



Reviewing the anticancer effects and underlying molecular targets of thymoquinone, a molecule with promising biological activities that was isolated 50 years ago, is of crucial importance for its clinical translation.

Thymoquinone: fifty years of success in the battle against cancer models

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Thymoquinone (TQ), the main active constituent of black seed essential oil, exhibits promising effects against inflammatory diseases and cancer. TQ, modulates signaling pathways that are key to cancer progression, and enhances the anticancer potential of clinical drugs while reducing their toxic side effects. Considering that TQ was isolated 50 years ago, this review focuses on TQ's chemical and pharmacological properties and the latest advances in TQ analog design and nanoformulation. We discuss our current state of knowledge of TQ's adjuvant potential and *in vivo* antitumor activity and highlight its ability to modulate the hallmarks of cancer.

Introduction

Thymoquinone (TQ) has been chemically synthesized for years by oxidation of thymol with hydrogen peroxide. This year marks the 50th anniversary of TQ's first isolation from a natural product when in 1963 it was identified in the essential oil of the *Nigella sativa* L. black seed, one of the most used plants in folk medicine in the Mediterranean region and West Asia [1]. Later, TQ was isolated from other plants with therapeutic properties namely *Eupatorium ayapana* [2], the leaves of several *Origanum* species [3], the heartwood essential oils of *Calocedrus decurrens* [4], the oil of different *Satureja* species [5], the aerial flowering parts of *Thymus vulgaris* L. [6] and from *Nepeta distans* Raul [7].

Nigella sativa commonly known in the Middle East as Habbatul Baraka or the 'seed of blessing' has curative potential as described in the Old Testament and by the prophet⁴ himself [1]. Black seed oil is traditionally used for enhancing immunity and combating inflammatory and respiratory diseases, among many disorders. Scientific research supports the folk medicine use of the oil as an antiinflammatory, antimicrobial, and antidiabetic agent [1].

Both TQ and its carbonyl polymer Nigellone have been mainly investigated for their anti-inflammatory and anticancer activities. For 10 years, we have studied the anticancer effects of TQ and provided evidence for its promise both *in vitro* and *in vivo*. What makes TQ interesting is that it

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is readily available from a plant source and is not toxic to normal tissues. Rather, TQ protects many organs from standard chemotherapy-induced damage, and enhances the efficacy of chemotherapeutic agents even in resistant cancers [1].

Nigella sativa and TQ's anti-inflammatory potential account for the observed analgesic, antidiabetic, and antihistaminic effects, and ability to alleviate respiratory diseases, rheumatoid arthritis, multiple sclerosis, and Parkinson's disease [8]. Three patents have been filed on behalf of TQ for the treatment of cancer, sepsis syndrome and urinary tract infections. The first was filed in 1999 for the treatment of parental and multi-drug resistant (MDR) human cancers (US 6218434 B1). Ten years later, a patent was filed for the treatment and prevention of sepsis syndrome (US 20100273893 A1) and a formulation containing TQ was patented for treating urinary tract inflammatory conditions in the following year (United States Patent Application: 20100028468). Moreover, TQ has demonstrated great potential against many microbes and parasites [8] and was shown to prevent atherosclerosis, osteoporosis, hypertension, and epilepsy [9,10]. A recent clinical study in children further supports TQ's antiepileptic role [11].

As of May 2013, the term "thymoquinone" brings up 345 search results in the Pubmed database. The first paper describing TQ's anticancer activity was published 35 years after its isolation. Approximately 70% of the papers on TQ appeared in the past five years, one-third of which describe its anticancer properties. This reflects increased recent interest in TQ's therapeutic properties, and underscores the necessity for reviewing its anticancer activities. For this reason, we sought to revisit the grounds which made TQ the promising anticancer agent it is today by emphasizing its effects on the universal cancer hallmarks and the underlying signaling mechanisms.

TQ isolation and chemical characterization

The yellow crystalline molecule TQ (2-methyl-5-isopropyl-1,4-benzoquinone) was isolated using thin layer chromatography on silica gel [12]. TQ has a basic quinone structure consisting of a para substituted dione conjugated to a benzene ring to which a methyl and an isopropyl side chain groups are added in positions 2 and 5, respectively (Fig. 1). The crystal structure of TQ was later determined by high-resolution X-ray powder diffraction [1]. For

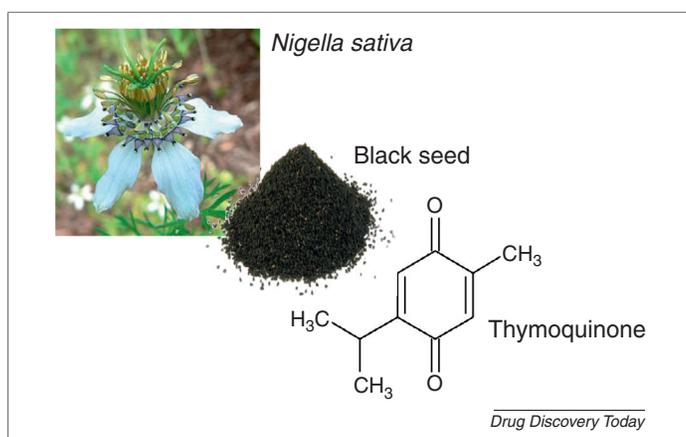


FIGURE 1

Illustration of the black seed and *Nigella sativa* plant, and the chemical structure of thymoquinone (TQ).

quantifying TQ, several methods including chromatography, high performance liquid chromatography (HPLC), and differential pulse polarography have been described [1]. TQ reacts readily with amino or thio groups of amino acids [13], and undergoes a series of oxido-reduction reactions (Fig. 2) resulting in the formation of semiquinone ($1e^-$ reduction) and thymohydroquinone ($2e^-$ reduction). The semiquinone can undergo redox cycling leading to the generation of reactive oxygen species (ROS).

Pharmacological properties of TQ

TQ was found to exert its biological functions by modulating the physiological and biochemical processes involved in ROS generation. In normal tissues, TQ acts as a strong antioxidant and inhibits the production of superoxide radicals and lipid peroxidation, or increases the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione (GSH), glutathione transferase and quinone reductases [9,10,14,15]. In tumors, however, TQ induces ROS generation [16,17] and decreases GSH levels in a dose-dependent manner [17,18]. Thus TQ has a dual role, and depending on the cellular microenvironment, it may act as an antioxidant or a pro-oxidant.

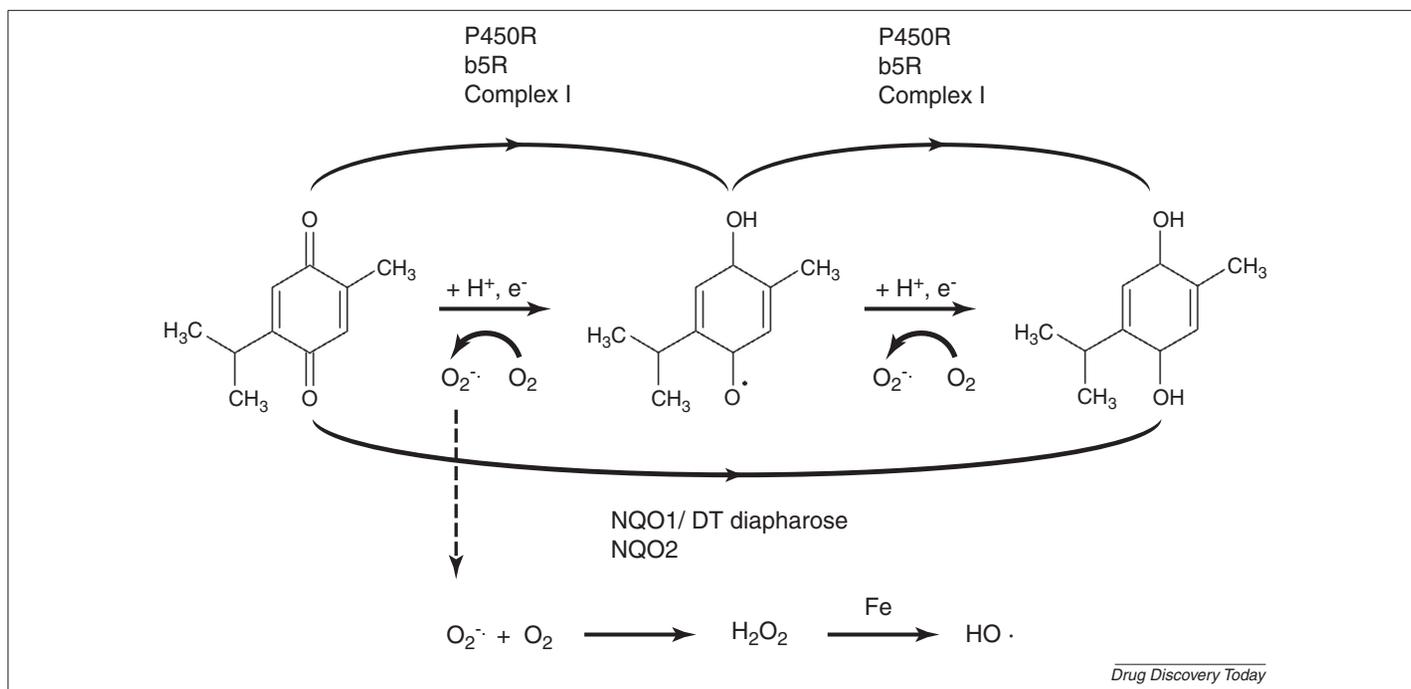
TQ targets several proteins and is one of the rare natural products that can inhibit protein-protein interactions [19]. It reacts with the antioxidant enzyme GSH, NADH and NADPH to yield the two strong antioxidants, glutathionyl-dihydrothymoquinone and dihydrothymoquinone [20]. It also binds to human α_1 -acid glycoprotein, bovine serum albumin, and the phosphoserine/phosphothreonine recognition site of the polo-box domain (PBD) [21]. TQ inhibits the activity of polo-like kinase 1 (Plk1) by blocking its PBD-dependent interaction, thus interfering with its localization [19]. GSH and proteins with PBDs are promising targets for cancer therapy [22].

Unfortunately, there are few studies on the pharmacokinetic and pharmacodynamic properties of TQ. In a recent study, TQ was detected in the plasma of rats for up to 12 hours after oral administration [23]. Using a streptavidin/biotin system of detection, TQ was found to accumulate in the nuclei of potoroo kidney cells [24]. There is strong evidence for the localization of a TQ analog in compartments of the nuclei and Golgi apparatus using a highly specific visualization method based on alkyne-azide cycloaddition [24]. The identification of all TQ binding targets as well as its distribution profile *in vivo* can greatly contribute to the better understanding of its mechanism of action, hence the need for further studies in this domain.

TQ analogs and nanoparticle formulations

Hydrophobic drugs commonly show poor bioavailability when given to animals due to their low absorption capacity, which results in low amounts of drug reaching the target tumor and increased toxicity to normal tissues. Considering that TQ is a hydrophobic molecule, many laboratories have attempted to synthesize more soluble TQ analogs and to encapsulate it in nanoformulations.

Two different studies have conjugated TQ with fatty acids to enhance its membrane penetration capacity and antitumor activity [9,10]. In both studies, the antitumor activity of TQ was significantly improved by attaching fatty acid derived groups to the molecule (Fig. 3a,b). Terpenes were also conjugated with TQ

**FIGURE 2**

Thymoquinone oxido-reduction reactions. One electron reduction of TQ is catalyzed by several reductases, including microsomal nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase, microsomal nicotinamide adenine dinucleotide (NADH) cytochrome b5 reductase, and mitochondrial NADH ubiquinone oxidoreductase (complex I). Alternatively, the cytosolic flavoenzymes NAD(P)H quinone acceptor oxidoreductases can catalyze the two electron reduction of TQ resulting in the direct formation of thymohydroquinone known for its antioxidant effects.

and the resulting analogs were found to exert greater antitumor activities than the parent drug while exerting no toxicity in nonmalignant foreskin fibroblasts [25]. The triterpene betulinic acid conjugate of TQ (Fig. 3d) was a potent derivative exhibiting up to 200-fold greater activity in HL-60 leukemia cells in comparison to the control. These results are promising in view of the fact that betulinic acid is currently undergoing clinical trials at the National Cancer Institute (NCI) [25]. Another group engineered 27 different TQ analogs by reacting quinone building blocks with various amines and testing their anticancer activity against several pancreatic cancer cell lines [26]. In this study, three analogs were found to be more effective than TQ and are being patented [26]. A synthetic derivative of TQ, poloxin (Fig. 3e), has been tested against HeLa cells and MDA-MB-231 human breast cancer cell lines and in mouse mammary xenografts [22]. Treatment with poloxin caused cell cycle arrest and apoptosis due to centrosome destabilization and mitotic spindle disruption. Interestingly, poloxin showed promising *in vivo* effects as evidenced by the reduced growth of mammary tumors [22].

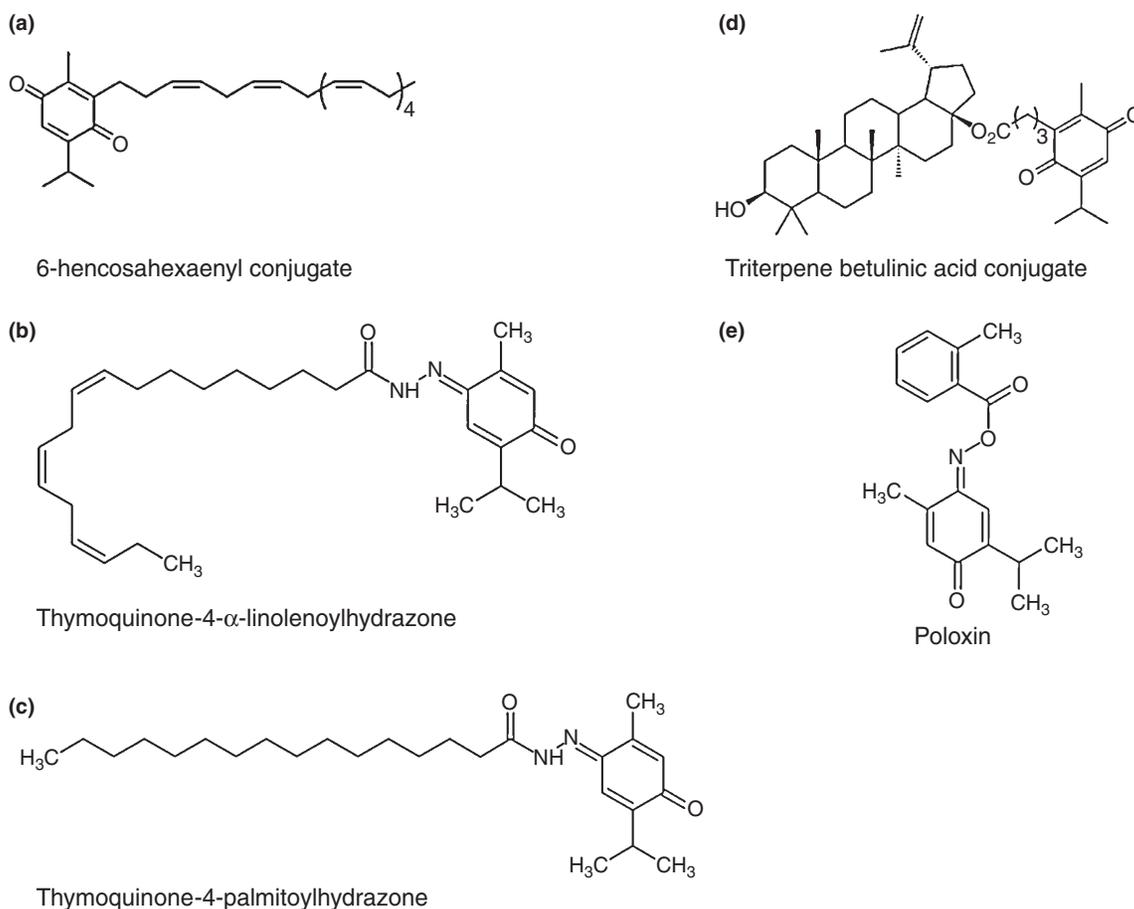
Nanoparticles (NPs) are drug delivery systems with properties that enable enhanced drug activity and reduced side effects. The encapsulation of water-insoluble drugs in nanoparticles increases their bioavailability, protects the drugs from exposure to external factors, limits their diffusion to normal tissues and prevents their rapid metabolism. Because of the leaky nature of tumor vasculature along with poor lymphatic drainage, nanoparticles often accumulate near the tumors which results in better targeting and reduced exposure of non-specific sites to the drug. TQ nanoparticles (TQ-NPs) are believed to hold greater promise than free TQ for two main reasons: (1) their enhanced activity due to better

targeting of the cancer hallmarks *in vitro*, and (2) their improved *in vivo* bioavailability and distribution.

So far, eight TQ nanoparticle formulations have been described including three that have been tested *in vitro* against breast, colon and prostate cancer cell lines, as well as leukemia and multiple myeloma. The types and characteristics of the different TQ-NPs are summarized in Table 1. In one study, TQ was encapsulated in nanoparticles using biodegradable poly (lactide-co-glycolide) (PLGA) and the stabilizer polyethylene glycol (PEG)-5000 [27]. The resulting NPs showed enhanced antiproliferative, anti-inflammatory, and chemosensitizing effects when compared to the free drug. Proliferation inhibition was enhanced from 15% to 85% in HCT-116 colon cancer cells, 30–88% in MCF-7 breast cancer cells, 30–85% in PC-3 prostate cancer cells, and 55–70% in U-266 multiple myeloma cells. Furthermore, TQ-NPs exhibited two-fold greater efficacy than free TQ in KBM-5 human leukemia cell line and had a greater sensitizing potential when applied before tumor necrosis factor (TNF) or paclitaxel.

Another study reported that the encapsulation of TQ in molecular micelles poly(D,L-lactide-co-glycolide) modified PLGA NPs resulted in slower release of the drug *in vitro* (Table 1) and more effective antitumor effects in MDA-MB-231 cells [28].

TQ liposomal carriers have been also prepared, characterized and tested against MCF-7 and T47D breast cancer cell lines and normal periodontal ligament fibroblasts [29]. As expected, the drug carriers were effective in inhibiting the proliferation of cancer cells and were non-toxic to normal fibroblasts. The TQ-liposomes were, however, less potent in inhibiting cell viability than free TQ which was due to the negligible release of TQ from the liposomes. Recently, two amphiphilic TQ nanoparticle formulations were



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FIGURE 3

Chemical structures of thymoquinone analogs: (a) is a fatty acid conjugate attached at C4; (b) and (c) are both hydrazone derivatives; (d) is a terpene analog; (e) is a synthetic derivative of TQ.

prepared, using the polyhydroxyalkanoates (PHA)–monomethoxy PEG (mPEG) and the poly([R]-3-hydroxyvalerate)-block-monomethoxy poly(ethylene glycol) (PHV-block-mPEG) diblock copolymers, respectively [30,31]. The formulations caused slower release of TQ after an initial burst release phase and were non-toxic to neuronal cells.

Additionally, two groups encapsulated TQ in solid lipid NPs (SLNs) and studied *in vitro* and *in vivo* drug release profiles [23,32]. Pathan *et al.* (2010) prepared a formulation having biphasic sustained release of TQ *in vitro* with an initial rapid release phase followed by a slower release one. Upon oral administration and quantification of TQ in the plasma of rats, a two-fold increase in bioavailability of TQ-SLNs was noted in comparison to the parental drug. Singh *et al.* (2013) also report a formulation with *in vitro* controlled drug release properties *versus* time dependent release by TQ suspension, and demonstrate more drug release by the carriers that could be possibly facilitated by the greater surface area. Similarly, upon SLNs administration, there was five-fold increase in bioavailability in the plasma of rats, and the amount of TQ in many organs was much higher. Biologically, the formulation had hepatoprotective effects and was successful in liver protection against cirrhosis. Chitosan nanoparticles of TQ were recently described [33]. TQ release was sustained after an initial burst phase.

In vivo, the nanoparticles distribution was assessed in different organs after intranasal or intravenous administration and showed high targeting of the brain after intranasal administration.

Modulation of cancer hallmarks by TQ

Cancer development is a progressive multistep process during which normal cells acquire traits that enable their transformation into malignant tumors. These traits, also called the hallmarks of cancer were described by Hanahan and Weinberg in 2000 and subsequently revised by them in 2011 [34].

The ten cancer hallmarks [34] are common to most tumors. Drugs which interfere with one or more of these hallmarks are considered promising anticancer therapeutics, with some even making it to the clinic. TQ holds great promise for clinical translation, considering that it affects all of cancer hallmarks except 'avoiding the immune system' which has not been investigated to date (Fig. 4).

Moreover, organs like the kidneys, liver, and heart are unaffected or even protected by TQ administration (Table 2) which modulates their antioxidant state as well as their metabolism, thus providing strong support for its further clinical development as a safe chemotherapeutic agent. In addition, normal cells including human pancreatic ductal epithelial (HPDE) cells [35], prostate

TABLE 1

Thymoquinone nanoparticle formulations and characteristics

Type	Method	Size in nm	EE %	LC %	Release	Pharmacokinetics/dynamics	Ref.
PLGA-PEG 5000 NPs	Nanoprecipitation	150–200	97.5	N/A	N/A	N/A	[27]
Micelles poly(D,L-lactide-co-glycolide) modified PLGA NPs	Emulsification solvent evaporation	100–200	11–35	N/A	Rapid release in the first 8 hours Lower than free TQ: 40–60% versus 80% at T = 1 hour	N/A	[28]
Amphiphilic PHA–mPEG copolymeric NPs	Emulsification solvent evaporation	110–160	29–57	N/A	Burst release (30–40%) in the early phase followed by sustained and decreased release compared to rapid TQ release (70% in 6–8 hours)	N/A	[30]
SLNs	Ultrasonication	N/A	59	28	Biphasic: initial rapid release phase followed by a slower sustained release phase	Two-fold increase in availability of TQ in plasma of orally administered rats	[23]
Amphiphilic diblock copolymer poly([R]-3-hydroxyvalerate)-block-monomethoxy poly(ethylene glycol) (PHV-block-mPEG)	Modified emulsion-solvent evaporation	200	27	N/A	Extended release of TQ after an initial burst phase	N/A	[31]
SLNs	Solvent injection	166	72	N/A	Controlled release versus time dependent release of TQ	Five-fold increase in availability of TQ in plasma of orally administered rats, higher concentration of drug in major organs, decrease of levels of liver damage enzymes	[32]
CS NPs	Ionic gelation	150–200	63	31	Burst rapid release followed by sustained slow release	High drug targeting to the brain after intranasal administration	[33]
-Liposomes -Triton X-100 modified liposomes	Thin film hydration technique	100	–90 –49.6	N/A	Negligible	N/A	[29]

EE: entrapment efficiency; LC: loading capacity; N/A: Not applicable.

epithelial (BPH-1) cells [36,37], and normal human intestinal (FHs74Int) cells [38] are resistant to TQ.

In an attempt to better understand and evaluate TQ's therapeutic potential, below we will summarize the cancer hallmarks and the target molecules modulated by this promising molecule (Fig. 5).

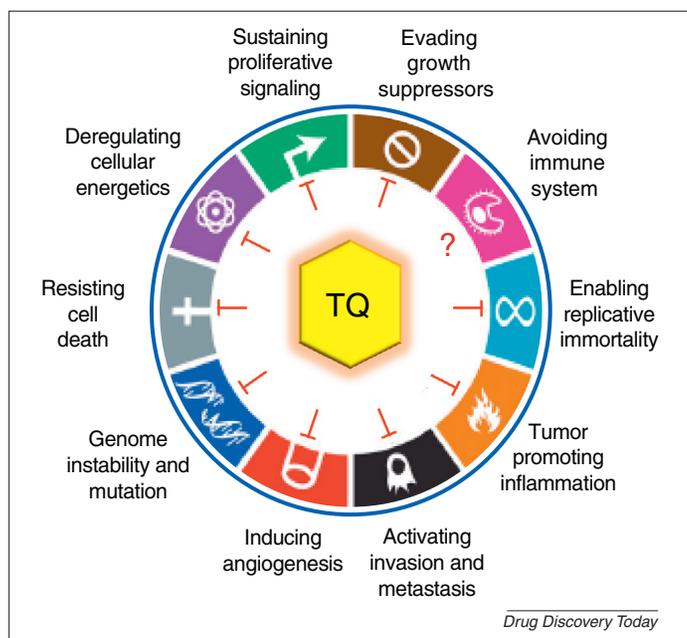
Sustaining proliferative signaling

One of the universal characteristics of cancer cells is their uncontrolled proliferation which causes tumors to increase in size and become difficult to treat. TQ inhibits the proliferation of many cancer cell lines, including several multi-drug resistant cell line variants which are up to ten times more resistant to the standard chemotherapy drugs doxorubicin (dox) and etoposide [39].

TQ's antiproliferative effect has been linked to its capacity to deregulate both the mitogen-activated protein kinase (MAPK) and protein kinase B (AKT/PKB) signaling pathways in multiple myeloma [40], squamous cell carcinoma [41], and human prostate cancer cell lines [42]. Other studies have described the effects of TQ

on either the AKT or the MAPK pathway separately. As expected, AKT phosphorylation was inhibited by TQ in breast tumors [43], as well as in primary effusion lymphomas [44]. In addition, the inhibition of AKT phosphorylation was associated with PTEN upregulation and ROS generation [43,44], while the downregulation of vascular endothelial growth factor (VEGF) was linked to the inhibition of ERK phosphorylation [42].

By contrast, we have documented that TQ treatment of DLD-1 human colon cancer cells activates the c-Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinases (ERKs) proteins of the MAPK family [38]. Two additional studies showed that JNK and p38 proteins are activated upon TQ treatment in prostate and pancreatic cancer cells, respectively [37,45]. Even though TQ effects on the MAPK pathway are conflicting with previous reports [41,42], it successfully attenuated cancer cell proliferation in these cell lines. The activation or inhibition of the MAPK protein family seems to depend on the cell type and possibly on the concentration of TQ used. Our recent attempt to identify key protein kinase targets of TQ in HCT-116 human colon cancer cells using a

**FIGURE 4**

The cancer hallmarks modulated by TQ. TQ affects nine of the ten cancer hallmarks identified by Hanahan and Weinberg. The effects of TQ on avoiding the immune system have not been investigated to date and are represented by (?). Modified from Hanahan and Weinberg [34], after their approval.

PEPSCAN array showed that the top TQ-regulated pathways were cell cycle control and cancer signaling molecules (R. Schneider-Stock *et al.*, unpublished). We identified 11 proteins regulated by TQ that are common in apoptosis, proliferation, and inflammation pathways and include the key molecules in the AKT-MEK-ERK pathway which is the central signaling network for current targeted therapies. Overall, there is evidence that TQ modulation of the AKT and MAPK pathways is strongly associated with its anti-proliferative potential. The exact mechanisms underlying the modulation of MAPK proteins by TQ are not fully understood and further investigations should be undertaken to decipher these mechanisms.

Evading growth suppressors

Cyclin dependent kinases (CDKs) and cyclins regulate the transitions between G1, S, G2 and M phases of the cell cycle. In almost all human cancers, the CDKs and cyclins levels are deregulated to induce transcription of cell cycle genes, avoid checkpoints and enable mitosis. TQ is effective in disrupting cancer cell evasion from growth suppressor's signals by targeting several of the CDKs, CDK inhibitors, as well as cyclins. We have shown that TQ induces G1 cell cycle arrest by inhibiting the expression of the CDK inhibitor p16 and downregulating cyclin D1 in human colon cancer HCT-116 cells [46]. Others have documented induction of G1 arrest by TQ *via* the increased expression of the CDK inhibitor p21 [1]. The increased expression of p21 is believed to block CDK2, CDK4 and CDK6, thus resulting in G1 arrest [46]. TQ also caused G0/G1 cell-cycle arrest in several cell models including lymphoblastic leukemia Jurkat [47], multiple myeloma [48], and skin papilloma SP-1 cells [49]. This arrest was associated with a decrease in cyclin D1 protein levels and increase in p16 protein expression levels.

Another study conducted on androgen dependent LNCaP prostate cancer cells revealed that TQ caused G1/S arrest and significantly increased p21 and p27 protein levels. This resulted in a decrease in androgen receptor, cyclin A, E2 Transcription Factor (E2F-1), and E2F-1-regulated proteins which are required for the progression from G1 to S phase [36]. Similarly, G2/M arrest in doxorubicin-resistant breast cancer cells involved an increase in p53 levels, and decrease in cyclin B1 and cdc25 levels [43]. In I-7 spindle carcinoma cells, we demonstrated that TQ causes G2/M cell-cycle arrest which is associated with an increase in the expression of the tumor suppressor protein p53 and a decrease in cyclin B1 protein expression [49].

Collectively, these studies show that TQ has the ability to arrest cells at different phases of the cell cycle leading to growth inhibition. The upregulation of the expression of the tumor suppressor p53 protein and its transcriptional target p21 are the main mechanisms through which TQ disrupts cancer evasion from growth suppressors.

Enabling replicative immortality

It is well established that cancers overcome proliferation barriers and become immortal. In normal cells, the telomere length dictates how many replication cycles the cell can undergo [34]. In the absence of telomerase activity, telomeres shorten and consequently lose their ability to protect the cell, leading to a state of crisis which triggers senescence or apoptosis. In most cancer cells, telomerase is activated allowing unlimited replication capacity, and ultimately an escape from senescence and apoptosis [34].

The inhibition of telomerase has been extensively studied as a possible target for cancer therapy [34]. TQ has been shown to inhibit telomerase and to induce telomere shortening and apoptosis in glioblastoma cell lines [50]. In the latter study, TQ inhibited telomerase telomere attrition and the cells that had DNA dependent protein kinases (DNA-PKcs) exhibited greater sensitivity to TQ than the deficient ones. Hence, telomere length shortening in response to TQ is dependent on the presence of DNA-PKcs [50].

Genome instability and mutation

Cancer development and progression is orchestrated by a large number of mutations and repair defects which occur because of genome instability. Compromised check points and sensitivity to mutagenic agents increase the rate of mutation. When the defect is in a tumor suppressor gene, the detection and repair of damaged DNA is halted. Hence, several researchers have assessed the effect of TQ on tumor suppressor genes [51]. Through its ability to induce apoptosis in numerous cancer cell lines, TQ does help the genome to overcome the instability acquired during tumor formation. TQ's mechanism of action has been linked to the modulation of the most studied tumor suppressor gene p53, also known as the guardian of the genome. Patients with inactive or mutated p53 show resistance to traditional chemotherapies. In colon cancer, TQ induction of DNA damage was p53-dependant [46]. In leukemia, osteosarcoma, and glioblastoma cell lines, TQ induced DNA damage in p53 independent manner [50,52,53]. In p53 mutant cells, Roepke *et al.* (2007) reported that the levels of the DNA damage sensor, gamma H2AX, and double-strand break repair complex member (NBS1) remained upregulated indicating the

TABLE 2

The mechanisms of protection of chemotherapy induced toxicity by thymoquinone

Agent	Toxicity	Model	Protection mechanism	Ref.
Doxorubicin	Cardiotoxicity Nephrotoxicity	Rat, mouse	-Scavenging superoxide anion radicals; reduction of serum LDH and creatine kinase levels -Lowering serum urea, triglycerides, total cholesterol, lipid peroxides and increasing non-protein sulfhydryl content and catalase activity	[73,74] [78]
Cyclophosphamide	Cardiotoxicity Hepatotoxicity Lung injury	Rat	-Decreasing oxidative and nitrosative stress (decrease in thiobarbituric acid reactive substances, total nitrate/nitrite levels and increase in reduced GSH, glutathione peroxidase, catalase, and ATP levels -Antioxidant effects, increasing liver enzymes activities to normal, decreasing bilirubin, urea, creatinine, lipids and lipid peroxidation -Inhibition of lipid peroxidation, stimulation of GSH	[76] [75] [77]
Carbon tetrachloride	Hepatotoxicity	Mouse	Antioxidant effects, reduction of serum enzymes and MDA levels in the liver and increase of the nonprotein sulfhydryl (-SH) concentration	[14]
Cadmium	Hepatotoxicity	Mouse	Protein oxidation reduction and recovering of antioxidants	[81]
Aflatoxin (B1)	Hepatotoxicity	Mouse	Restoring MDA and GSH levels	[82]
tert-Butyl hydroperoxide	Hepatotoxicity	Rat	Decreasing leakage of cytosolic enzymes and restoring GSH levels	[83]
Acetaminophen	Hepatotoxicity	Mouse	Decreasing alanine aminotransferase, total nitrate/nitrite, lipid peroxides and reversing GSH and ATP decrease	[84]
Cisplatin	Nephrotoxicity	Rat	Decreasing MDA, 8-isoprostane, multidrug resistance-associated proteins and increasing organic cation transporters	[72]
Vancomycin	Nephrotoxicity	Rat	Decreasing MDA levels and restoring the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)	[85]
Mercuric chloride	Nephrotoxicity	Rat	Restoring antioxidant enzymes activities and decreasing serum creatinine	[86]
Gentamycin	Nephrotoxicity	Rat	Decrease in blood urea nitrogen, creatinine, thiobarbituric acid-reactive substances, total Nitrate/nitrite (NOx) and recovery of GSH, GPx, CAT and ATP levels	[87]
Toluene	Neurotoxicity Lung injury	Rat	-Morphologically attenuates neurodegeneration enlargement of intercellular spaces in Sertoli and spermatid cells -Inhibition of the inflammatory response (iNOS) and increase of expression of surfactant protein D	[88] [92]
Alcohol	Gastrotoxicity	Rat	Decreasing thiobarbituric acid-reactive substances, GSH levels and increase superoxide dismutase and glutathione-S-transferase activities	[89]
Azoxymethane	Genotoxicity	Rat	Decrease of MDA levels/Not defined	[90]
DMH	Erythrocyte damage	Rat	Restoring MDA levels, catalase, glutathione peroxidase, and superoxide dismutase activities to normal	[91]
Methotrexate	Testicular injury	Mouse	Decrease of antioxidant and myeloperoxidase activities and reduction of interstitial space dilatation	[93]

Thiobarbituric acid-reactive substances (TBARS) are an index of lipid peroxidation; malondialdehyde (MDA) is an index for liver damage.

beginning of a DNA repair mechanism. In accordance, Muhtasib *et al.* (2008) studied the effect of TQ on the cell-cycle checkpoint kinase CHEK1 in colorectal cancer. In the absence of transcriptional repression, normally established by the presence of p53, CHEK1 levels were significantly increased after TQ treatment confirming the onset of DNA repair.

The expression of another tumor suppressor gene, phosphatase and tensin homolog (PTEN), was upregulated in breast cancer cells in response to TQ exposure [43]. In this study, TQ induced DNA damage along with increases in p53 and p21 protein levels which led to cell cycle arrest in the G2/M phase. Similarly, in prostate cancer cells, TQ upregulated the expression of GADD45 alpha, a growth arrest and DNA damage-inducible gene, indicating its possible involvement in drug-induced apoptosis [37].

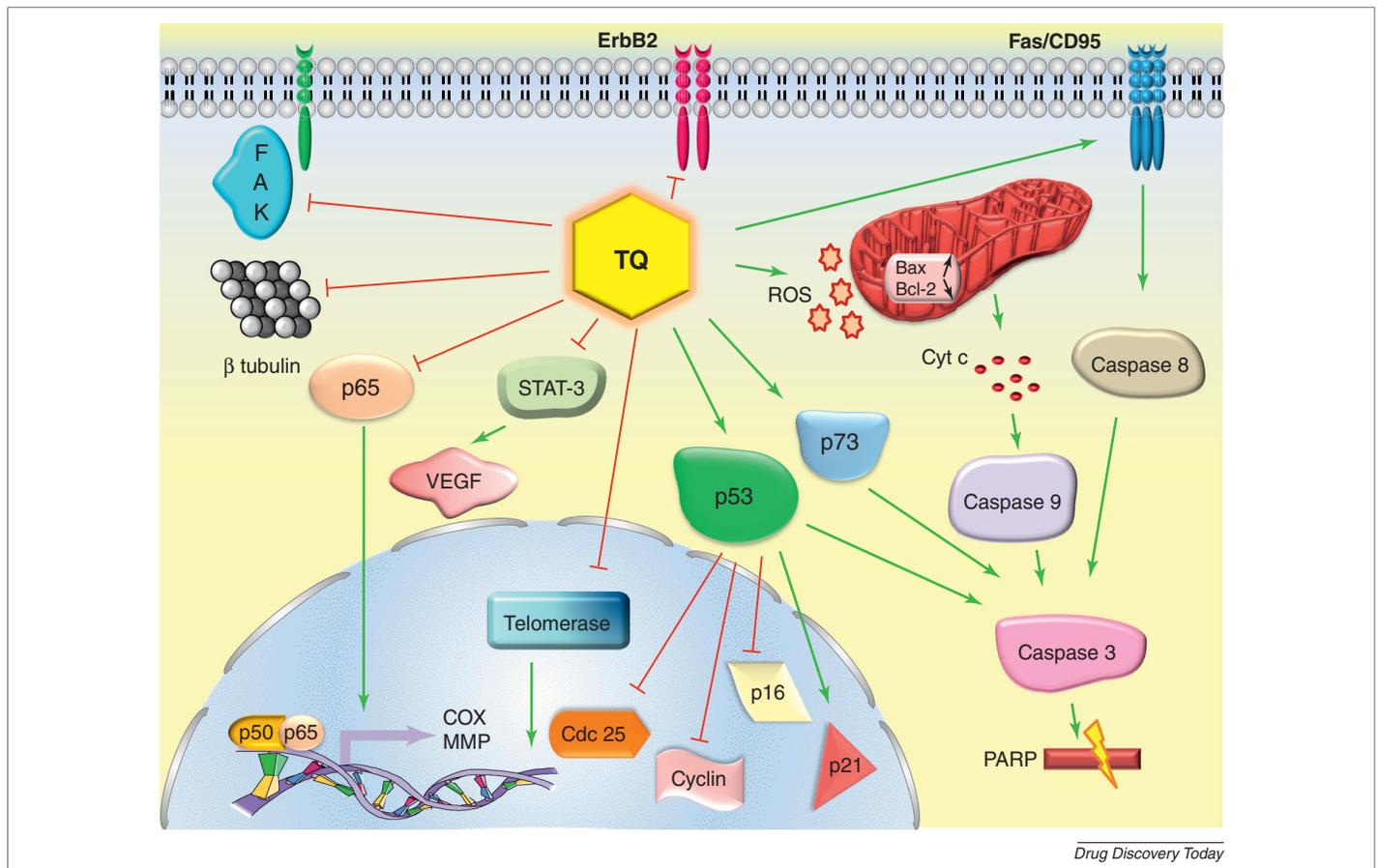
Tumor promoting inflammation

Chronic inflammation accounts for approximately 20% of all human cancers. In fact, some of the most common inflammatory mediators namely prostaglandins (PGs), leukotrienes (LTs) and interleukins are known to play an important role in promoting tumor progression by activating cell proliferation, angiogenesis,

migration, and invasion. Only three studies have so far assessed the effect of TQ on inflammation in cancer cells. However, when investigated in multiple inflammation models, TQ was able to alter the expression levels and activities of all aforementioned inflammatory mediators, which highlights its great potential in regulating inflammation-driven cancer hallmarks.

In inflammation models, the inhibition of PGs involved the inhibition of cyclooxygenase-2 (COX-2) enzyme which produces PGs from arachidonic acid upon its stimulation [54]. The production of LTs was similarly reduced by attenuation of the activities of the LT-C4-synthase and the 5-lipoxygenase (5-LOX) enzyme which convert the precursor of the arachidonic acid to LTs [55]. The production of IL-1 alpha and beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10 and IL-13 were all decreased in response to TQ in different cell types [9,10]. The pro-inflammatory cytokine TNF alpha was also identified as a TQ target in rat basophil cells [56]. TNF alpha is a potent activator of the nuclear factor- κ B (NF κ B). Hence, treatment with TQ disrupted the transactivation of NF κ B also in these cells.

In cancer cells, TQ was shown to modulate the inflammatory signals mainly through the NF κ B pathway [57]. NF κ B plays a major



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FIG. 5

Key signaling pathways and molecules modulated by TQ. TQ prevents cancer cells from evading growth suppressors by targeting cdk (cdc 25 inhibition), cdk inhibitors (p21 activation and p16 inhibition), as well as inhibiting cyclins. It prevents replicative immortality by inhibiting telomerase activity. TQ also induces the generation of ROS and triggers p53 or p73 dependent apoptosis. Apoptosis by TQ occurs mostly via the intrinsic pathway through Bax upregulation and Bcl-2 downregulation, causing mitochondrial disruption, release of cytochrome c and caspase activation or may involve the extrinsic pathway by activation of FAS/CD95 death receptor. TQ also triggers apoptosis by the degradation of α/β tubulin in cancer cells. The modulation of inflammatory signals by TQ occurs mainly through the inhibition of the NF κ B pathway whereby the nuclear translocation of the p65 protein and the expression of genes coding for COX2 and MMP enzymes are inhibited. TQ also reduces angiogenesis by VEGF downregulation that is associated with STAT-3 deactivation. Finally, TQ inhibits invasion and metastasis of cancer cells by downregulation of FAK on one hand, and downregulation of NF κ B and MMPs on the other.

role in development and progression of 15% of all solid tumors. Over 500 genes are regulated by NF κ B: interleukins, COX-2, and inducible nitric-oxide synthase (iNOS) being among the main targets of NF κ B. Composed of the two subunits p50 and p65, NF κ B is only active after translocation into the nucleus and dimerization. I κ B inactivates NF κ B by retaining it in the cytoplasm. In response to proinflammatory mediators, I κ B gets phosphorylated by IKK which causes its degradation and subsequent activation of NF κ B.

In myeloid leukemia cancer cells, TQ inhibited NF κ B activation in a dose- and time-dependent manner [57]. TQ prevented I κ B degradation by TNF alpha as well as IKK activation. As expected, TQ inhibited nuclear translocation of p65 and binding to DNA. Similarly, in pancreatic ductal adenocarcinoma cells, TNF-induced NF κ B activation and nuclear translocation was reduced by TQ [58]. The expression levels of TNF alpha, IL-1 beta, COX-2 and that of a novel target, the monocyte chemoattractant protein-1 (MCP-1) were also decreased at the transcriptional level [58]. Both TQ and TQ-NPs were shown to inhibit NF κ B and decrease MMP-9 levels in KBM-5 cell line with TQ-NPs being more potent inhibitors in comparison to free TQ

[27]. In summary, TQ holds great potential for inhibiting pro-inflammatory mediators especially by regulating the TNF alpha-NF κ B pathway. Nevertheless, more studies that describe TQ effects on cancers caused by chronic inflammation are needed.

Resisting cell death

In normal cells, apoptosis is a highly regulated process which takes place in response to cellular abnormalities, namely the lack of antiapoptotic proteins (Bcl-2, XIAP, survivin, CHEK1), elevated proapoptotic signals (Bax) and/or DNA damage (H2AX) [34]. Avoiding apoptosis is a key strategy by which cancer cells can proliferate and develop.

Apoptosis is induced by TQ in many cancer cell lines mainly by the activation of the intrinsic pathway, although the involvement of the extrinsic apoptotic pathway through the Fas/CD95 death receptor in malignant plasma cells has been documented [40]. The increase in p53 levels [16,43,46,59] and ROS generation [17,37,38,44,47] are the main triggers for TQ-induced apoptosis in cancer cells, even though p53 independent mechanisms have been also reported [47,52,53,60–62].

We were the first to show that treatment with TQ increases p53 protein levels in HCT-116 human colon cancer cells. Similar effects were later reported in cervical squamous carcinoma cells and in the MCF-7/Dox cell line [43,59]. The mechanism of p53 upregulation in MCF-7/Dox cells involved an increase in the expression of the tumor suppressor PTEN followed by the downregulation of AKT [43]. We later demonstrated that the inhibition of p53 in HCT-116 colon cancer cells can attenuate apoptotic cell death [46]. Human colon cancer HCT-116 containing p53 were much more sensitive to TQ than p53 null cells as a result of the much higher levels of the survival protein CHEK1 in the latter cells [16]. The expression of p53 or the silencing of CHEK1 in the p53 null HCT-116 cells sensitized them to TQ, confirming the involvement of p53 in TQ-triggered apoptosis.

By contrast, TQ was shown to trigger apoptosis in myeloblastic leukemia and osteosarcoma cell lines lacking p53 or harboring mutant p53 [52,53]. TQ also triggered p53-independent cell death in lymphoblastic leukemia cell lines [47]. It was established that the p73 signaling pathway was responsible for cell death by TQ, and that the mechanism of anticancer activity involved the repression of UHRF1, the antiapoptotic and epigenetic integrator upstream of p73 [47]. Later, it was determined that the downregulation of the cyclic nucleotide phosphodiesterases was responsible for UHRF repression and p73 upregulation [62]. The upregulation of p73 in response to TQ was also reported in human astrocytoma and Jurkat cell lines. Interestingly, in these two cell lines the upregulation of p73 was associated with α/β tubulin degradation while no effects were observed in normal human fibroblasts [61].

The increase in Bax/Bcl-2 ratio causes mitochondrial membrane disruption which is associated with the release of cytochrome c from mitochondria and subsequent caspase activation. TQ has been shown to induce apoptosis by the deregulation of the Bcl-2 protein family [49,59,63] followed by mitochondrial disruption and release of cytochrome c [17,52,63], downregulation of anti-apoptotic proteins such as the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP) and survivin [48,51,57,63], the activation of caspases [17,43,52,53,63] and consequently the cleavage of the effector of apoptosis, poly(ADP-ribose) polymerase (PARP) [17,52,63]. An increase in the Bax/Bcl-2 ratio in response to TQ has been observed in MDA-MB231 human breast cancer [51], MCF-7/DOX [43], myeloblastic leukemia HL-60 [52] and neuroblastoma cell lines [63]. TQ treatment of DLD-1 human colon cancer cells [38] and MCF-7/DOX cells [43] led to the excessive loss of the mitochondrial potential. We have shown significant inhibition of Bcl-2 protein expression by TQ in HCT-116 human colon cancer cells [46], and enhanced Bax/Bcl-2 ratio caused by a decrease in Bcl-xL protein expression in I7 spindle carcinoma cells [46]. In MDA-MB231 cells, TQ decreased the protein expression levels of Bcl-2, Bcl-xL and survivin by upregulating the PPAR- γ signaling pathway [51]. In a recent study, TQ was found to downregulate several proteins of the Bcl-2 family including BAG-1, Bcl2, Bcl2A1, Bcl2L1 and BID in androgen receptor (AR)-independent (C4-2B) and AR naïve (PC-3) prostate cancer cells [37]. Moreover, in multiple myeloma, TQ was found to induce the expression of Src homology-2 phosphatase 2 and inhibit STAT3 activation and the expression of Bcl-2, Bcl-xL, survivin and Mcl-1 proteins which are regulated by STAT-3, leading to PARP cleavage and apoptosis

[48]. The deregulation of mitochondrial proteins in response to TQ led to the release of cytochrome c in neuroblastoma [63], primary effusion lymphoma [44], leukemia [17] and p53-null myeloblastic leukemia [52].

Caspase cleavage by TQ has been observed in a wide range of cancer cells such as neuroblastoma [63], human myeloblastic leukemia [52], human breast carcinoma [43,51], primary effusion lymphoma [44], lymphoblastic leukemia [47], human colon cancer [38], hepatic cancer [18] and osteosarcoma cell lines [53]. The activation of caspases was found to result in the cleavage of PARP in leukemia [17], human breast carcinoma [43] and p53 null myeloblastic leukemia cells [52]. However, caspase independent cell death mechanisms have also been reported in prostate cancer cell lines [37].

Inducing angiogenesis

To sustain their growth and survival, tumors are known to upregulate several angiogenesis factors [34]. VEGF is a key pro-angiogenic molecule which enables oxygen and nutrient uptake by tumors and stimulates the formation of new blood vessels from existing ones. *In vitro*, TQ's antiangiogenic potential was supported by its inhibitory effects on VEGF in prostate cancer, as well as in multiple myeloma and KBM-5 leukemia cell lines [27,42,48]. Mechanistically, VEGF downregulation in multiple myeloma was associated with STAT-3 deactivation, while AKT and ERK deactivation was observed in prostate cancer cell lines. TQ did not inhibit vascular endothelial growth factor receptor 2 (VEGFR2) in prostate cancer cells but rather inhibited VEGF-induced ERK and AKT activation [42]. In addition, TQ was shown to regulate ENA-78 and Gro- α ; two cytokines which play an important role in neo-angiogenesis in non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) cell lines [64]. Taken together, these results indicate that TQ is capable of interrupting crucial steps required for tumor survival and metastasis.

Activating invasion and metastasis

The most deadly aspect of cancer is its ability to spread or metastasize to distant sites. Targeting the ability of cancer cells to invade and metastasize can halt cancer cell dissemination and enable the containment of tumors, thereby increasing overall patient recovery and survival. We were the first to report that TQ inhibits the invasion of cancer cell lines [65]. *In vitro*, using a matrigel artificial basement membrane invasion assay, we observed up to 50% reduction in the invasion of mouse colon cancer cells upon treatment with TQ [65]. In later studies, TQ was found to inhibit the invasion of NSCLC NCI-H460 cell line by 85% as compared to the control [64]. Wound healing and Transwell experiments also supported the inhibitory effect of TQ on invasion and migration of pancreatic cancer cells [45]. The analysis by confocal microscopy of these cells after phalloidin staining showed a decrease in the number of cellular projections reflecting a decrease in motility [45]. Further investigations revealed a reduction in the expression levels of two proteins that are overexpressed and associated with metastatic tumors namely; the growth receptor ErbB2 and the focal adhesion kinase (FAK) which controls cell migration. FAK downregulation by TQ was also reported as a possible mechanism for the inhibition of migration, invasion and adhesion of glioblastoma cells [66].

NFκB and MMP-9, two potential TQ targets, have been also reported as markers of metastasis. As expected, the inhibition of pancreatic cancer cell metastasis by TQ was associated with the downregulation of both NFκB and MMP-9 protein levels [67]. Furthermore, MMP-9 and MMP-2 were both downregulated in glioblastoma cells upon TQ treatment [66].

Deregulating cellular metabolism

In vitro, TQ has been shown to regulate the metabolic activity of several cancer cell line models including adenocarcinomic human alveolar basal epithelial A549 cells [68], SH-SY5Y human neuroblastoma cells [69], SW626 colon [70] and ES-2 ovarian cancer cells [71]. The modulation of cellular energetics was further implicated in TQ's protective effects *in vivo* when combined with different chemotherapeutic drugs (Table 2). The key metabolic targeting mediated by TQ includes the reduction of MDA [68,72], LDH [73,74] and different liver enzyme levels or activities [75], and the stimulation of GSH [76,77]. TQ was also found to reduce the levels of triglycerides [78], cholesterol [78], bilirubin [78], lipids [75] and creatinine [75]. More importantly, TQ has been shown to modulate ATP levels [76].

In vivo toxicity and anticancer activity of TQ

TQ toxicity has been assessed in several animal models in addition to a Phase I clinical trial [79,80]. Only one study investigated the efficacy of TQ in adult patients with solid tumors or hematological malignancies who had failed or relapsed from standard therapy [80]. In animal models, the histopathological analysis of liver, kidney, heart and lungs of mice and rats given TQ enabled the determination of nontoxic doses [79]. In mice, TQ's LD₅₀ following oral and intraperitoneal administration were 870.9 mg/kg and 104.7 mg/kg, respectively. In rats the LD₅₀ was 794.3 mg/kg and 57.5 mg/kg for oral and intraperitoneal administration, respectively [79]. It is worth noting that the anticancer properties of TQ are observed at much lower doses (5–20 mg/kg) than the corresponding LD₅₀ [64,65]. In addition, the oral administration of TQ to 21 patients who had relapsed from therapy against a wide array of malignant tumors was well tolerated at doses up to 2600 mg/day [80]. For a treatment period ranging from 1 to 20 weeks, TQ administration to these patients did not cause any side effects.

Several studies have shown that TQ retards the carcinogenic process in animals and abrogates the toxic effects of several chemotherapeutic agents mainly through its antioxidant and anti-inflammatory properties. TQ exhibited protective effects against doxorubicin- and cyclophosphamide-induced cardiotoxicity [73–76], and attenuated hepatotoxicity induced by carbon tetrachloride, cadmium, aflatoxin (B1), *tert*-butyl hydroperoxide, acetaminophen and cyclophosphamide [14,75,81–84]. In recent studies, TQ was found to reduce the nephrotoxicity of several drugs such as cisplatin, vancomycin, mercuric chloride, gentamicin and doxorubicin [72,78,85–87], and protect from the neurotoxicity of toluene [88]. TQ also inhibits alcohol induced gastrotoxicity [89] and genotoxicity induced by azoxymethane and benzo(a)pyrene [90]. Damage to erythrocytes caused by the carcinogen 1,2-dimethylhydrazine [86], lung injury by toluene and cyclophosphamide [77,92], in addition to toluene and methotrexate-induced testicular injury [77,93] were all reduced by TQ.

The models and mechanisms where these effects were demonstrated are summarized in Table 2.

The anticancer properties of TQ have also been demonstrated in several animal models. TQ delayed tumor onset, decreased tumor burden and enhanced survival in the 20-methylcholanthrene (MC) induced fibrosarcoma animal model [15]. The mechanism of anticancer action involved the reduction of lipid peroxide accumulation in the liver and increase in the activities of key antioxidant enzymes. Cytotoxic activity of TQ was also documented in squamous cell carcinoma animal models [94]. In the hormone refractory prostate cancer xenograft model, reduction of androgen receptor, E2F-1, and cyclin A as well as induction of apoptosis were associated with tumor growth inhibition by TQ [36]. Furthermore, TQ inhibited VEGF-induced angiogenesis in a xenograft prostate cancer model. TQ is also a potent anticancer agent against 1,2-dimethyl hydrazine-induced colon cancer and in xenograft colon cancer models [65]. In a xenograft metastatic model of human pancreatic cancer, TQ inhibited tumor metastasis and invasion by downregulating the expression levels of NFκB, MMP-9 and XIAP [67,95]. NFκB and XIAP levels were also decreased after administration of TQ in animals bearing bladder cancer [96].

Adjuvant potential of TQ

Combination therapies are being increasingly employed in the battle against cancer, particularly against resistant tumors. It would be ideal to combine the toxic chemotherapeutic drugs with non-toxic natural drugs for greater efficacy and fewer unpleasant side effects. TQ has been used in combination with hormones, chemotherapeutic agents, and with ionizing radiation. Extensive evidence from *in vitro* and *in vivo* studies indicates that TQ improves the therapeutic efficacy of many chemotherapeutic agents by enhancing their antitumor activity and/or decreasing their toxicity to normal cells. The mechanism(s) by which TQ reduces the side effects of chemotherapeutic drugs are summarized in Table 2.

In vitro, TQ was found to enhance the anticancer effects of cisplatin in many solid tumors [64,97]. TQ also enhances the efficacy of dox against resistant breast cancer [43], epigallocatechin gallate (EGCG) against prostate cancer [98], gemcitabine and/or oxaliplatin against pancreatic cancer [35], as well as 5-fluorouracil (5-FU) against gastric and colon cancers [70,99]. In rodent studies, TQ protects from the toxicity of dox [74], ifosfamide, benzo-pyrene and cisplatin [100,101]. However, to date, no studies have investigated the effects of combining TQ with targeted therapies used in the clinical setting.

TQ in combination with selenomethionine and lycopene, in the presence of estrogen also caused damage to cervical cancer cells [102]. Similar effects were observed when TQ was combined with estrogen, testosterone, and parathyroid hormones for the treatment of prostate cancer [103]. Furthermore, combination of TQ with buthionine sulfoximine (BSO), an inhibitor of GSH, enhanced apoptosis of HEp-2 human laryngeal carcinoma cells [18].

In vitro and *in vivo* studies documented that TQ enhances the death of squamous cell carcinoma cells when combined with diosgenin [41]. *In vivo*, the combination of TQ with cisplatin or with gemcitabine and/or oxaliplatin enhanced cell death in lung

cancer xenografts in mice and in pancreatic cancer models, respectively [35,64].

Finally, TQ appears to be a candidate for application with radiotherapy as shown by the positive antitumor effects following its administration with a single dose of ionizing radiation (2.5 Gy) for the treatment of breast cancer [104]. The mechanism of tumor sensitization is very similar to the single drug treatment effects on the different cancer hallmarks described earlier. Altogether, these studies highlight the strong adjuvant potential of TQ and emphasize the need for translating this molecule to the clinical setting.

Limitations of clinical translation of TQ

The main limitations for translating TQ to the clinic is its poor bioavailability, in addition to the lack of knowledge about its toxicity in humans and the lack of understanding of its exact molecular targets. Systems and network biology studies can help determine the potential molecular targets of TQ, as well as understand the mechanisms by which they are regulated. Several programs and servers can be used for identifying potential binding partners, and virtually visualizing candidate sites of interaction. A recent attempt in our laboratory showed that TQ interacts with several proteins, namely oxido reduction, gene regulation, signaling and DNA binding proteins. The binding of TQ to these proteins was very comparable to that of clinically used drugs (unpublished data). Validating the binding of TQ to these candidate sites will greatly improve our understanding of the mechanism of anticancer action of TQ.

In a phase I clinical study, the administration of TQ at very high doses was not toxic to cancer patients who relapsed from standard chemotherapy [79]. Unfortunately, TQ did not add therapeutic value, which could be due to its failure to exhibit anticancer effects in relapsed aggressive tumors. It is also possible that TQ's poor dissolution and bioavailability, in addition to its high capacity to bind to plasma proteins [105] prevented it from reaching the tumor site. More studies are needed to investigate the

bioavailability of TQ *in vivo*, and to determine the pharmacologically achievable concentrations which are effective for the treatment of tumors and that do not show any apparent toxicity to humans. It is important to note that TQ analog formulations or encapsulation could be used to circumvent these problems given that such formulations are designed to enhance drugs' bioavailability and ultimately activity [106]. Furthermore, nanoparticles can enhance tumor targeting *in vivo* especially in aggressive cancers owing to their vasculature architecture by a phenomenon referred to as passive targeting [106].

Concluding remarks

TQ is a natural product that can be used for the treatment of a wide number of diseases. Over the past five years, there has been a significant increase in research on TQ, particularly describing its anticancer effects. The attractive feature of TQ is that it is not only able to selectively target tumor cells, but it can also protect normal tissues from the toxic side effects of chemotherapeutic agents when used as an adjuvant. The mechanism of anticancer action of TQ involves differential triggering of ROS in cancer *versus* normal cells. TQ disrupts key signaling pathways that are of crucial importance for tumor growth and development.

More importantly, TQ has proven to be an effective anticancer molecule in animal models *in vivo*. The latest advances in TQ analog drug design and methods of delivery have further improved its activity, thus increasing its chances of reaching the clinic. In conclusion we believe that TQ should be considered seriously for cancer clinical development, especially in combination with chemotherapeutic agents.

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