and inflammation. We found that the dose of TQ-NP ($10 \mu\text{M}$) that suppressed TNF-induced NF- κ B activation by more than 80%, TQ inhibited less than 20%. These results are similar to those recently observed with curcumin nanoparticles [40]. When examined for suppression of NF- κ B-regulated gene products cyclin D1, MMP-9, and VEGF, once again TQ-NP were significantly more potent than TQ. It is very likely that enhanced suppression of proliferation activity by TQ-NP

was due to enhanced suppression of cyclin D1 expression, which is closely linked to the G1/S transition. Our results also predict that TQ-NP will exhibit enhanced anti-metastatic activity, as both MMP-9 and VEGF have been closely linked with tumor metastasis.

Because NF- κ B activation has been linked with chemoresistance, we also examined the effect with TQ-NP on sensitivity to TNF and the chemotherapeutic agent paclitaxel. By both the annexin V method and the Live/Dead assay, TQ-NP were more active than TQ in potentiating the cytotoxic effects of TNF and paclitaxel. These results provide further evidence that TQ-NP have greater anti-cancer activity than free TQ.

Several studies have reported the anti-cancer activity of TQ not only *in vitro* but also *in vivo* [19–21]. The use of TQ-NP as described here should provide superior effects as compared to TQ. More studies are needed to investigate the potential of TQ-NP in animals. Nanoparticles of numerous natural products including ellagic acid [29], curcumin [30], quercetin [41], and coenzyme Q [27] have been reported, as have nanoparticles containing such chemotherapeutic agents as camptothecin [42], doxorubicin [32], or paclitaxel [43].

We recently reported silk fibroin and chitosan polymers blended non-covalently to encapsulate curcumin using the capillary-microdot technique [44]. These curcumin nanoparticles showed higher efficacy against breast cancer cells and had the potential to treat breast tumors by local, sustained, and long-term therapeutic delivery as a biodegradable system.

TQ-NP can be employed not only for treatment but also for prevention of cancer as recently reported with green tea polyphenols [45]. More recently Abraxane™, an albumin nanoparticle conjugate of paclitaxel, has been approved by FDA as anti-cancer agent in this emerging class of drug formulations [33]. Thus future studies will reveal the potential of TQ nanoparticles in animal studies and in humans.

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