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Molecular evidence of cryptotanshinone for treatment and prevention of human cancer

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Abstract

Cryptotanshinone is one of the major tanshinones isolated from the roots of the plant *Salvia miltiorrhiza Bunge* (Danshen). Danshen has been widely used in traditional Chinese medicine for treatment of a variety of diseases, including coronary artery disease, acute ischemic stroke, hyperlipidemia, chronic renal failure, chronic hepatitis, and Alzheimer's disease, showing no serious adverse effects. Recent studies have shown that cryptotanshinone not only possesses the potential for treatment and prevention of the above-mentioned diseases, but also is a potent anticancer agent. Here we briefly summarize the physical and chemical properties and the pharmacokinetic profiles of cryptotanshinone, and then comprehensively review its anticancer activities as well as the underlying mechanisms.

Keywords

Cryptotanshinone; tanshinones; cancer; cell proliferation; cell death; mTOR

1. Introduction

Phytochemicals, naturally occurring substances in plants, have attracted considerable public and scientific interest to find their activities to relieve human diseases, especially the two commonest killers in the industrialized world, cardiovascular disease and cancer [1]. *Salvia miltiorrhiza Bunge*, also known as red sage or Chinese sage, is a deciduous perennial plant in the genus *Salvia*, highly valued for its roots, called Danshen, in traditional Chinese medicine [2, 3]. It typically grows to 30-60 cm high, with branching stems, oval leaves and purple flowers, and mainly distributes in northeastern China, Korea and Japan [4], at 90-1200 m elevation, preferring grassy places in forests, hillsides, and along stream banks

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[5]. Danshen is one of most widely used herbs in China and now largely exported to other countries [6, 7].

Danshen is typically characterized by dilating blood vessels and removing blood stasis, and thus has been clinically used to treat ischemic diseases including angina pectoris (chest pain) and ischemic stroke in China [4, 8]. Danshen has also been frequently used to treat hyperlipidemia, hepatic fibrosis, chronic renal failure, and gynecological diseases, such as dysmenorrhea, amenorrhea, and lochioschesis [9]. Modern studies have revealed that Danshen is an effective platelet aggregation inhibitor decreasing blood hypercoagulability and increasing coronary blood flow, and scavenges free radicals in ischemic diseases, thus reducing cellular damage from ischemia [3]. Furthermore, clinic trials have also indicated that Danshen is an effective agent for prevention and treatment of Alzheimer's disease [3]. Danshen contains fat-soluble diterpenoids and water-soluble phenolic acids, as well as flavonoids and triterpenoids. Up to date, it has been identified that salvianolic acid B is one of the main hydrophilic extracts, whereas tanshinone I, tanshinone IIA, dihydrotanshinone, and cryptotanshinone (Fig.1) are the major lipophilic ingredients in Danshen. Most of Danshen extracts exhibit certain medicinal effects *in vitro* and *in vivo* [3]. In particular, recent studies have revealed that cryptotanshinone not only has potential to prevent ischemia, atherosclerosis, and Alzheimer's disease, but also possesses diverse properties, such as antibacterial, anti-inflammatory, antioxidative [10], antidiabetic, anti-obesic, and anticancer activities (Table 1) [11]. Here, we briefly summarize the physical and chemical properties, as well as the pharmacokinetic profiles of cryptotanshinone, and then comprehensively review its anticancer activities and the underlying mechanisms.

2. Physical and chemical properties of cryptotanshinone

Cryptotanshinone, also named cryptotanshinon or tanshinone C, is a cell-permeable diterpene quinone, one of the major tanshinones (including tanshinone I, tanshinone IIA, dihydrotanshinone and cryptotanshinone) (Fig.1) derived from the roots of *Salvia miltiorrhiza Bunge* [12, 13]. Cryptotanshinone is an orange-brown powder, which is now commercially available. Its chemical name is 1,2,6,7,8,9-hexahydro-1,6,6-trimethyl-(R)-phenanthro(1,2-b)furan-10,11-dione (CAS registry number: 35825-57-1), with a molecular formula of C₁₉H₂₀O₃, a molecular weight of 296.36 and a melting point of 184°C. Cryptotanshinone is not water-soluble, but soluble in dimethyl sulfoxide, methanol, ethanol, chloroform and ether, and turns red when interacting with concentrated sulfuric acid [12, 13]. Cryptotanshinone belongs to phenanthraquinone derivatives, and photochemical reaction would occur, when exposed to light [14]. This is evidenced by the findings that the content of cryptotanshinone was found to be obviously different in the methanol extract of Danshen or in cryptotanshinone methanol solution before and after illumination [14]. Additionally, the solubility of cryptotanshinone varies slightly between pH 2 to pH 8, but largely between pH 10 to pH 12 [15], implying that cryptotanshinone is highly sensitive to alkaline conditions as well. The fluorescence property and the molecular structure of cryptotanshinone are undergoing considerable change in different media [15]. When in strong acidic or in strong basic medium, the fluorescence is quenched completely and shifted to a higher frequency [15]. If the strong acidity is adjusted to neutrality, the fluorescence intensity can be retrieved [15]. The reaction process [15] is illustrated in Fig.2. Therefore, it is suggested that cryptotanshinone be stored and protected from light and under dry and cool (2-8°C) condition.

Synthetic cryptotanshinone was firstly achieved by Hiroshi Kakisawa and Yoshinobu Inouye, two Japanese chemists, in 1969 [16]. Synthesis of cryptotanshinone took multiple steps, involving Diels-Alder adduct reaction between 3-methylbenzofuran-4,7-quinone and 6,6-dimethyl-1-vinylcyclohexene in ethanol at room temperature, followed by air oxidation

and further adduction with concentrated sulfuric acid in ethanol, catalyzed by 5% Pd-C (Palladium on carbon) (Fig.3) [16].

3. Pharmacokinetic profiles of cryptotanshinone

Pharmacokinetic studies of cryptotanshinone have been carried out in rats, mice and pigs [17-20]. In rats, cryptotanshinone was found to mainly distribute in liver, lung, brain and heart, while little was detected in spleen and kidney [17]. Only 0.34% of the dose was recovered in the 48-h urine as unchanged form after a single oral administration, indicating that a great part of cryptotanshinone was metabolized in the body [17]. In addition to cryptotanshinone, the following metabolites, including tanshinone IIA, hydroxylated forms of tanshinone IIA, and a glutamic acid-tanshinone IIA conjugate, were isolated and identified from urine and bile samples (Fig.4) [17].

In pigs, cryptotanshinone was given intravenously at a single dose of 10 mg/kg, and cryptotanshinone concentration in serum was measured by high performance liquid chromatography. Half time ($t_{1/2}$) of cryptotanshinone distribution in the body was 2.36 min, and $t_{1/2}$ of its elimination was 64.78 min [18, 19], also suggesting that cryptotanshinone distributed and was metabolized quickly. In addition, tanshinone IIA was detected after 4.6 min [18, 19], further highlighting that cryptotanshinone could be converted to tanshinone IIA by the metabolism transformation very rapidly in the body. Oral administration of 20 mg/kg cryptotanshinone in pigs resulted in non-detectable cryptotanshinone and tanshinone IIA in serum. However, when the dose increased to 40 mg/kg, 0.043 $\mu\text{g/ml}$ of cryptotanshinone in serum was detected at 1 h of post-administration [18, 19]. These data indicate that the bioavailability of cryptotanshinone is very low when orally administered. The biliary and urinary excretion of both cryptotanshinone and its metabolite tanshinone IIA was minimal (0.3% of total dose over 48 h) after oral, intramuscular, or intravenous dosing, but the fecal recovery was 12% [19]. It remains elusive why such low bioavailability of cryptotanshinone occurs. Possibly, this is related to first-pass metabolism and poor intestinal absorption [20]. Using Caco2 cells, P-glycoprotein-overexpressed MDCKII cells and single-pass rat intestinal perfusion, Zhang et al found that cryptotanshinone was a substrate of P-glycoprotein, an ATP-dependent efflux pump, that pumped cryptotanshinone into the luminal side, resulting in low bioavailability [20].

Due to the low bioavailability of cryptotanshinone, a lot of efforts have been tried to increase serum level of the compound. For example, hydroxypropyl- β -cyclodextrin-included complex of cryptotanshinone was prepared for oral administration [21]. After a single oral dose, cryptotanshinone in the inclusion complex was absorbed slowly and the C_{max} and AUC_{0-t} were dose-dependent, as detected by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) [21]. The oral bioavailability of cryptotanshinone in rats was increased by 2.5-fold (2.05% for parent cryptotanshinone *versus* 6.90% for cryptotanshinone inclusion complex) at 60 mg/kg [21]. After a single oral dose of cryptotanshinone inclusion complex at 53.4 mg/kg, the bioavailability of cryptotanshinone was 11.1% \pm 1.8% in dogs [21]. The $t_{1/2}$ values of cryptotanshinone in rats and dogs were 5.3-7.4 and 6.0-10.0 h, respectively [21]. Compared with the parent cryptotanshinone, the oral inclusion complex has a higher bioavailability and longer half life. However, excretion data in rats did not show significant change between the two preparations [21, 22]. Of interest, oral administration of liposoluble ethanol extract of Danshen, in comparison with the equivalent dose of single cryptotanshinone or tanshinone IIA administration, increased the plasma concentrations of cryptotanshinone and tanshinone IIA by about 8 and 10 folds, respectively [23], suggesting that a drug-drug interaction may occur between the coexisting tanshinones, which affects their absorption, transformation and metabolism.

Besides, biotransformation also affects metabolism of cryptotanshinone. It has been described that cryptotanshinone was able to be biotransformed by *Cunninghamella elegans* to three new products, respectively identified as (3R,15R)-3-hydroxycryptotanshinone, (3S,15R)-3-hydroxycryptotanshinone, and (4S,15R)-18-hydroxycryptotanshinone, that are identical to three of the minor hydroxylated metabolites *in vivo* (Fig. 5) [24]. Although the activities of the hydroxylated forms of cryptotanshinone remain to be defined, the above finding suggests that microbial biotransformation be a feasible approach for large preparation of certain useful metabolites, if the amount of the metabolites is traceable in the body.

4. Therapeutic potential of cryptotanshinone for human cancer

Danshen has been used to treat coronary artery disease and ischemic stroke in China for centuries. Modern studies have demonstrated that cryptotanshinone from Danshen extracts has potent pharmacological activity against cardiovascular and cerebrovascular diseases [3]. Increasing evidence has implicated that cryptotanshinone possesses more activities, such as anti-Alzheimer's disease, anti-bacteria, anti-inflammation, anti-oxidation, anti-diabetes, anti-obesity, anti-fibrosis, and anticancer as well [25]. Therefore, cryptotanshinone has received more and more attention. Here we focus on reviewing the current findings regarding the anticancer activities of cryptotanshinone and the underlying mechanisms. In particular, cryptotanshinone targeting the phosphatidylinositol 3'-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway will be discussed.

4.1. Inhibition of cell proliferation

In vitro studies have demonstrated that cryptotanshinone inhibits cell proliferation in a variety of cancer cell lines, including rhabdomyosarcoma, melanoma, cervical, colon, breast and prostate cancer cells [11, 26-28]. The inhibitory effect on cell proliferation is through inducing a cell cycle arrest either at G₁/G₀ or G₂/M phase, depending on cell lines [11, 26-28]. It has been described that cryptotanshinone induced a G₁/G₀ cell cycle arrest in rhabdomyosarcoma (Rh30) and prostate cancer (DU145) cells by downregulating expression of cyclin D1 and phosphorylation of retinoblastoma protein (Rb) [11, 26], but induced a G₂/M cell cycle arrest in melanoma (B16) cells via upregulating expression of Cdc25c, cyclin A1 and cyclin B1 [27]. Cryptotanshinone also upregulated expression of p21^{Cip1} and p27^{Kip1}, two inhibitors for G₁ or G₁/S cyclin dependent kinases [11, 27]. In addition, Aurora A kinase, regulating mitosis and overexpressing in numerous cancer cells, was also proved to be an important anticancer target [29]. Cryptotanshinone inhibiting expression of Aurora A kinase led to significant suppression of prostate cancer cells *in vitro* and in mice [28].

4.2. Induction of cell death

Cryptotanshinone also induces cell death in cancer cells [27, 28, 30]. The underlying mechanisms remain to be elucidated. Studies have shown that cryptotanshinone induced apoptosis in prostate cancer (DU145) and rhabdomyosarcoma (Rh30) cells by downregulating expression of anti-apoptotic proteins including survivin and Mcl-1 [26, 30]. It has been speculated that this might be related to inhibition of the transcriptional activity of the signal transducer and activator of transcription 3 (Stat3) [26], as Mcl-1 and survivin are positively regulated by Stat3 at transcriptional level [31, 32]. Stat3, regulated by Janus kinases (JAKs), is constitutively activated in most human malignant tumors, and involved in the proliferation, angiogenesis, immune evasion and anti-apoptosis in cancer cells [33, 34]. Therefore, Stat3 has been regarded as a potential target for cancer therapy [33, 34]. However, cryptotanshinone inhibited Tyr705 phosphorylation of Stat3 independent of the JAKs [26]. Instead, by computational modeling, cryptotanshinone was found to bind to the

SH2 domain of Stat3 and subsequently block Stat3 dimerization, nuclear translocation and Stat3-dependent transcription activity [26].

Mostly recently, we have demonstrated that cryptotanshinone induction of cell death was independent of activation of caspase cascade, as treatment with cryptotanshinone neither altered caspase3/7 enzyme activities, nor induced cleavage of caspase 3 or poly ADP-ribose polymerase (PARP) in Rh30 and DU145 cells [30]. It appears that cryptotanshinone induced caspase-independent cell death by activation of c-Jun N-terminal kinase (JNK) and p38 and inhibition of extracellular-signal-regulated kinases 1/2 (Erk1/2) in Rh30 and DU145 cells. This is strongly supported by the findings that: i) Inhibition of p38 with SB202190 or JNK with SP600125 attenuated cryptotanshinone-induced cell death. ii) Silencing p38 or c-Jun also in part prevented cryptotanshinone-induced cell death. iii) Expression of constitutively active mitogen-activated protein kinase kinase 1 (MKK1) conferred resistance to cryptotanshinone inhibition of Erk1/2 phosphorylation and induction of cell death [30]. Furthermore, we observed that cryptotanshinone-induced cell death was attributed to induction of reactive oxygen species (ROS), as N-acetyl-L-cysteine (NAC), a ROS scavenger, attenuated cryptotanshinone activation of p38/JNK, inhibition of Erk1/2, and induction of cell death [30]. Similarly, cryptotanshinone induction of ROS triggered endoplasmic reticulum (ER) stress and activated mitogen-activated protein kinase (MAPK) cascade, leading to apoptosis in hepatoma (HepG2) and breast carcinoma (MCF7) cells [35]. Through inducing ER stress, cryptotanshinone also synergized the anticancer activity of TNF- α , Fas, cisplatin, etoposide or 5-fluorouracil [35], suggesting a therapeutic potential for human hepatoma and breast cancer.

4.3. Inhibition of cell motility and invasion

Cancer metastasis is a process by which cancer cells spread to other parts in the body, which is a leading cause of death in cancer patients [36]. Tumor cell migration and invasion are prerequisite for metastasis [36]. It has been described that cryptotanshinone inhibited complement 5a (C5a) or macrophage inflammatory protein-1 α (MIP-1 α)-induced motility in RAW264.7 and primary human macrophages [37, 38]. The anti-migratory effect of cryptotanshinone was attributed to its inhibition of C5a or MIP-1 α -induced F-actin polymerization and filopodia formation [37]. Furthermore, cryptotanshinone inhibition of C5a or MIP-1 α -induced PI3K-p110 γ membrane translocation as well as phosphorylation of Akt and Erk1/2 also contributed to the anti-migratory activity [38]. Moreover, in our studies we have observed that cryptotanshinone inhibited cell motility in rhabdomyosarcoma (Rh30) cells as well, although the underlying mechanism is unknown.

In addition, cryptotanshinone was found to inhibit basic fibroblast growth factor (bFGF)-stimulated invasion in bovine aortic endothelial cells (BAECs) in culture [39]. Interestingly, under the same condition, tanshinone IIA did not show such activity [39]. Analysis of the structure-activity relationship revealed that the only structural difference between the two tanshinones was double bond at C-15 position of the dihydrofuran ring [39]. Therefore, it has been proposed that the absence of double bond at C-15 position is the key for the inhibitory effect on cell invasion [39]. In addition, cryptotanshinone was found to inhibit tumor necrosis factor- α (TNF- α) induced invasion in human aortic smooth muscle cells by inhibition of expression of matrix metalloproteinase 9 (MMP-9) at transcriptional level [40]. This was possibly related to cryptotanshinone inhibition of TNF-induced activation of Erk1/2, p38 and JNK signaling pathways, which led to downregulation of the transcriptional activities of activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) [40]. Apparently, the effect of cryptotanshinone on MAPK cascade in human aortic smooth muscle cells [40] is in sharp contrast to that in cancer cells (Rh30 and DU145), as described above [30]. Further studies are required to determine whether this is due to different cell lines or stimuli used.

4.4. Anti-angiogenesis

Angiogenesis is a process that new capillaries forms with endothelial cells and sprouts from preexisting vessels [41]. It is regulated by a number of factors involving endothelial cells and other cell types (e.g. monocytes/macrophages, fibroblasts, smooth muscle cells, pericytes), cytokines, molecules of the extracellular matrix and adhesion molecules [42]. More importantly, due to playing a crucial role in tumor growth and metastasis, angiogenesis has been regarded as a promising target for cancer therapy [41, 42]. Bevacizumab (Avastin, Genentech/Roche), a humanized monoclonal antibody that inhibits vascular endothelial growth factor (VEGF) and slows angiogenesis, has been used to treat various types of cancer, including colorectal, lung, breast, kidney and ovarian cancer, as well as glioblastoma [43]. Increasing evidence suggests that cryptotanshinone possesses anti-angiogenic activity as well. For instance, cryptotanshinone was found to inhibit proliferation in BAECs in culture, with an IC_{50} of 10 μ M [39]. Also, cryptotanshinone at 10 μ M inhibited bFGF-stimulated invasion and tube formation in BAECs [39]. Furthermore, cryptotanshinone inhibited formation of microvessels in chick embryo chorioallantoic membrane [44]. Obviously, it is necessary to perform more *in vivo* studies in animals to validate the anti-angiogenic activity of cryptotanshinone. Also, further investigations are required to understand how cryptotanshinone functions as an anti-angiogenic agent.

4.5. Anti-lymphangiogenesis

Lymphangiogenesis refers to the formation of lymphatic vessels from pre-existing lymphatic vessels, which not only plays a critical role in regulating physiological homeostasis, metabolism and immunity, but also promotes tumor metastasis [45, 46]. Anti-lymphangiogenesis has been considered as a potential tactic for cancer treatment and prevention [45, 46]. Recently, we have demonstrated that cryptotanshinone inhibited tube formation in murine lymphatic endothelial cells, an *in vitro* model of lymphangiogenesis, suggesting that cryptotanshinone has anti-lymphangiogenic activity [47]. The anti-lymphangiogenic effect of cryptotanshinone was partly attributed to its downregulating protein expression of VEGF receptor 3 (VEGFR-3), as overexpression of VEGFR-3 conferred resistance to cryptotanshinone inhibition of the tube formation [47]. Furthermore, cryptotanshinone functioned as an anti-lymphangiogenic agent, in part by suppressing VEGFR-3-mediated Erk1/2 phosphorylation, and in part by inhibiting protein expression and activities of the small GTPases, such as Rac1 and Cdc42 [47]. More *in vivo* studies are expected to substantiate the *in vitro* results.

4.6. Anti-oxidant activity

Oxidative stress generates plenty of free radicals, involved in multiple diseases such as aging [48], cancer [49], obesity [50], and Alzheimer's disease [51]. Studies have shown that cryptotanshinone exhibited inhibitory effects on xanthine oxidase activity [52] and pyrogallol autoxidation [53]. Cryptotanshinone improved the ability of learning and memory in APP/PS1 transgenic mice, protected rat cortical neurons from glutamic acid-induced neurotoxicity [54], and relieved acute liver damage induced by carbon tetrachloride [10], by suppression of lipid peroxidation. Cryptotanshinone was found to enhance the activity of anti-oxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase, relieving carbon tetrachloride-induced liver injury *in vivo*, and reducing production of intracellular ROS *in vitro* [10]. Moreover, cryptotanshinone was able to directly clear hydroxyl free radicals *in vitro* [55]. Cryptotanshinone (10 μ g/ml, corresponding to 3.37 μ M) protected primary rat hepatocytes from bile acid-induced apoptosis by inhibiting JNK phosphorylation [56]. Also, cryptotanshinone (1 μ M) directly inhibited hydrogen peroxide-induced NF- κ B luciferase reporter gene activity in human umbilical vein endothelial cells [57]. The aforementioned data strongly suggest that cryptotanshinone is a potent anti-oxidant. However, our recent studies have shown that cryptotanshinone is also an oxidant, as

treatment with cryptotanshinone was able to induce ROS in a time- and concentration-dependent manner in rhabdomyosarcoma (Rh30) and prostate cancer (DU145) cells [30]. Of note, in our studies, cryptotanshinone did not significantly increase ROS level in Rh30 and DU145 cells until 5 μ M. Therefore, CPT acts as an anti-oxidant at low concentrations (<5 μ M), whereas likely exerts oxidized activity at high concentrations (>5 μ M). From publications, it appears very common that a natural product may act as an anti-oxidant or oxidant, depending on concentrations and environmental conditions [58]. Typical examples include (-)-epigallocatechin-3-gallate (EGCG) [59, 60], curcumin [61-63], and resveratrol [64, 65]. Definitely, the oxidant or anti-oxidant issue of cryptotanshinone warrants further investigation.

4.7. Targeting androgen receptor signaling

Development and progression of prostate cancer are intimately associated with androgen receptor (AR) signaling [66]. Anti-androgen agents that reduce or prevent androgen binding to AR are widely used to suppress AR-mediated prostate cancer growth [66, 67]. Recent studies have shown that cryptotanshinone targets AR signaling as well [68-70]. For instance, on days 16-18 of pregnancy, rats were injected subcutaneously with testosterone propionate continuously for 3 days, and then treated with cryptotanshinone by gavage for 14 days. It was found that cryptotanshinone reduced the serum level of 17 α -hydroxy progesterone in male offspring, suggesting inhibition of androgen synthesis [71]. Further studies revealed that cryptotanshinone reversed the reproductive and metabolic disturbances in prenatally androgenized rats via regulating protein expression of insulin receptor substrate-1/2, PI3K p85 α , glucose transporter-4, Erk-1, and 17 α -hydroxylase in the ovaries [69]. Similarly, cryptotanshinone also reduced the levels of androgens by downregulating expression of the key enzymes for androgen synthesis in Akt^{-/-} mice [72].

In addition, cryptotanshinone was also found to inhibit the growth of AR-positive prostate cancer cells [68]. This was attributed to cryptotanshinone inhibition of the transcriptional activity of AR, thereby downregulating expression of several AR-target genes at the mRNA and the protein levels [68]. Mechanistically, cryptotanshinone disrupted the interaction between AR and lysine-specific demethylase 1 (LSD1), and suppressed the AR-LSD1 complex to the promoter of AR target genes without influencing the protein degradation and translocation of AR [68]. Cryptotanshinone enhanced the mono-methyl and di-methylation of histone H3 lysine 9 (H3K9), a repressive histone marker that is demethylated and activated by LSD1 [68].

Furthermore, cryptotanshinone, similar to dihydrotestosterone (DHT) in structure, was also found to inhibit prostate cancer cell growth by suppressing DHT-induced AR transactivation, which resulted in reduced expression of the DHT-mediated AR target genes (PSA, TMPRSS2, and TMEPA1) in both androgen responsive prostate cancer cells, LNCaP cells and castration resistant CWR22rv1 cells [70]. At molecular level, cryptotanshinone blocked DHT-induced AR dimerization and AR-coregulator complex formation [70]. Moreover, cryptotanshinone effectively inhibited CWR22Rv1 cell growth and expression of AR target genes in a xenograft model [70]. Collectively, these findings highlight that cryptotanshinone is a promising therapeutic agent for prostate cancer.

5. Inhibition of mTOR signaling

mTOR, a conserved and ubiquitously expressed serine-threonine kinase, lies downstream of type I insulin-like growth factor (IGF-1) receptor and PI3K, and functions at least as two complexes (mTORC1 and mTORC2) in mammalian cells [73, 74]. In response to growth factors, mTORC1 and mTORC2 phosphorylates p70 ribosomal protein S6 kinase 1 (S6K1)/ eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and Akt, respectively

[73, 74]. Although the cellular functions of the mTOR complexes remain to be determined, current data indicate that mTOR is a central controller for cell growth, proliferation, survival, motility, invasion, angiogenesis and lymphangiogenesis [73-75]. As discussed above, cryptotanshinone functions as an anticancer agent by inhibiting cell proliferation, inducing cell death, suppressing cell motility/invasion, and inhibiting angiogenesis/lymphangiogenesis. In particular, cryptotanshinone has been found to target certain proteins, such as Stat3 [26], cyclin D1 [11], NF- κ B [40], MMP9 [40], Akt [38, 76, 77], VEGF [78], VEGFR-3 [47], and small GTPases [47], which all are directly or indirectly regulated by mTOR [79-88]. This prompted us to hypothesize that the effects of cryptotanshinone on those proteins and the cellular events may be a consequence of inhibition of mTOR. Our recent findings [11] support this hypothesis. In the studies, we found that treatment of serum-starved Rh30 cells with cryptotanshinone for 2 h inhibited IGF-1-stimulated phosphorylation of S6K1 and 4E-BP1, in a dose- and time-dependent manner [11]. After 2 h exposure, cryptotanshinone obviously inhibited IGF-1-stimulated phosphorylation of S6K1 (Thr389) starting at 2.5 μ M; and at 10 μ M, cryptotanshinone profoundly suppressed this phosphorylation event within 2 h in Rh30 cells [11]. Similarly, cryptotanshinone also inhibited IGF-1-stimulated phosphorylation of 4E-BP1 in the cells [11]. Furthermore, cryptotanshinone did not apparently affect expression of total protein levels of S6K1 and 4E-BP1 [11]. In addition, we found that cryptotanshinone also inhibited IGF-1-stimulated phosphorylation of mTOR at Ser2448, a site phosphorylated by S6K1 [89], in a dose- and time-dependent manner [11]. Similar results were also noticed in DU145 and MCF-7 cells, and Rh30 cells grown in the normal culture medium containing 10% fetal bovine serum [11]. Interestingly, other tanshinones, including tanshinone I, tanshinone IIA and dihydrotanshinone, did not obviously affect phosphorylation of S6K1, 4E-BP1 and mTOR [11], which was in good agreement with the findings that cryptotanshinone, but not tanshinone I, tanshinone IIA and dihydrotanshinone, potently inhibited cancer cell proliferation [11]. Besides, expression of constitutively active mTOR conferred high resistance to cryptotanshinone inhibition of cyclin D1 expression and cell proliferation in Rh30 and DU145 cells [11].

However, in the studies, we also observed that cryptotanshinone did not affect expression of total Akt, but increased phosphorylation of Akt (Ser473) in Rh30 and DU145 cells [11]. Our result was in good consistence with the findings in C2C12 myotubes [76] and primary rat cortical neurons [77], but was in great contrast to the observation in macrophages [38]. Treatment with cryptotanshinone resulted in elevated phosphorylation of Akt (Ser473) in C2C12 cells [76]. Similarly, cryptotanshinone (5 μ M) rapidly and transiently activated phosphorylation of Akt (Ser473) in primary rat cortical neurons [77]. Time course studies revealed that cryptotanshinone induced obvious activation of Akt as early as at 5 min, and maximal at 30 min before returning to the baseline at 3 h in primary rat cortical neurons [77]. Dose response studies indicated that cryptotanshinone was able to activate Akt at 0.1 μ M, and the activation reached to the maximal level at 5 μ M in the neurons [77]. Cryptotanshinone did not affect the expression either in C2C12 cells or in primary rat cortical neurons [76, 77]. In addition, it has been described that cryptotanshinone activation of Akt appeared to be mediated by PI3K in primary rat cortical neurons, since this could be blocked by LY294002 and wortmannin, two PI3K inhibitors [77]. In contrast, cryptotanshinone-induced Akt activation in C2C12 cells was independent of PI3K, and instead, this was through activation of AMP-activated protein kinase (AMPK), as Compound C, an AMPK inhibitor, profoundly attenuated cryptotanshinone-induced Akt activation [76]. It remains unclear how cryptotanshinone activates Akt in Rh30 and DU145 cells. However, cryptotanshinone was found to inhibit complement 5a (C5a) or macrophage inflammatory protein-1 α (MIP-1 α)-induced phosphorylation of Akt in RAW264.7 and primary human macrophages [38]. This was likely associated with inhibition of PI3K, as

cryptotanshinone inhibited C5a or MIP-1 α -induced PI3K-p110 γ membrane translocation [38].

Taken together, current data suggest that cryptotanshinone inhibits mTORC1-mediated phosphorylation of S6K1 and 4E-BP1, but may inhibit or activate mTORC2-mediated Akt, depending on cell lines. It would be important to identify the underlying molecular mechanisms. The new data would help understand how cryptotanshinone acts as a new anticancer agent. The findings may also lead to design of novel tumor-selective treatments.

Conclusions and perspectives

Unlike tanshinone IIA that has been investigated comprehensively for a long time, cryptotanshinone was really concerned only in recent years, albeit they are very similar structurally. Based on what mentioned above, cryptotanshinone exerts diverse activities *in vitro* and *in vivo*. Increasing evidence indicates that cryptotanshinone is a potential anticancer agent. However, because of its poor bioavailability, cryptotanshinone has not been in clinical trials for any cancer therapy. Some attempts have been made to improve its bioavailability, but none of them proves effective enough. Clearly, more efforts are needed to solve the problem. Beside modification of its structure to increase the solubility and stability, nano-cryptotanshinone is a promising strategy. In addition, it is essential to further elucidate the molecular mechanisms by which cryptotanshinone functions as an anticancer agent. As mTOR is a master kinase that regulates cell growth/proliferation, survival, and motility, autophagy, as well as angiogenesis/lymphangiogenesis, understanding how cryptotanshinone inhibits mTOR signaling would shed new insights on the design and development of novel therapies.

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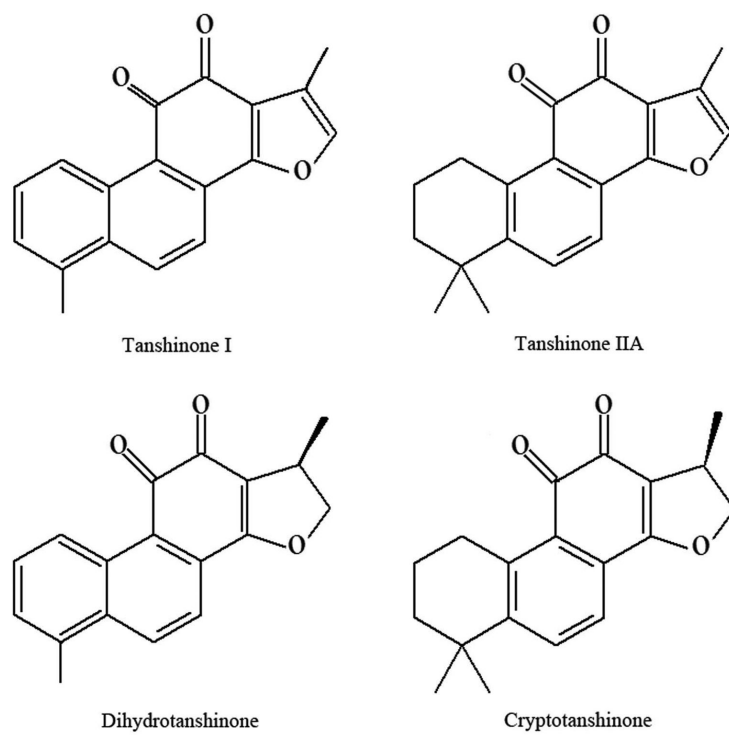


Fig.1. Structures of tanshinone I, tanshinone IIA, dihydrotanshinone and cryptotanshinone

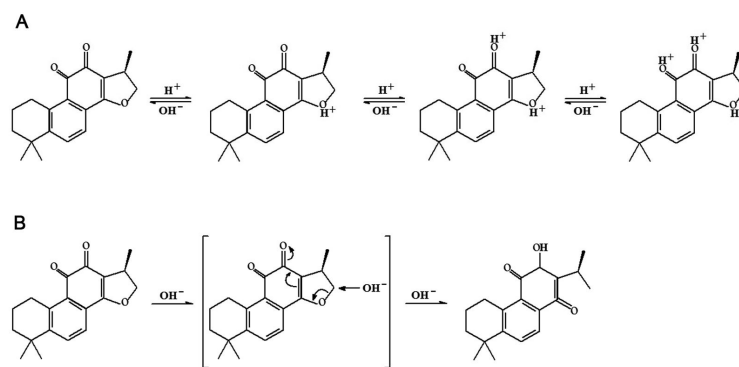


Fig.2. Tautomerism of cryptotanshinone under acidic (A) and strong alkaloid (B) conditions.

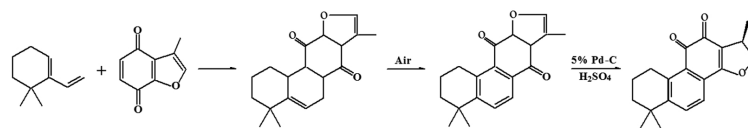


Fig.3.
The route of synthesis of cryptotanshinone.

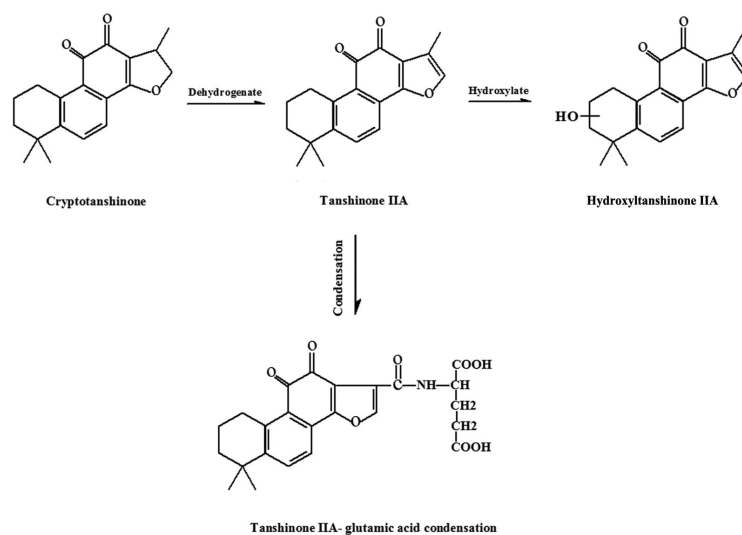


Fig.4.
Chemical structures of major metabolites of cryptotanshinone in rodents.

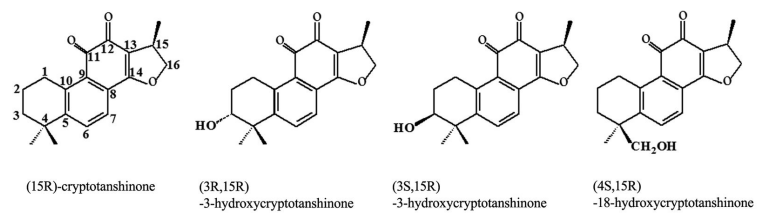


Fig.5. Chemicals from biotransformation of cryptotanshinone by *Cunninghamella elegans*.

Table 1

The overview of biological activity of cryptotanshinone

Biological activity	Targets	Possible application in diseases
Cardiovascular effect		
Vasorelaxation	Ca ²⁺ Endothelin-1	Coronary artery disease Acute ischemic stroke Chronic renal failure
Cerebrovascular effect		
Neuroprotection	ACHE* BuCHE*	Alzheimer's disease
Antibacterial activity	Superoxide radicals	G ⁺ & G ⁻ bacterial infection
Anti-inflammatory activity	NF- κ B, COX-1, IL-1, IL-2 TLR4*, TAK1*	Inflammation
Antioxidant activity	Superoxide radicals SOD, CAT, GPx	Aging; Cancer; Obesity Alzheimer's disease
Antidiabetes	AMPK, PPAR γ	Diabete; Obesity Hyperlipidemia
Anti-fibrosis	MMPs, JNK	Liver, lung, heart, kidney fibrosis
Anticancer	Detailedly discussed below	

ACHE: Acetylcholinesterase; BuCHE: Butyrylcholinesterase; TLR4: Toll-like receptor 4; TAK1: TGF- β -activated kinase 1;