Chemopreventive and Anticancer Effects of Thymoquinone: Cellular and Molecular Targets

Rohini Gomathinayagam[1], Ji Hee Ha[1], Muralidharan Jayaraman[1], Yong Sang Song[2], Ciro Isidoro[3], Danny N. Dhanasekaran[1]

[1]Stephenson Cancer Center, Department of Cell Biology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA, [2]Department of Obstetrics and Gynecology, Cancer Research Institute, College of Medicine, Seoul National University, Seoul, Korea, [3]Laboratory of Molecular Pathology and NanoBioImaging, Department of Health Sciences, Università del Piemonte Orientale, Novara, Italy

Thymoquinone (TQ) is a bioactive component derived from the seeds of *Nigella sativa* that are commonly as black cumin. Evidences indicate that the medicinal properties of TQ have been recognized for more than 2000 years. TQ has been shown to possess potent chemopreventive properties that include anti-inflammatory and anti-neoplastic activities. Recent studies have unraveled the multiple mechanisms through which TQ exerts its chemopreventive and anticancer activity in different cancer cells in a contextual manner. The present review aims to provide a brief compendium on the molecular mechanisms through which TQ inhibits signaling pathways underlying cancer genesis, progression, and metastasis.

Key Words *Nigella sativa*, Thymoquinone, Neoplasms, Chemoprevention, Antineoplastic agent

INTRODUCTION

Carcinogenesis is a multistep process that involves the transformation of a normal cell to a malignant one. Clinically, the series of events from pre-malignancy to malignancy is progressively observed as dysplasia, hyperplasia, tumorigenic malignancy and metastasis. Recent studies have identified that both genetic and environmental factors play a critical role in cancer development and progression. It has also been realized that cancers could be prevented or at least cancer initiation and/or progression could be delayed by naturally-occurring, dietary compounds [1-4]. Most, if not all, of the dietary compounds are derived from plants and often denoted as phytochemicals. Recent cell line-based ex vivo and animal model-based in vivo studies have further validated the chemopreventive as well as tumoricidal effects of these phytochemicals [5-7]. Phytochemicals come in many different, structurally-defined, flavors such as polyphenols, flavonoids, terpenoids, saponins, anthocyanins, phytosterols, isothiocyanates, or organo-sulfur compounds. Irrespective of their structural differences, many of these compounds possess

significant chemopreventive and chemotherapeutic effects against many cancers [1,8-10]. As these chemopreventive/anticarcinogenic agents are quite diverse in their structural chemistry and bioactivity, assessing their efficacy based on their signaling mechanisms is critical for the development of potential prophylactic and/or therapeutic agents for cancer [11].

Thymoquinone, abbreviated hereafter as TQ, is one such phytochemical, derived primarily from the seeds of *Nigella sativa* [12]. The seeds of *N. sativa*, commonly known as black cumin, have been used as a herbal medicine for more than 2000 years [12-14]. While various formulations of *N. sativa* have been used to treat many different diseases including cancer, TQ has been identified as the principal bioactive component that contributes to health benefits of *N. sativa* [12-15]. Defining the etiological features associated with cancer, ten critical alterations in cell physiology have been defined as the hallmarks or of cancer. They are: (1) sustained proliferative signaling; (2) insensitivity to growth inhibitory signals; (3) invasion and metastasis; (4) limitless replicative potential; (5) tumor angiogenesis; (6) resistance to cell death; (7) eva-

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sion of immune destruction; (8) tumorigenic inflammation; (9) genomic instability; and (10) reprogramming of energy metabolism [16-18]. TQ has been shown to exert its chemopreventive and anticancer effects by inhibiting all of the major pathways involved in the manifestation of cancer hallmarks and their enabling characteristics [19]. Recent studies have provided insights into the mechanism by which TQ modulates the different cancer-enabling characteristics in order to suppress cancer cell growth, proliferation, invasive migration, and cancer progression. While the mechanistic roles of phytochemicals such as curcumin, epigallocatechin gallate, and resveratrol in chemoprevention have been extensively reviewed [20-22], such information is rather limited in the case of TQ. Considering the multi-targeted chemopreventive and anticancer effects of TQ, the focus of this review is to provide a comprehensive update on the molecular mechanisms underlying the chemopreventive, chemotherapeutic, and anticancer effects of TQ.

REDOX CYCLING AND ANTICANCER ACTIVITY OF TQ

TQ, bearing the chemical formula $C_{10}H_{12}O_2$, is chemically defined as 2-isopropyl-5methyl-1,4-benzoquinone (Fig. 1). Although TQ was originally isolated from *N. sativa*, it is also present in many other plants [23]. As a typical quinone compound, TQ can undergo both enzymatic and non-enzymatic redox cycle reactions, giving rise to pro-oxidant as well as anti-oxidant derivatives [24,25]. One electron reduction of TQ by an enzyme complex consisting of NADPH cytochrome p450 reductases, NADH cytochrome b_5 reductase, and NADPH ubiquinone oxidoreductase can generate semiquinone and TQ-dianion with pro-oxidant activity. In addition, neutral pH and low intracellular concentration of TQ appear to favor the formation of pro-oxidative semiquinone [26].

As opposed to the one-step one electron reduction that generates the pro-oxidative derivatives of TQ, a two electron reduction of TQ by NADPH quinone oxidoreductase or further reduction of semiquinone by NADPH cytochrome p450 reductases, cytochrome b_5 reductase, and NADPH ubiquinone
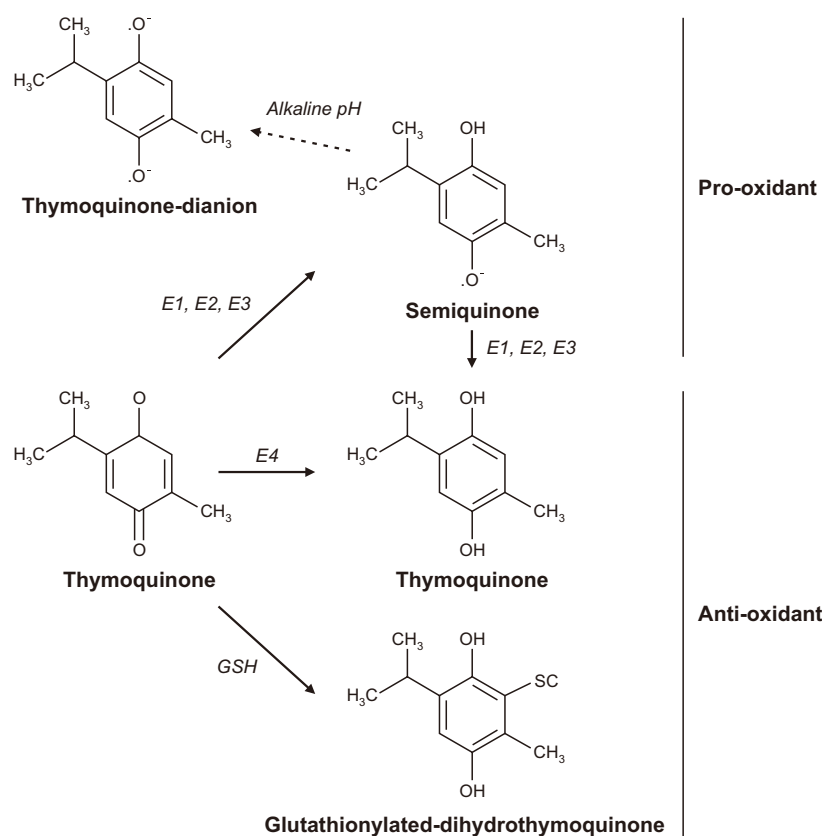

Figure 1. Redox cycle of thymoquinone (TQ). One electron reduction of TQ by NADPH cytochrome p450 reductases (*E1*), cytochrome b_5 reductase (*E2*), and NADPH ubiquinone oxidoreductase (*E3*) generates semiquinone with pro-oxidant activity. In alkaline pH (mostly in vitro) semiquinone is converted further into TQ-dianion with pro-oxidant activity. One-step two electron reduction of TQ by NADPH cytochrome p450 reductases (*E1*), cytochrome b_5 reductase (*E2*), and NADPH ubiquinone oxidoreductase (*E3*) generates thymohydroquinone with anti-oxidant activity. TQ can also undergo a non-enzymatic reduction through its interaction with glutathione (*GSH*) forming glutathionyl-dihydrothymoquinone with ant-oxidant activity.

oxidoreductase can generate thymohydroquinone with potent anti-oxidant activity [25-28]. TQ can also undergo a non-enzymatic reduction through its interactions with glutathione forming glutathionyl-dihydrothymoquinone in physiological conditions [29]. It has been observed that the glutathionyl-dihydrothymoquinone can act as an anti-oxidant capable of scavenging organic free radicals [29,30].

It appears that the cellular and/or physiological context(s) determines whether TQ acts as a pro-oxidant or an anti-oxidant in vivo. Since TQ has been used in a wide range of concentrations in many of the reported ex vivo (2 to 100 μM) as well as in vivo (5 to 20 mg/kg) studies [23], it has become challenging to distinguish the pro versus anti-oxidant effects of TQ in eliciting the observed anticancer response. Nevertheless, it should be stressed that both the pro-oxidant and the anti-oxidant derivatives of TQ have been observed to elicit anticancer response through different signaling mechanisms [31]. Thus, the redox cycling of TQ indeed plays a major role in the contextual modulation of different oncogenic pathways by TQ.

PRO-OXIDANT ROLE OF TQ IN CHEMOPREVENTION

A number of studies have documented the role of the pro-oxidant activity of TQ in its chemopreventive and anticancer activities [26,32]. Conversion of TQ into semiquinone is accompanied by the release of reactive oxygen species (ROS) that include superoxide, peroxide, and hydroxyl radicals. TQ-generated ROS causes oxidative stress, mitochondrial and nuclear damage, and subsequent cell-death. This has been demonstrated in many different cancer cells including those from adult T-cell leukemia [33], prostate [34], hepatocellular carcinoma (HCC) [35], skin [36,37], breast [38], kidney [39,40], renal cell carcinoma [39], and ovarian cancer [41]. The pro-oxidant property of TQ is also observed to modulate the ionic status of the transition metal ion copper, so as to promote cell death in prostate cancer cells [34]. In addition, the ROS generated by TQ is also involved in modulating multiple signaling pathways to promote its anticancer activity. In HCC, the pro-oxidant activity of TQ has been shown to be involved in inducing apoptosis by stimulating the increased expression of several pro-apoptotic proteins including TNF-related apoptosis-inducing ligand (TRAIL), TRAIL-receptor 2 (TRAILR2)/death receptor 5, caspase 9, caspase 8, caspase 3, and B-cell lymphoma-x-Small (Bcl-xS) while concomitantly inhibiting the expression of the antiapoptotic B-cell lymphoma-2 (Bcl-2) [35,42].

Similarly, TQ-induced ROS generation is involved in promoting apoptosis in primary effusion lymphoma cells by inhibiting AKT [43]. In prostate cancer cells, TQ-generated ROS has been demonstrated to play a role in the downregulation of androgen receptors as well as anti-apoptotic proteins along with increased expression of apoptosis-inducing factor-1 to induce growth arrest and apoptosis [44]. In A431 epidermoid carcinoma cells, TQ-induced apoptosis involves ROS-dependent inactivation of Src kinase (Src) and the downstream transcription factor, STAT3. In light of the previous findings that ROS can rapidly inactivate Src [45], the pro-oxidative activity of TQ targets cancer cells by more than one mechanism.

ANTI-OXIDANT ROLE OF TQ IN CHEMOPREVENTION

The anti-oxidant activity of TQ has been shown to play a chemopreventive role in many cancer models including prostate, colon, and hepatic cancers [46-49]. TQ has been reported to inhibit tumor development and progression induced by oxidative-stress induced colon tumorigenesis by 1,2-dimethyl-hydrazine in murine models of colon cancer [47,48]. In this model, the anti-oxidant role of TQ involves its inhibition of the tumorigenic generation of superoxide anions and nitric oxide as well as its role as a scavenger of carcinogenic free radicals [47]. Furthermore, TQ has been shown to induce the expression of several cytoprotective anti-oxidant enzymes involving catalases, superoxide dismutase, reduced glutathione, glutathione peroxidase, glutathione-S-transferases, and glutathione reductase that can combat the oxidative stress-induced tumorigenesis [50,51].

Anti-oxidant activity of TQ has also been attributed to its inhibitory effect on diethylnitrosamine-induced hepatic carcinogenesis through a similar upregulation of anti-oxidant enzyme levels [49]. In addition, the anti-oxidant effect of TQ is also associated with the ability of TQ to suppress the adverse effects of chemotherapeutic agents such as cyclophosphamide [52], cisplatin [53,54], and doxorubicin [55] in non-cancerous cells and tissues [26]. Thus, TQ-mediated increase in the levels of anti-oxidant enzyme plays a significant role in the oxidative stress-induced tumorigenesis and chemically induced carcinogenesis as well as the cytoprotection against chemotherapeutic toxicity [56].

EFFECT OF TQ ON CANCER CELL PROLIFERATION

TQ inhibits cell proliferation by controlling key cell cycle checkpoints and inducing cell cycle arrest at G0/G1, G1/G2, or G2/M phases through a variety of mechanisms (Fig. 2). In Jurkat cells representing acute T-cell leukemia, TQ induces G0/G1 phase cell cycle arrest [57]. TQ induces G1/S cell cycle arrest in many other cancer cells including those of colorectal cancers [58], lung cancer [59], HCC [60], and prostate cancer [61]. In cell-lines derived from cholangiocarcinoma, TQ induces G2/M cell cycle arrest [62-64]. In many breast cancer cell lines including those derived from triple negative breast cancer, TQ induces G0/G1, G1/S, or G2/M phase cell cycle arrest in a cell type dependent manner [65-70].
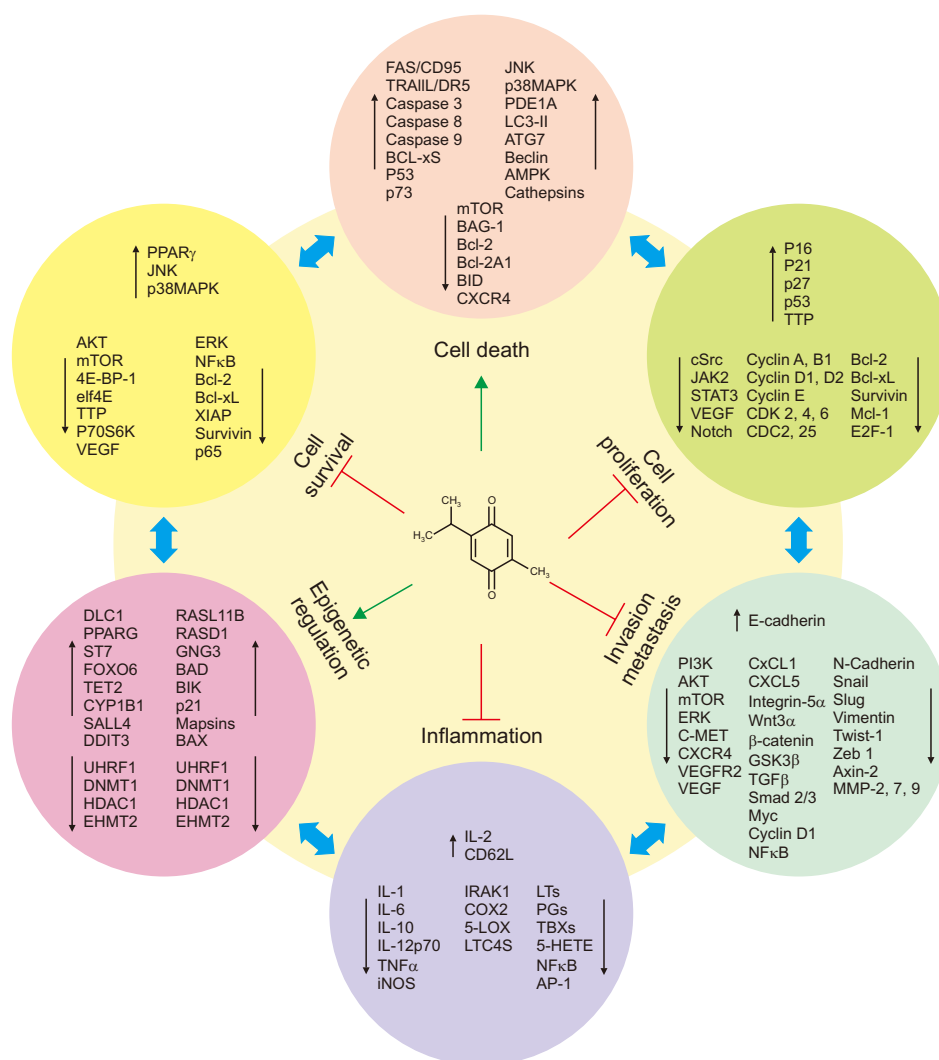
Figure 2. Anticancer activity of thymoquinone (TQ). As a multi-target chemopreventive phytochemical, TQ exerts its anticancer activity through the modulation of expression as well as activation profile of critical signaling molecules involved in cancer cell growth and progression. Signaling molecules upregulated or downregulated by TQ in its anticancer activity is depicted by the upwards ↑ or downwards ↓ arrows. By regulating the overlapping pathways involved in cell proliferation, cell survival, cell death, invasive metastasis, and inflammatory signaling through multiple mechanism including epigenetic regulation, TQ strongly inhibits cancer growth, progression, and metastasis (detailed in the text). JNKs, c-Jun N-terminal kinases; MAPK, modulation of the mitogen-activated protein kinase; eIF4E, eukaryotic translation initiation factor 4E; TTP, tristetraprolin; ERK, extracellular signal-regulated kinases; Bcl-2, B-cell lymphoma-2; Bcl-xS, B-cell lymphoma-x-Small; XIAP, X-linked inhibitor of apoptosis; TRAIL, TNF-related apoptosis-inducing ligand; DR5, death receptor 5; PDE1A, phosphodiesterase 1A; AMPK, AMP-activated kinase; BID, BH3-interacting domain death protein; CXCR4, CXC-motif chemokine receptor-4; JAK2, Janus kinase; CDK, cyclin-dependent kinases; CDC, cell division cycle; E2F-1, Early 2 Family-1; UHRF1, ubiquitin-like-containing PHD and RING finger domains; DNMT1, DNA methyltransferase 1; HDAC1, histone deacetylase 1; EHMT2, euchromatic histone lysine methyltransferase 2; IL, interleukin; iNOS, inducible NOS; IRAK1, interleukin-1 receptor-associated kinase 1; 5-LOX, 5-lipoxygenase; LTC4S, leukotriene C4 synthase; LTs, leukotrienes; PGs, prostaglandins; TBX, thromboxanes; 5-HETE, 5-hydroxyeicosatetraenoic acid; PI3K, phosphatidylinositol-3-kinase; VEGFR, VEGF-VEGF-receptor; CXCL, CXC-motif ligand; GSK3β, glycogen synthase kinase 3β; MMP, matrix metalloproteinase.

The primary mechanism through which TQ exerts its anti-proliferative effects involves the downregulation of cyclin A [61], cyclin D1 [37,66,71-73], cyclin D2 [73], cyclin E [66], and cyclin-dependent kinases (CDK) along with the increased expression of CDK inhibitors, such as p16 [71], p21 [61,74], and p27 [61,66]. For instance, TQ has been shown to induce G1 cell cycle arrest as well as radiosensitization [75] by in-hibiting key proteins, such as CDK2 and CDK4/CDK6 [65-67]. On the other hand, TQ-treated LNCaP prostate cancer cells demonstrated a decrease in E2F-1 transcription factor regulated proteins and cyclin A with G1/S phase cell cycle arrest [61]. In neuroblastoma cells, TQ-induced G2/M cell cycle arrest is associated with the decreased expression of proliferating cell nuclear antigen, cyclin B1, and CDK1 along

with the increased expression of p53 and p21 [76]. In doxo-rubicin-resistant breast cancer cells and spindle carcinoma cells, TQ induces G2/M arrest that can be associated with the decreased expression of cyclin B1 and cell division cycle 25/Cdc25 phosphatase levels and increased expression of p53 [77,78]. In HCC cells, TQ has been shown to induce G0/G1 cell cycle arrest by inhibiting the expression of cyclin D1, CDK2 and Bcl-2 while increasing the expression of p21 through a pathway involving the downregulation of Notch signaling [60].

STAT3-signaling is another signaling node targeted by TQ for the inhibition of cell proliferation. TQ modulates STAT3 signaling in colon cancer cells and multiple myeloma cells so as to inhibit oncogenic signaling and proliferation [70,73,79]. In these cells, TQ has been shown to inhibit the expression and/or the activities of STAT3, Src, Janus kinase-2 phosphorylation, cyclin D1, Bcl-2, B-cell lymphoma-x-Large (Bcl-xL), survivin, myeloid cell leukemia sequence 1/Mcl-1 and VEGF [70,73,79]. TQ can also inhibit cell proliferation by stimulating the expression of tristetraprolin (TTP), a tumor suppressor involved in the stability of diverse mRNAs [80]. TTP exerts its tumor suppressive role by binding to the ade-nylate-uridylate-rich elements at the 3'-untranslated region of many oncogenic mRNAs and accelerating their rapid decay by recruiting an enzyme involved in the shortening of their poly(A) tails [81,82]. Interestingly, the list of mRNAs targeted by TTP for accelerated decay includes those of cyclins, CDKs, and E2F-1 [82], all of which show decreased expression following TQ-treatment. Thus, TTP appears to provide a pivotal mechanism through which TQ can downregulate cyclins and CDKs to induce cell cycle arrest.

TQ-MEDIATED INHIBITION OF CANCER CELL SURVIVAL

A large number of studies have established the inhibitory role of TQ in cancer cell survival [15,31]. Cell survival depends on the balance between cell proliferation and apoptosis. TQ has been found to inhibit the survival of many cancer cells derived from breast cancers [65,67,77,83], pancreatic cancer [84], gastric cancer [85], primary effusion lymphoma [43], prostate cancer [86,87], kidney cancer [88], squamous cell carcinoma [89], renal cell carcinoma [90], and cholangiocarcinoma [63], primarily through attenuation of the activity of AKT, a crucial kinase associated with cell survival. TQ inhibits the activation of AKT through ROS-dependent mechanisms and/or through activation of the lipid phosphatase, phosphatase and tensin homolog (PTEN). In primary effusion lymphoma cells, it has been shown that the inhibition of AKT by TQ requires the generation of ROS [43]. Results from breast, gastric, and pancreatic cancer cells indicate that TQ stimulates the expression of PTEN. PTEN, in turn, dephosphorylates and downregulates the activity of phosphatidylinositol-3-kinase (PI3K) with the resultant decrease in the activation of downstream AKT and

its effectors [77,84,85]. Considering the critical role of AKT in the vast array of cell survival signaling pathways, the inhibitory effect of TQ on the activity of AKT is reflected on multiple signaling nodes downstream of AKT. In breast cancer, the inhibition of AKT by TQ results in the dysregulated phosphorylation of mTOR [84,91], eukaryotic translation initiation factor 4E/eIF4E, eukaryotic translation initiation factor 4E-binding protein 1/4E-BP1, and ribosomal protein S6 kinase β1/p70S6 kinase [66,86], contributing to an overall reduction in cell survival.

In conjunction with the inhibition of the PI3K-AKT pathway, TQ-mediated inhibition of cell survival involves the upregulation of pro-apoptotic proteins, such as Bcl-2-Associate X protein (Bax) along with the downregulation of anti-apoptotic proteins, such as Bcl-2, Bcl-xL, X-linked inhibitor of apoptosis (XIAP), and survivin [66,84]. In addition to AKT-signaling, TQ also attenuates NF-κB signaling to reduce cell survival. For instance, TQ has been shown to reduce the expression levels of AKT through a pathway involving NF-κB, microRNA-603, and eukaryotic elongation factor-2 kinase. Interestingly, in a parallel pathway, TQ has also been shown to induce the expression of pro-apoptotic proteins via PPAR-γ in breast cancer cell lines [92]. TQ has been reported to induce the expression of PPAR-γ in diverse cellular contexts [92,93]. In breast cancer cells, TQ-mediated activation of PPAR-γ leads to the downregulation of the expression of the pro-survival genes Bcl-2 and BCL-xL [92]. Based on in-silico analyses, it has also been postulated that TQ can potentially interact with PPAR-γ [92], thereby promoting PPAR-γ-mediated inhibition of cell survival pathways.

Additionally, TQ-mediated inhibition of cell survival often involves the modulation of the mitogen-activated protein kinase (MAPK)-family of kinases including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. While ERKs are often associated with cell survival and proliferation, JNK and p38 MAPK are primarily involved in apoptotic signaling pathways. Consistent with this general signaling paradigm, TQ has been shown to inhibit the activity of ERKs in many cancer cells, such as those derived from myeloma [94] squamous cell carcinoma [89], prostate cancer [95,96], breast cancer [97], and breast cancer xenografts [38] while activating JNKs and/or p38MAPKs in colon [98], prostate [44], and pancreatic cancer cells [99]. TQ also controls cancer cell survival by modulating the signaling activity of NF-κB. In HS766T pancreatic ductal adenocarcinoma cells, TQ inhibited the translocation of the NF-κB transcription factor into the nucleus. Other effects of TQ on the NF-κB pathway include its inhibitory role in TNF-induced degradation of the inhibitory subunit of NF-κBα, inhibition of p65 activation via phosphorylation, and nuclear translocation [100,101]. TQ also potentiated the anticancer effect of bortezomib in multiple myeloma cells by downregulating the VEGF, Bcl-2 and p65 expression [102]. Thus, the inhibition of cancer cell survival by TQ is mediated by its modulatory effects on multiple

pro-survival signaling nodes in addition to the activation of anti-survival mechanisms. It should be pointed out that these multitargeted effects of TQ are not mutually exclusive. Rather, they form an array of overlapping, permissive, and/or synergistic effects that collectively contribute to the cumulative inhibition of cancer cell survival.

INDUCTION OF APOPTOTIC CELL DEATH IN CANCER CELLS BY TQ

In addition to its inhibitory effect on cell proliferation and survival, TQ promotes apoptosis of cancer cells (Fig. 2). TQ-induced apoptosis often results in restoring the dysregulation of apoptosis-associated gene expression [103]. TQ promotes both intrinsic and extrinsic apoptotic cell death through multiple mechanisms. A plethora of evidences indicate that TQ induces intrinsic apoptotic cell death by downregulating the expression of Bcl-2-family of anti-apoptotic proteins as well as through the induction of mitochondria-dependent activation of caspases. This has been exemplified in many cancer cells including those of neuroblastoma [104], myeloblastic leukemia [105], breast carcinoma [77], primary effusion lymphoma [43], lymphoblastic leukemia [57], colon cancer [98], hepatic cancer [106], and osteosarcoma cell lines [107]. TQ's ability to downregulate the expression of Bcl-2 family of anti-apoptotic proteins is well characterized in prostate cancer cells in which TQ-treatment leads to the drastic reduction in the expression of several anti-apoptotic proteins belonging to the Bcl-2 family including Bcl-2, B-cell lymphoma 2-related protein A1/ Bcl-2A1, Bcl-xL, and BH3 Interacting-domain Death agonist protein/BH3 Interacting-domain Death agonist protein leading to the activation of caspases [44].

The mechanisms underlying the activation of caspases by TQ is very much cell type dependent. In bladder cancer cells, it has been shown that TQ induces endoplasmic reticulum (ER) stress and subsequent action of an ER-stress related signaling pathway leading to mitochondrial dysfunction and caspase 9-mediated activation of caspase 3 [108]. In leukemia-derived HL60 cells, TQ activates caspase 3 via the upstream caspase 8 and mitochondrial release of cytochrome c [105]. A similar mitochondrial pathway involving cytochrome-release and caspase 9-caspase 3 activation has been ascribed to TQ-induced apoptosis in both the p53-null MG63 and p53-mutated MNNG/HOS osteosarcoma cell lines [107]. In addition, TQ-associated ROS activity also plays a role in inducing apoptotic cell death via p53. In HCT116 colorectal cancer cells and MCF-7 breast cancer cells, it has been shown that the ROS-activity of TQ inflicts DNA-damage, which leads to the upregulation p53, and the increased expression of p53-target genes involved in apoptosis or growth inhibition [78,109,110]. TQ has been shown to induce apoptosis of p53-null or p53-mutated cancer cells through activation of caspases, independently of p53.

Pertaining to the role of TQ in extrinsic apoptosis, it has been noted that TQ promotes Fas/cluster of differentiation-95-death receptor-induced apoptosis in multiple myeloma cells [94]. TQ-treated multiple myeloma cells exhibited significant downregulation of CXC-motif chemokine receptor-4 (CXCR4) expression along with the inhibition of chemokine CXC-motif ligand (CXCL)12-mediated chemotaxis that led to the increased sensitivity of these cells to Fas-/ CD-95-receptor mediated cell death [94]. Studies using HCC cell lines as well as HCC tissue from a murine model have shown that TQ stimulates TRAIL and TRAILR-2-mediated apoptotic signaling by eliciting the increased expression of TRAIL, TRAILR2, caspase 9, caspase 8, caspase 3, and Bcl-xS. TQ potentiates this pathway further by decreasing the expression of Bcl-2 in HCC. TQ-generated ROS has been implicated in initiating this pathway in HCC [35,42].

TQ has also been observed to induce apoptosis through several other alternative apoptotic signaling pathways. One such mechanism involves the activation of tumor suppressor p73, a structural and functional homologue of p53 [111]. In lymphoblastic leukemia derived Jurkat cells, TQ has been shown to increase the levels of p73 either via ROS-mediated DNA-damage pathway or by inhibiting the expression of phosphodiesterase 1A [57,69]. It has been well characterized that p73 can induce apoptosis through transactivation of many different proapoptotic genes, involved in both intrinsic and extrinsic apoptotic pathways [111,112]. In addition, p73 is known to induce apoptosis through transcription-independent mechanisms including the epigenetic de-repression of proapoptotic genes [112].

INDUCTION OF AUTOPHAGIC CELL DEATH IN CANCER CELLS BY TQ

In addition to pro-apoptotic mechanisms, TQ promotes cell death in cancer cells via autophagy. Autophagy is emerging as a double-edged sword in cancer biology [22,113,114]. In a context-specific manner, autophagy suppresses tumor growth in specific instances or promotes cancer cell growth and survival. The anticancer effects of TQ has been documented in in both of these pathobiological contexts. TQ-mediated autophagic cell death involves the context-specific inhibition or induction of autophagy. In the head and neck squamous cell carcinoma-derived SASVO3 cell-line, TQ induces cancer cell death via pathways involving both caspase-dependent apoptosis and microtubule-associated protein 1A/1B-light chain 3/ LC3-II activation-dependent autophagy [115]. In these cells, TQ inhibits the activation of mTOR, a negative regulator of autophagy while simultaneously stimulating the expression of autophagic markers, such as LC3-II and Beclin-1. This is also consistent with the observation that TQ induces autophagic cell death in renal carcinoma derived 786-O and ACHN cell lines via the upregulation of AMP-activated kinase along with the downregulation of mTOR activity and increased expression of LC3-II [116]. Increased autophagic cell death has also

been observed to synergize with the caspase-dependent apoptotic cell death in SASVO3 cells. A similar role for TQ in inducing both apoptotic and autophagic cell death with the resultant potentiation of the anti-proliferative effects of Gemcitabine has been noted in MCF7 breast cancer cells [117]. In LoVo colon cancer cells, TQ can induce autophagic cell death through pathways involving the activation of JNK and p38 MAPK [118]. In these cells TQ promotes mitochondrial outer membrane permeabilization-mediated mitophagy and increased expression of several autophagic as well as mitophagic proteins that results in autophagic cell death [118].

In cells that utilize autophagy as a survival mechanism, such as those from glioblastoma multiforme (GBM), TQ promotes cell death by inhibiting autophagy. Using GBM-derived U87MG cell line, it has been shown that TQ enhances the anti-proliferative effects of Temozolomide through inhibition of autophagy [119]. In this cell line, TQ induced cell death by inhibiting the expression of autophagy-associated proteins Beclin-1 and autophage-related 7/ATG7 at the transcriptional level. It has also been observed that TQ-induced cell death through the inhibition of autophagy in GBM-derived cell lines could also involve a cathepsin-B-dependent mechanism [120]. In GBM-derived U87MG and Gli36ΔEGFR cell lines, TQ-induced lysosomal membrane permeabilization leads to the inhibition of autophagic flux, release of cathepsins to cytosol, and the resultant execution of cell death by cathepsin-B.

EFFECT OF TQ ON CANCER CELL MIGRATION, INVASION, AND TUMOR ANGIOGENESIS

Many of the signaling nodes targeted by TQ in inhibiting the survival and proliferation of the cancer cells are also involved in cancer cell migration, invasion, and tumor angiogenesis (Fig. 2). TQ was demonstrated to inhibit epithelial to mesenchymal transition (EMT) in many cancer cells including those from breast cancer [121], bladder cancer [122,123], cervical cancer [124], gastric cancer [91], and prostate cancer [125]. TQ has been shown to reverse EMT in bladder cancer as well as gastric cancer cells by strongly attenuating the PI3K/AKT/mTOR signaling activity and downregulating the expression of key signaling components of the EMT pathway, such as N-cadherin, Snail, Slug, vimentin, Twist1, and β-catenin [91,122]. In bladder cancer cells, TQ promotes the reversal of EMT by inhibiting the Wnt3α/β-catenin/glycogen synthase kinase 3β (GSK3β) signaling axis [123]. By inhibiting this signaling axis, TQ also downregulates the expression of downstream Myc, Axin-2, matrix metalloproteinase (MMP)-7, cyclin D1 and c-MET that are critically involved in EMT and invasive migration of cancer cells. Similarly, it has been shown that TQ inhibits EMT-specific transcription factors such as Twist1 and Zeb 1 in cervical cancer cells [124]. In prostate cancer cells, TQ represses the expression of EMT markers by inhibiting TGF-β/SMAD 2/3 signaling [125]. Thus, TQ reverses EMT

through targeted inhibition of several upstream signaling nodes that promote the expression of the critical players of EMT in a cancer and/or cell-type specific manner. The potent inhibitory effect of TQ on EMT results in the attenuation of migration and invasion of cancer cells irrespective of their etiology or cell-type. This is further substantiated by the in vivo mouse model studies. In a breast cancer xenograft mouse model, it was observed that TQ drastically reduced Twist1-induced metastasis by inhibiting the expression of EMT associated N-cadherin and stimulating the expression of E-cadherin [121].

The inhibitory effects of TQ also span other signaling nodes in invasive cell migration. It has been recognized that TQ hinders the migration and metastasis of glioblastoma cells by suppressing the activities of Focal adhesion kinases and ERKs, thus inhibiting the downstream MMP-9, MMP-2 that are involved in the invasive migration of cancer cells [126]. In the case of breast cancer cells, TQ exerts its inhibitory effect on metastasis by downregulating the stimulators of EMT and invasive migration such as TGF-β, MMP-9, MMP-2, and integrin-5α while upregulating the expression of the inhibitors of metastasis such as E-cadherin [127]. TQ also inhibits bone metastasis of breast cancer cells by blocking the activation of NF-κB, thereby repressing the CXCR4-signaling axis [128]. TQ-induced TTP can also play a role in attenuating the expression of metastasis-specific genes, as in the case of proliferation-specific genes by accelerating the decay of the metastasis-specific mRNAs [80,82]. It has been shown that TQ inhibits the migration, invasion, and EMT of renal cell carcinoma cell lines by inducing autophagy [116].

In addition to its effect on EMT and invasive metastasis, TQ potently downregulates the expression of the key antiangiogenic molecules, thus impeding tumor angiogenesis. Both in vitro and in vivo studies have shown that TQ blocks tumor angiogenesis effectivity by blunting the activity of VEGF in many different cancers. TQ attenuates tumor angiogenesis by inhibiting the expression of VEGF as well as the signaling pathways stimulated by VEGF. TQ also downregulates the expression VEGF in several different cell lines derived from gastric carcinoma [129], osteosarcoma [130], colon carcinoma [131], cholangiocarcinoma [63], prostate cancer cells [95], multiple myeloma cells [101,102], and leukemia [70]. In addition to VEGF, TQ has been shown to inhibit the expression of several other angiogenic chemokines, such as CXCL5/ENA-78 and CXCL1/Gro-alpha cytokines in non-small lung cancer and small cell lung cancer cell lines [59]. Both in vivo and invitro analyses have shown that the effect of TQ on VEGF expression is primarily due to its inhibitory effect on the activation of NF-κB, which is involved in the regulation of several angiogenic genes including VEGF [130,131]. In addition to its direct effect on the expression of VEGF that can have significant negative impact on the angiogenic signaling in the endothelial cells in the tumor vasculature, TQ also inhibits the signaling pathways downstream of VEGF-VEGF-receptor

(VEGFR) in tumor associated endothelial cells. Using both a prostate cancer xenograft based in vivo mouse model and a human umbilical vein endothelial cells (HUVEC) based ex vivo model, it has been shown that TQ inhibits tumor angiogenesis and arrests prostate cancer growth [95]. TQ also inhibits the proliferation as well as migration of human endothelial cells. Using HUVEC cells, it has also been demonstrated that TQ inhibits VEGFR-mediated activation of ERK and AKT in these cells, rather than by directly inhibiting VEGFR2 receptor activation [95]. Thus, TQ mediated inhibition of angiogenesis occurs at two different tiers in the two distinct cellular components of the tumor microenvironment. In cancer cells TQ downregulates the expression of VEGF and other angiogenic growth factors. In tumor associated endothelial cells, TQ inhibits the activation of signaling pathways downstream of VEGFR.

ANTI-INFLAMMATORY ROLE OF TQ

Inflammatory signaling has been identified as a causative factor in the genesis and progression of many cancers. As an anticancer agent, TQ potently inhibits both the cellular and the organismal inflammatory responses [132,133] (Fig. 2). Inflammatory response mainly involves the synthesis and release of the eicosanoids, such as prostaglandins (PGs), thromboxanes (TBX), and leukotrienes (LTs) that act as the mediators of inflammation. While COX converts arachidonic acid (AA) into PGs and TBXs, lipoxygenase (LOX) converts AA into LTs. Experimental evidence indicates that TQ inhibits the generation of the eicosanoids by inhibiting the COXs as well as LOXs [134]. In the case of LTs, TQ shuts down the synthesis of LTs by inhibiting the activities of 5-LOX, 5- and LT C4 synthase [135]. TQ inhibits the synthesis of PGs and TBXs by downregulating the expression of COX2 by targeting the upstream interleukin-1 receptor-associated kinase 1 (IRAK1) [136]. By targeting the activity as well as the degradation of IRAK1, TQ downregulates the transcriptional activities of NF-κB and activator protein 1/AP1, which are collectively required for the transcriptional activation of COX-2 expression. In fact, through inhibition of the IRAK1-mediated signaling, TQ also downregulates the expression of many other inflammatory cytokines and signal mediators, such as interleukin (IL)-1, IL-6, TNFα, and inducible NOS [136]. It is noteworthy that the anti-oxidant activity of TQ can also play a role in attenuating cellular inflammatory response. Cytokine-induced generation of ROS has been shown to play a significant role in the synthesis of the inflammatory eicosanoids from AA through the activation and/or expression of the critical upstream signaling molecules including AKT and NF-κB [137]. Since the anti-oxidant activity of TQ is known to scavenge the free radicals [47], this could lead to the attenuation of inflammatory response through the inhibition of eicosanoid synthesis.

IMMUNOMODULATORY ROLE OF TQ

Owing to the overlapping pathways involved in inflammatory response and immunomodulation, TQ has significant immunomodulatory effects. Potentially, TQ could suppress inflammation-induced immuno-suppression through its negative impact on the pro-inflammatory eicosanoid synthesis and NF-κB mediated gene expression [138]. While several lines of evidence attest to this potential of TQ, they are primarily derived from the use of *N. sativa* extract or oil rather than from the use of TQ alone [133,139,140]. Thus, there is a paucity of information on the immunomodulatory role of TQ in relation to its anticancer activity. However, a small set of studies using TQ has indicated that TQ can modulate both innate and adaptive immune responses [133]. With its potent inhibitory effect on the activity or the expression of different inflammatory cytokines and their effector molecules, TQ can modulate different aspects of cellular as well as humoral immunity [133]. Cellular immune responses modulated by TQ include dendritic cell maturation, NK-cell cytotoxicity, phagocytic activity, chemotaxis, and T cell activation [141,142]. The effect of TQ on the specific immune response appears to be context-specific. For instance, TQ inhibits lipopolysaccharide-induced maturation of dendritic cells by blunting the expression of IL-10, IL-12, and TNFα along with the activation of caspase 3/8 and increasing annexin V binding [141]. On the contrary, TQ rescued T cell activation and the associated immune response in the *Streptozotocin*-treated mouse model by stimulating IL-2 and T cell proliferation [143].

An immunomodulatory role of TQ was also demonstrated by the observation that TQ stalls and overcomes pesticide-induced reduction in immunoglobulin levels, leukocyte numbers, and phagocytic activities of macrophages in in a rat model system [144]. Although these studies do not directly correlate with the potential anticancer immunity of TQ, they do point out the immunoregulatory function of TQ and its therapeutic potential in cancer immunotherapy. A case in point is the observation that TQ enhances the survival of antigen-specific CD8-positive T cells along with the sustained expression of the homing receptor cyclical expression of L-selectin/L-selectin, which could have a significant impact in expanding cluster of differentiation-8/CD8- and/or L-selectin-positive T cells for adoptive T cell therapy [145].

EPIGENETIC MODULATION OF GENE EXPRESSION BY TQ

TQ-mediated anticancer activity has also been shown to involve epigenetic suppression or activation of specific genes [57,146,147]. TQ-induced p73 has been shown to represses the expression of the anti-apoptotic epigenetic integrator known as ubiquitin-like-containing PHD and RING finger domains (UHRF1) and its partner DNA methyltransferase 1 (DNMT1) [57,69]. Since UHRF1-DNMT1-histone deacety-

lase (HDAC) complex is very much involved in epigenetic regulation of gene expression, the effect of TQ on p73 and UHRF1 provide an epigenetic basis through which TQ can modulate the expressions of diverse pro- and anti-apoptotic proteins. Results from ex vivo studies using cell lines derived from T-cell acute lymphoblastic leukemia and breast cancer have brought to light the ability of TQ to attenuate the expression of epigenetic regulator UHRF1 and its partners DNMT1, HDAC1, and euchromatic histone-lysine N-methyltransferase 1 [146]. Since UHRF1 is involved in silencing several tumor suppressor genes and pro-apoptotic genes in many cancers, TQ-mediated decrease in UHRF1 levels leads to the de-repression and increased expression of several tumor suppressor genes and pro-apoptotic genes in TQ-treated cells (Fig. 2). This has been demonstrated in Jurkat cells in which TQ activates the expression of several tumor suppressor genes (*DLC1*, *PPARG*, *ST7*, *FOXO6*, *TET2*, *CYP1B1*, *SALL4*, and *DDIT3*) *as well as* proapoptotic genes (*RASL11B*, *RASD1*, *GNG3*, *BAD*, and *BIK*) through such epigenetic mechanism [146].

In addition to the UHRF1-mediated mechanism, a direct role for TQ in physically interacting with DNMT1 and inhibiting its DNA-methylation activity has also been proposed [148]. TQ has also been shown to inhibit HDAC activity, possibly through direct interactions with HDACs [64]. In MCF7 breast cancer cells, TQ inhibits the deacetylation activity of HDAC and reactivates the expression of p21, Mapsins, and Bax, thereby ushering the cells into G2/M cell cycle arrest. It has also been observed that the chemical structure of TQ can also contribute to epigenetic regulation of gene expression [147]. Thus, TQ can donate or accept a methyl group from DNA, thus playing a mediatory role in DNA-methylation and demethylation respectively.

CONCLUDING REMARKS AND PERSPECTIVES

As presented here, detailed experimental analysis of the biological activities of TQ has clearly established it as a multi-target inhibitor that exerts its chemopreventive and anticancer activities by inhibiting multiple pathways involved in tumorigenesis, tumor progression, and metastasis. It should be stressed here that none of these pathways are mutually exclusive. Often, they are inter-connected and overlapping (Fig. 2). The cumulative effects lead to the potent inhibition of cancer cell growth and progression by TQ. As discussed above, TQ has been shown to inhibit the cellular machinery and molecular targets underlying almost all of the hallmarks of cancer. Thus, the ability of TQ to inhibit multiple signaling pathways involved in cancer growth and progression highly underscores its potential as an ideal candidate for adjuvant therapy. Of particular interest are the findings that TQ can overcome resistance to various chemotherapeutic drugs and potentiate their efficacies while reducing their innate cytotoxicity [140]. Such chemo-potentiating effects of TQ in different cancer cells have been observed with 5-fluorouracil in gastric cancer and colorectal cancer models [149,150]; Bortezomib in multiple myeloma cell line [102]; Cisplatin in colon carcinoma [131], ovarian carcinoma [151], esophageal carcinoma [152], and lung cancer [59]; Doxorubicin in leukemia [33], melanoma, cervix, and breast cancer cell lines [153]; Docetaxel in prostate cancer [86,87]; Gemcitabine in breast [117] and pancreatic cancer [84] models; Oxaliplatin in osteosarcoma [154], pancreatic [155], and ovarian cancer [156] models; Paclitaxel in triple negative breast cancers [157]; Tamoxifen in breast cancer [67,158]; Temozolomide in glioblastoma [159]; and Topotecan in colorectal cancer [160] and acute myelogenous leukemia cell lines [161]. In addition to these chemopotentiation and chemosensitization effect, TQ has also been observed to have a radiosensitization and radioprotection effects in many cancers, such as breast [75] and head and neck cancers [162]. Collectively, these studies point to the potential role of TQ as an adjuvant therapeutic agent for many cancers.

However, it should be noted that the efficacy of TQ alone or in combination therapies could be context-specific. For example, TQ treatment along with paclitaxel reduced the potency of paclitaxel as evidenced by an increase in IC$_{50}$ values for paclitaxel in the combination regiment. TQ has been shown to antagonize the therapeutic efficacy of paclitaxel in breast cancer cell lines [163]. It is also of importance to note that although TQ-cisplatin combination treatment showed synergy in an in vitro oral squamous cell carcinoma model, it contrarily increased cytotoxicity to normal oral epithelial cells [54]. In a similar vein, a recent in vivo study, while emphasizing the potency of TQ in combination therapy with cisplatin in ovarian cancer xenograft tumor growth [151], gives cautions that the long-term treatment with TQ leads to an increase in the NF-$_\kappa$B activity and attenuation of its therapeutic efficacy [164]. The observed attenuation of the therapeutic activity of TQ has been attributed to "microenvironmental effects". This is further substantiated by the observations that TQ could synergize with the pro-oncogenic activity of lysophosphatidic acid, which is present in ovarian cancer tumor microenvironment [165]. Specifically, TQ has been demonstrated to augment lysophosphatidic acid-induced glycolytic shift in ovarian cancer cells. Thus, context-specific effects of TQ need to be considered in the development of TQ-based anticancer therapeutic strategy. Nevertheless, mounting evidence from the in vitro and in vivo studies collectively underscore and validate the role of TQ as a potent chemopreventive as well as therapeutic agent. Despite its well-established role as a multi-targeted chemopreventive agent, detailed human studies are still lacking. To date, there has been only one Phase 1 clinical trial with TQ [31]. While the results from the Phase I trial quite significantly established the safe dosage for administration, the anticancer effect of TQ at the administered tolerant dosage could not be successfully established. This has been pri-

marily attributed to the physicochemical characteristics of TQ and its relatively poor bioavailability. However, with the development of the second generation TQ analogues [166,167] and novel nanoparticle-based delivery systems [168,169], it should be possible to embark on new human clinical trials and establish the role of TQ in effective therapeutic regimen for many cancers.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

ORCID

Rohini Gomathinayagam,
  https://orcid.org/0000-0001-7626-3403
Ji Hee Ha,
  https://orcid.org/0000-0002-6281-1904
Muralidharan Jayaraman,
  https://orcid.org/0000-0003-3274-877X
Yong Sang Song,
  https://orcid.org/0000-0001-7115-4021
Ciro Isidoro,
  https://orcid.org/0000-0002-5494-3034
Danny N. Dhanasekaran,
  https://orcid.org/0000-0001-6350-8926

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