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Review Article

Shikonin: A Review with a Focus on Anti-inflammatory and Anticancer Mechanisms

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Abstract

Shikonin (β-alkannin) is one of the main active components isolated from the Chinese herb *Lithospermum erythrorhizon*. Shikonin shows pleiotropic medical properties, such as antibacterial, wound healing, anti-inflammatory, antithrombotic, neuroprotective and antitumor effects. In this article, we discuss recent studies on the activities of shikonin with an emphasis on its effects on the intracellular signaling pathways relevant to anti-inflammatory and anticancer effects. Shikonin has a beneficial feature that it can induce apoptosis in many cancer cells despite its relatively low toxicity to normal cells. The effects of shikonin on many signaling pathways and processes including the NF-κB pathway, the PI3K/Akt pathway, the nuclear factor E2 (erythroid-derived 2)-related factor 2(Nrf2) pathway, the reactive oxygen species (ROS) production, and the aerobic glycolysis represent the presence of the multiple target. Similarities between the anti-inflammatory and anticancer mechanisms emerge, but shikonin differentially regulates the ROS levels depending on the cell type. This feature appears to enable shikonin to regulate the cell fate in a manner dependent on the cell context and experimental setting. Further analyses on shikonin are expected to provide insights into the mechanisms by which how cells integrate the ROS signaling in the decision of the cellular outcome, such as survival, autophagy and apoptosis.

Introduction

Shikonin (5,8-dihydroxy-2-[(1R)-1-hydroxy-4-methylpent-3-enyl]naphthalene-1,4-dione) is a naphthoquinone compound isolated from the Chinese herbs *Lithospermum erythrorhizon, Arnebia euchroma* and *Onosma paniculata*, which have been used for the relief of wounds, burns, dermatitis, and bleeding since ancient times in the traditional Chinese medicine [1-3]. In 1918, Kuroda purified and crystallized a coloring compound from *Lithospermum erythrorhizon*, and named shikonin [4,5]. Shikonin and its derivatives have intrigued scientists from diverse fields as it and its derivatives were shown to confer many medicinal properties such as antibacterial, wound healing, anti-inflammatory, antithrombotic, neuroprotective and antitumor effects. Several excellent review articles on such multifaceted effects of shikonin have been published [1,6,7]. The present article is aimed to review recent studies on the anti-inflammatory and anticancer effects of shikonin. Shikonin modulates the activity of many enzymes and protein-protein interactions, making the analysis of specific targets challenging. Nonetheless, several studies have revealed specific target molecules of shikonin. Moreover, an increasing number of studies utilize anti-oxidative agents and specific inhibitors to probe the signaling molecules and the specific pathways modulated by shikonin. In this article, we give emphasis on such molecules and pathways in an attempt to propose a unified view on the anti-inflammation and anticancer effects of shikonin.

Prior to discussing specific findings, it would be instructive to list some data showing the relationship between the shikonin dose and the cellular outcome. Among the six cell lines tested by Gupta et al. [8], the most sensitive were Colo205 (human colon cancer) and K562 (myelogenous leukemia) cells that showed LD_{50} (at 48 h) of 0.17 and 0.20 μM, respectively. The less sensitive were MCF-7 (human breast cancer) and Hela (human cervical carcinoma) cells with LD_{50} of 3.1 and 1.2 μM, respectively. Thus, the sensitivity to shikonin significantly differs among cancer cells. For non-cancer cells, shikonin shows cytotoxicity at relatively high concentrations. For example, for the retinal pigment epithelial cells primary culture, the 24 h cell viability was unaffected by 1 μM, but was slightly decreased by 3 μM and decreased to 40% by 10 μM shikonin [9]. Likewise, 1 μg/ml (3.5 μM) shikonin only slightly decreased the viability of TGF-β1 stimulated human skin fibroblast [10]. However, Nie et al. reported that mouse lung fibroblasts showed IC_{50} (48 h) of 0.87 μM shikonin, representing high cytotoxicity at a low concentration range [11]. It is interesting that unstimulated fibroblasts have similarity in sensitivity with cancer cells. The relationship between such proliferative potential of normal cells and their sensitivity to drug has not been fully addressed.

Obviously, the effects that occur at <1.0 μM shikonin should be far more desirable than the effects attainable at >10 μM. Nonetheless, even when the individual molecular events are controlled with low efficacies (e.g., 10 μM), the cellular processes, such as cell migration, autophagy, and apoptosis, may well be controlled at high efficacies (~ 1 μM), due to the synergy of many activities.

The cell death pathway triggered by shikonin also depends on the cell and shikonin dose used. Han et al. showed

that, in leukemic cell lines HL60 and K562, shikonin induced an apoptosis at ≤ 2.5 μM, and necroptosis at ≥ 10 μM [12,13]. (For necroptosis, we refer the readers to Linkermann et al. [14].) In human breast cancer MCF-7 and human embryonic kidney HEK293 cells, shikonin induced necroptosis irrespective of drug concentration. In other cases, switching between autophagy and apoptosis has been focused. Using hepatocellular carcinoma BEL7402 and Huh7 cells, Gong et al. found that 6 μM (24 h) and 2.5 μM (12 h) shikonin treatment induced apoptosis and autophagy, respectively, both in a manner dependent on shikonin-induced ROS production [15].

In the following we first discuss the diverse effects, targets and modalities of shikonin in the anti-inflammatory and anticancer interventions. Later, we focus on several signaling molecules and pathways including reactive oxygen species (ROS). Rather than providing a comprehensive summary of the broad targets regulated by shikonin, this review is focused on certain findings that provide molecular-level insight into the anti-inflammatory and anticancer effects of shikonin.

Diverse targets in anti-inflammatory effects of shikonin

A number of pro-inflammatory processes have been shown to be inhibited by shikonin [6,7]. In this section, we discuss a variety of studies that showed anti-inflammatory effects of shikonin and the signaling pathways affected. The effects on the NF-κB pathway is discussed in detail in the next section. Overall, these studies strongly suggest the presence of many targets of shikonin, as previously suggested [6,7].

Early observations include those reported by Hayashi that showed that topical application of shikonin and acetylshikonin blocked the increase in vascular permeability induced by histamine [16]. In vivo analyses by Hayashi showed overall pharmacological similarity between shikonin and acetylshikonin. Tanaka et al. demonstrated shikonin-mediated inhibition of the capillary permeability induced by an intradermal injection of histamine and edema caused by a thermal injury to the skin of rats [17]. Later, besides the inhibition of plasma extravasation, Wang et al. reported shikonin-mediated inhibition of mast cell degranulation [18].

Shikonin also modulates the production of eicosanoids. In the models of ear edema induced by croton oil in mice and paw swelling induced by yeast in rats, shikonin inhibited leukotriene B_4 biosynthesis [19]. Hsu et al. showed that the inhibition of eicosanoid production by acetylshikonin was attributable to the attenuation of the membrane recruitment of cytosolic phospholipase A_2 (at 3 μM) and to inhibition of the cyclooxygenase (ram seminal vesicles) and 5-lipoxygenase (human recombinant) activity, although the latter activities were seen at relatively high shikonin concentrations [20]. Intriguingly, at low concentrations, (≤ 1 μM) shikonin rather promoted phosphorylation and the membrane association of cytosolic phospholipase A_2. This exemplifies a complex feature of shikonin that its effect can reverse in a dose-dependent manner. Shikonin showed a moderate degree of inhibition with IC_{50} of 24.3 μM against leukotriene B_4 biosynthesis in human neutrophil granulocytes stimulated by calcium ionophore A23187 [21].

Acetylshikonin inhibits the respiratory burst in neutrophils with high efficacy. Wang et al. observed that, in a system containing acetylshikonin (>1 μM) and purified NADPH oxidase, acetylshikonin enhanced the activity, but in neutrophils it inhibited the membrane translocation of $p47^{phox}$, whose phosphorylation is essential to the activation of NADPH oxidase. They also demonstrated that protein tyrosine phosphorylation in neutrophils activated by formylmethionyl-leucyl-phenylalanine (fMLP)/dihydrocytochalasin B (CB) stimulation was blocked with high efficacy (~90% inhibition with 1 μM acetylshikonin), implicating these effects for the inhibition of respiratory burst in neutrophils by acetylshikonin [22]. It is possible that the inhibition of protein tyrosine phosphorylation by shikonin in association with the inhibition of NADPH oxidase assembly may have a mechanism similar to the early seminal finding on the ROS-mediated tyrosine phosphorylation, that is, the finding that growth factor-induced receptor phosphorylation requires NADPH oxidase-derived ROS [23]. In a similar system, phospholipase C activity was inhibited by acetylshikonin with IC_{50} of 21.4 μM. Acetylshikonin also inhibited the Ca^{2+} release from internal Ca stores with IC_{50} of 5.3 μM [24]. It is possible that the shikonin effects on the other targets than phosphatidylinositol signaling jointly assist and confer the high efficacy to the overall shikonin effect on Ca^{2+} mobilization.

Regarding the ROS-scavenging effect and the NADPH oxidase inhibition, in an analysis using electron paramagnetic resonance spectrometry, Yoshida et al. found potent activity of shikonin to scavenge $O_2^{\cdot -}$ and alkyl-oxy radical. Shikonin also inhibited NADPH oxidase activity in their cell-free reconstitution assay with IC_{50} of 1.1 μM [25]. This inhibition of NADPH oxidase was likely via impairing the assembly of the NADPH oxidase complex; when added after the NADPH oxidase activation, shikonin did not affect the enzyme activity. This finding is in accordance with Wang et al. that showed that NADPH oxidase complex assembly on membrane can be inhibited by shikonin at a low dose [22].

Chen et al. analyzed the direct effects of shikonin on several combinations of chemokine/receptor. In a system in which radiolabeled cytokines bind to chemokine receptor CCR1-expressing HEK/293 cells, shikonin inhibited the binding of RANTES and macrophage inflammatory protein-1α (MIP-1α) with concentrations of shikonin at 2.63 and 2.57 μM, respectively [26].

Shikonin inhibited the phorbol 12-myristate 13-acetate (PMA)-induced cyclooxygenase-2 (COX-2) expression in chondrosarcoma SW1353 cells and in human mammary epithelial 184B5/HER cells [27]. Their mutagenesis analysis did not show significance of the NF-κB binding site or the nuclear factor for IL-6 (NF-IL6) site in the *COX-2* promotor, but showed a crucial role for the cAMP response element (CRE). Shikonin and its six derivatives were equally effective in suppressing the TNF-α mediated induction of NF-κB activity with IC_{50} of 0.7 μg/ml (~2.4 μM). They further showed that shikonin inhibited the activation of extracellular signal-regulated kinase (ERK)1/2 and AP-1 in PMA-

stimulated 184B5/HER cells at relatively high concentrations (2.5 − 5 μg/ml).

Staniforth et al. showed that shikonin and derivatives can suppress the activity of the TNF-α promotor [28]. Analysis with a reporter gene transfection system showed that shikonin inhibits the binding of TFIID complex (TATA Box-binding Protein (TBP)) to TATA box in TNF-α promoter. This was likely due to the inactivation of TBP either by directly altering the association of TBP with TFIID or indirectly through host mechanisms that inhibits the TBP activity. However, in the authors system, p-ERK1/2 and p-NF-κB p65 increase induced by particle-mediated injury/stress was not blocked by shikonin.

Takano-Ohmuro et al. found that shikonin inhibits the histamine release from basophils induced by anti-IgE antibody with IC_{50} of 2.6 μM. Using recombinant kinase assay, they further showed that shikonin inhibits Syk protein tyrosine kinase (IC_{50} = 7.8 μM), a kinase important for the degranulation response [29].

Several more studies allowed us to infer novel targets for shikonin. The binding of lipopolysaccharide (LPS) to TLR4 requires an accessory protein called myeloid differentiation 2 (MD2 (lymphocyte antigen 96)) that participates in the LPS–TLR4 complex formation [30]. Recently, Zhang et al. showed that shikonin interferes with the LPS–MD2 interaction, although >10 μM shikonin was necessary for >50% inhibition [31]. Notably, using purified or recombinant materials, Gupta et al. sought novel direct targets of shikonin and found that inhibition of IL-8-binding to chemokine receptor CXCR2 with IC_{50} of 1.4 μM in human neutrophils. They also showed that shikonin inhibits CRTh2 (chemoattractant receptor homologous molecule expressed on Th2 cells) with IC_{50} of 1 μM, and activates nuclear factor E2 (erythroid-derived 2)-related factor 2 (Nrf2), a key factor for anti-oxidative factor in redox system, with EC_{50} of 7 μM [8].

Zorman et al. reported that Shikonin decreased NLRP3 inflammasome activation triggered by nigericin, an activator of the NLRP3 inflammasome. A half of IL-1β release was suppressed by ~1–2 μM shikonin. The authors also found that shikonin directly inhibits caspase-1 (down to 70% of activity by ~ 5 μM shikonin) [32].

As one can expect from such anti-inflammatory effects of shikonin, an increasing number of studies have reported the beneficial effects of shikonin in models of autoimmune diseases. As it is beyond our scope to list them all, we just list a couple of studies here that addressed the relatively new targets. For collagen-induced arthritis (CIA), beneficial shikonin effects were reported [33]. Dai et al. advanced this study and showed that shikonin increased expression of IL-10 and TGF-β transcripts and decreased the expression of IL-17A in the synovium and spleen. Dendritic cells in the spleen of shikonin-treated mice had lower expression of TLR4 and MyD88 [34]. Shikonin treatment increased the expression of lncRNAs NR_024118 in the joint of diseased mice of CIA, through increasing the acetylation of H3 in the promoter of the lncRNAs [35]. Knockdown analysis showed a mediating role of NR_024118 in the protective effect of shikonin in the CIA model. Zhang et al. showed that shikonin improves lesions in mice with imiquimod (TLR7/8 agonist)–induced psoriasis [36]. Shikonin promoted iTreg cell induction in vivo and in vitro in association with increased expression of IL-10 and TGF-β. These were in association with a decrease in p-Akt.

Inhibition of NF-κB signaling and beyond

NF-kB pathway

NF-κB plays a central role in innate immunity. Stimulations via TLRs lead to NF-κB activation. Phosphorylation of IκB is a key event that mediates this process; phosphorylation of IκB leads to its degradation in the proteosome, and the degradation releases p50, enabling its translocation into nucleus and formation of NF-κB, which in turn activates transcription of genes for TNF-α, IL-1β and IL-6.

Shikonin is likely to block NF-κB activation through multiple steps. Andújar et al. showed that shikonin interferes with IκB-α degradation in macrophages, thereby inhibiting the translocation of NF-κB to the nucleus, in a model of phorbor ester-induced oedema [37]. A similar inhibition has been observed in several LPS-induced acute inflammation models [e.g, 38-40]. Of note, IκB kinase-β (IKK-β) is responsible for phosphorylation and degradation of IκB-α, thereby mediating pro-inflammatory stimuli that lead to NF-κB activation. Specifically, phosphorylated IκB-α is recognized by $SCF^{\beta\text{-}TrCP}$ E3 ligase and transferred to the proteasome for degradation, thereby releasing NF-κB allowing its translocation into nuclei and transcription of pro-inflammatory genes [41]. Li et al. showed the direct inhibition of recombinant IKK-β activity by shikonin down to ~50% activity with 0.25 μM shikonin. This represents a remarkably high efficacy of shikonin. In the same system, they also showed shikonin-induced suppression of the JNK signaling [42].

As in tumor cells, the ubiquitin–proteasome system is also activated in LPS-mediated inflammatory cells, implicating proteasome as a target of anti-inflammatory therapeutics [43,44]. Shikonin exerts anti-inflammatory effect via proteasome inhibition. Lu et al. showed that shikonin specifically inhibits the proteasomal chymotrypsin-like activity but not the trypsin-like or the caspase-like activity. In LPS-stimulated rat macrophages, 1 μM shikonin showed significant accumulation of ubiquitinated proteins [45]. Considering such high efficacies of shikonin against proteasome and IKK signaling, these features are likely to play a central role in the anti-inflammatory activity of shikonin.

MAPKs

Do mitogen-activated protein kinases (MAPKs) pathways mediate shikonin suppression of the NF-κB pathway? A challenge is that experiments to discriminate the direct and indirect effects of shikonin are not done in many studies. In particular, ROS add complexity; shikonin can modulate the ROS levels in cells and ROS are likely to mediate a significant part of shikonin effects on MAPKs. The

following observations nonetheless show us a general trend in the shikonin effect on MAPKs in several settings.

MAPKs consist of extracellular signal-regulated kinase (ERK), the c-Jun NH2-terminal kinase (JNK), p38 kinase and the big MAP kinase 1 (BMK1/ERK5) pathway. In general, MAPKs pathways have profound effects on NF-κB activity. Several studies reported negative effects of shikonin on the MAPKs pathways, implicating the MAPKs as mediators of the negative effects of shikonin against NF-κB. For example, using LPS-stimulated RAW264.7 macrophage-like cells, Cheng et al. showed that shikonin derivatives inhibited the NF-κB pathway by prevention of IκB degradation, and also that shikonin derivatives suppressed the phosphorylation of ERK1/2 [46]. Several studies supported the significance of the ERK1/2 inhibition by shikonin associated with its inhibitory effect on NF-κB. Nam et al. showed that two shikonin derivatives isobutyrylshikonin (IBS) and isovalerylshikonin (IVS) decreased LPS-induced IκB-α phosphorylation and NF-κB DNA binding activity in rat microglia [47]. In this setting, the derivatives inhibited the LPS-induced phosphorylation of ERK1/2 and Akt, with a slight inhibition of JNK and p38. In some other settings, p38 can also be regulated by shikonin. For instance, in an ischemic injury model used by Wang et al., shikonin inhibited the activation of NF-κB, and phosphorylation of p38, in association with the inhibition of expression of TLR4 and TNF-α [48]. In a LPS/D-galactosamine-induced liver injury mouse model used by Tian et al., for all of ERK1/2, JNK and p38, phosphorylation induced by the insult was partially inhibited by shikonin [49]. Overall, in these LPS-induced inflammation models, negative regulation of MAPKs by shikonin appears to contribute to the inhibitory effect of shikonin on NF-κB.

Thus far, we considered MAPK pathways in the anti-inflammatory shikonin effects, but shikonin inhibitory effects on MAPKs have been observed in association with its anticancer effects as well. For just one example, using microarray hybridization Zhao et al. showed that shikonin and four derivatives (IBS, 2-methylbutyrylshikonin, IVS and β,β-dimethylacrylshikonin) show strong suppression of c-MYC expression in U937 histiocytic leukemia cell line [50]. As MAPKs and Akt are known to be upstream regulators of c-MYC, the authors also examined MAPKs and showed that while p-p38 was unaffected, both p-Akt and total Akt were reduced, and besides, p-ERK1/2 was inhibited whereas p-JNK was activated by shikonin and the derivatives. This set of data is consistent with the view that, in general, in malignant cells the p-Akt level can be suppressed by shikonin (as we discuss below), and that the JNK activation is associated with an increase in the ROS level. In general, the association between JNK and ROS has been documented [51].

From these findings, it is likely that shikonin inhibits MAPKs in many inflammation models and cancer cells, likely assisting the inhibition of NF-κB pathway. In particular, ERK1/2 suppression by shikonin may occur in a relatively wide range of systems [52-54]. However, identification of the specific MAPKs regulated by shikonin is a challenging problem as they depend on the cells and experimental conditions (e.g., the dose and time period of shikonin treatment).

For full understanding of shikonin effects on MAPKs, more studies are needed to investigate the effects on ROS, MAPKs and NF-κB and on their interrelationship. It is important to note that shikonin increases the concentration of ROS, which serve as an activator for MAPKs pathways [55]. While in the above we discussed the shikonin-induced *suppression* of p-ERK, Chen et al. showed that shikonin increased the phosphorylation levels of ERK1/2, JNK1/2 and p38 in both human prostate cancer PC-3 and DU145 cells and that the upregulation of the ERK cascade by shikonin correlated well with levels of ROS [56]. Moreover, Gong et al. showed that 2.5 μM (12 h) shikonin caused the accumulation of ROS and p-ERK and thus induced autophagy in a tumor xenograft model in hepatocellular carcinoma BEL7402 and Huh7 cells. Intriguingly, the same cells underwent apoptosis with 6 μM (24 h) shikonin without the increase in p-ERK [15]. These findings suggest that ROS level is the decider to switch cells between autophagy and apoptosis, and MAPKs may be following or fulfilling the decided cell fate. However, more details should be investigated in such regulation of cell fate.

Among MAPKs, it is generally known that the activation of JNK is facilitated by ROS, and is often implicated in transcriptional activation of pro-apoptotic genes [51]. This is likely of physiological relevance as TNF receptor-associated JNK activation is considered to be mediated in part by ROS [55]. Thus, further analyses are also necessary to understand the direct and indirect ROS effects on MAPKs. Another issue is that, as NF-κB is also redox-sensitive and basically plays a prosurvival role, suppressing both apoptosis and necrosis [57], it is not clear how the shikonin-induced suppression of NF-κB influences the ROS types (i.e., superoxide, peroxide and hydroxyl radicals) and their levels.

STAT3

In several inflammation models, shikonin showed an inhibitory effect on the STAT3 pathway. STAT3 is a member of the signal transducers and activators of transcription (STAT) proteins. Phosphorylation of STAT3 has been implicated in cell growth, proliferation, survival, differentiation, apoptosis, metastasis, and angiogenesis. STAT3 has been shown to be activated in several cancers [e.g., 58], and its critical role in malignancies has been suggested [59].

Shikonin effect on the STAT3 pathway has been analyzed in several studies, and all showed inhibitory effects. In the model of ulcerative colitis induced by dextran sodium sulfate, shikonin showed beneficial effects and decreased expression of COX-2 and pro-inflammatory cytokines as well as p-STAT3 [60]. As another example, shikonin inhibited IL-17-induced vascular endothelial growth factor (VEGF) expression, IL-17R, p-JAK2 and p-STAT3 expression, while upregulated the expression of negative signal transducer SOCS1 (suppressor of cytokine signaling-1) in keratinocyte line HaCaT [61].

Interestingly, Qiu et al. designed several shikonin derivatives that binds to the SH2 domain of STAT3, which is crucial for dimerization of STAT3, and found a compound

named PMM-172 has anti-proliferative activity against breast cancer MDA-MB-231 cells with IC_{50} value of 1.98 μM [62]. Furthermore, in Guo et al., the underlying mechanism by which shikonin sensitizes lung cancer A549 cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced cytotoxicity was analyzed (TRAIL and other death-inducing ligands are also discussed in the section "Anticancer effects - ROS-centered view"). Combined treatment with shikonin and TRAIL induced the downregulation of anti-apoptotic proteins and the upregulation of pro-apoptotic proteins, which occurred in parallel with the activation of the JNK pathways and the inhibition of the STAT3 and Akt pathways [63]. Overall, these suggest that the inhibition of STAT3 activation offers promise in both anticancer and anti-inflammatory interventions.

It is important to note that STAT3 is also regulated (in many cases, activated) by ROS [64]. ROS accumulation causes oxidative inhibition of the protein tyrosine phosphatases (PTPs) including SHP1 and SHP2. This inhibition activates STAT3, promoting preneoplastic cell proliferation. Given the general shikonin effect to enhance ROS level in cancer cells, it seems likely that the shikonin-mediated suppression of STAT3 represents a direct effect of shikonin on the STAT3 pathway, rather than its indirect effect via ROS. Overall, compared with MAPKs, the high levels of p-STAT3 due to the ROS effects might be more commonly seen in cancer cells, and therefore this provides a rationale for the development of STAT3 inhibitors in cancer therapeutics.

Anticancer effects - ROS-centered view

ROS and apoptosis

Shikonin can induce apoptosis and regulate the proliferation of cancer cells. As is the case with many anti-cancer drugs, shikonin induces ROS production in cancer cells. Exposure of cancer cells to ROS generating agents exhaust the cellular antioxidant capacity, causing cell cycle arrest and inducing apoptosis [65]. In this section, we first briefly review recent advances in research of apoptosis and ROS in cancer biology, and later we discuss anticancer effects of shikonin.

Cancer cells exhibit a higher ROS level than normal cells [65]. This feature may remain unnoticed as cancer cells also express antioxidant enzymes that counteract ROS. Nonetheless, the enhancement of ROS generation and/or decrease of antioxidant defense that may push cancer cells beyond the breaking point is now considered an important therapeutic strategy, as such treatment may activate cell death pathway in cancer cells [65]. It is known that apoptosis has at least two major pathways: extrinsic and intrinsic pathways. Briefly, the extrinsic pathway is mediated by ligation of death-inducing ligands such as Fas ligand, TNF-α, and TNF-related apoptosis-inducing ligand (TRAIL) to their respective receptors. The ligand-receptor interaction recruits adaptor protein and pro-caspase, leading to death-inducing signaling complex (DISC) formation that expedites further activation of caspase cascade and apoptosis [65]. ROS can facilitate the extrinsic pathway of apoptosis via negatively regulating the cellular FLICE-inhibitory protein (c-FLIP) that competitively blocks the DISC formation [66-69]. The intrinsic pathway, on the other hand, is the mitochondrial pathway of apoptosis. ROS triggers cytochrome c (Cyt c) (and other activators of caspase) release from mitochondria. Cyt c serves to form apoptosome that activates caspase signaling and apoptosis induction [70].

A variety of drugs including shikonin have been shown to exhibit anticancer effects via enhancing ROS production (Table 2 of [65]). Such drugs are also known to sensitize cancer cells to chemotherapeutic agents, and such sensitization has been shown to occur via induction of endoplasmic reticulum (ER) stress and DNA damage, upregulation of death receptor 5 (DR5) and downregulation of c-FLIP, all of which have pro-apoptotic effects. Such sensitization is intensely tested against TRAIL-based cancer therapies. TRAIL is secreted by immune cells including T cells, NK cells and dendritic cells upon activation. Some cancer cells show resistance to TRAIL-induced apoptosis, and how such resistance of cancer cells to TRAIL-based pro-apoptotic therapies might be overcome is an important current problem [71].

A number of drugs are known to induce ROS, thereby sensitizing cancer cells to TRAIL-induced apoptosis [65]. To name but one example, Gatsinzi and Iverfeldt focused on neuroblastoma SK-N-AS cells that exhibit resistance to TRAIL-induced apoptosis [72]. Among the five drugs tested, curcumin and oridonin sensitized the cells to TRAIL at concentrations at which they showed no inhibitory effect on NF-κB activity. This sensitization was dependent on ROS. Such studies demonstrate the importance of ROS in cancer sensitivity to chemotherapy. While NF-κB generally shows an anti-apoptotic property [57], enhancement of ROS, rather than inhibition of NF-κB, appeared to be more promising at least for for sensitization of neuroblastoma SK-N-AS cells to TRAIL [72]. In Zhou et al., shikonin could inhibit cells viability and induce apoptosis of cholangiocarcinoma (CCA) cells, effects enhanced by TRAIL treatment via ROS mediated JNK signaling pathways, involving up-regulation of DR5 expression [73].

Aside from the shikonin-mediated sensitization to TRAIL, many studies of shikonin have focused on the mechanisms for the shikonin-induced ROS production and for the subsequent apoptosis induction. Duan et al. reported the important role of cytosolic thioredoxin reductase (TrxR1), antioxidant enzyme, as a target of shikonin for ROS generation [74]. Their in vitro analysis showed that shikonin effectively inhibits TrxR1 with an IC_{50} value around 1.6 μM, at selenocysteine 498, likely through forming a shikonin-TrxR1 covalent adduct. For leukemia HL60 cells, IC_{50} (12h) of cellular TrxR1 inhibition was <2 μM. Their data further suggested that shikonin treatment further turns TrxR1 to an NADPH oxidase to directly generate superoxide anions. Their analyses using overexpression and knockdown of TrxR1 strongly suggested that the negative shikonin effect on TrxR1 plays a critical role in the shikonin-induced ROS production that leads to apoptosis.

In Chang et al. shikonin increased ROS generation and ERK activation, and reduced Bcl2, which caused the cells to

undergo apoptosis in osteosarcoma cells [75]. For a more recent example, in Liang et al. shikonin induced ROS-based mitochondria-mediated apoptosis in colon cancer [76]. In Xia Han et al., shikonin showed cytotoxic effects on human colon cancer SNU-407 cells with IC_{50} (48 h) of 3 μM. Shikonin induced apoptosis in the cell line mediated by ERK, JNK and p38 MAPK. Their further analysis on phosphorylated PERK (PKR-like ER-associated kinase) and phosphorylated eIF2α and other ER stress markers suggested that the mechanism of shikonin-induced apoptosis is mediated by the PERK/eIF2α/CHOP (C/EBP-homologous protein) stress response pathway in the ER [77].

Yang et al. analyzed the specific source in the cell for the ROS production. In glioma cell lines, shikonin-induced ROS production occurred via complex II, and, interestingly, NADPH oxidase and lipoxygenase also served as ROS generators in shikonin treatment. ROS production by shikonin led to Nrf2 translocation to nucleus [78].

As we mentioned above, NF-κB is a prosurvival and an anti-apoptotic factor. One of important ways in which NF-κB activity influences ROS is via increased expression of antioxidant proteins [79]. However, NF-κB can also promote the production of ROS especially in inflammation via expression of target genes including NADPH oxidase NOX2 ($gp91^{phox}$), COX-2 and a couple of cytochrome p450 enzymes. Much remains unknown about the ROS production in different cellular compartments when the nuclear translocation of NF-κB is inhibited by shikonin.

Other mechanisms for shikonin anticancer activities

Anticancer effects of shikonin are known to be also mediated by many other mechanisms, besides those mediated by ROS. Here, only a few notable studies are discussed. Rios et al. showed that in Caco-2 cells shikonin has a marked dose-dependent apoptotic mechanism, specifically, an increase in caspase 3 (a pro-apoptotic protein) and the inhibition of Bcl2 (anti-apoptotic protein) [80].

Recent finding showed that shikonin is a potent inhibitor of epidermal growth factor receptor (EGFR). Shikonin suppressed the phosphorylation of EGFR, causing EGFR degradation. This feature and the effect to enhance the level of ROS production due to thioredoxin inhibition jointly increased the efficacy of shikonin to induce cell cycle arrest and apoptosis in gefitinib-resistant non-small cell lung cancer (NSCLC) cells [81].

Jang et al. showed that shikonin inhibits the migration and invasion of breast cancer cells by suppressing AP-1 transcription factor-mediated matrix metalloproteinase 9 (MMP-9) expression [82].

Wiench et al. observed that shikonin was specifically accumulated in the mitochondria of U937 and SK-BR-3 cells, and this accumulation was associated with a shikonin-dependent deregulation of cellular Ca^{2+} and ROS levels. In U937 cells, ROS levels after treatment with 0.6 μM shikonin are comparable to those after incubation with 50 μM H_2O_2, our positive control [83].

Deng et al. focused on effect on TIPE2 (tumor necrosis factor (TNF)-alpha-induced protein 8-like 2) in osteosarcoma U2OS and SaOS-2 cells [84]. They showed that TIPE2 mediates the suppressive effects of shikonin on matrix metalloproteinase 13 (MMP-13) expression. Shikonin induced expression of TIPE2. This is interesting as TIPE2 is considered an anti-inflammatory factor and its expression level in cancer cells is generally low [85].

Roles of matrix metalloproteinases in cancer progression has been well documented [e.g., 86]. Shikonin inhibited MMP-2 and -9 expression in association with decreases in phosphorylation of Akt and mTOR and increases in ERK, p38 and JNK phosphorylation [56]. The author also showed the mediating role of ROS in these effects.

Shikonin effects on signaling pathways

Nrf2/HO-1 and NF-κB pathways

As we discussed in above, the enhancement of ROS production is considered important for the anticancer effect of shikonin. On the other hand, in most cases with non-cancer cells, shikonin exhibits anti-oxidant and anti-apoptosis activities. This is a beneficial feature of shikonin. Potential benefits of the anti-oxidant function of shikonin at 10 μM was shown, for example, in a model of 6-hydroxydopamine-mediated neurotoxicity to undifferentiated PC12 cells [87] and in a model of the cerebral and hepatic ischemia/reperfusion injury (IRI) [88,89]. Tong et al. showed shikonin-induced reduction of ROS content and pro-apoptotic signaling in β-amyloid peptide $A\beta_{1-42}$ –treated PC12 cells [90]. This ROS reduction was observed at 3.47 μM shikonin. Interestingly, shikonin-induced increases in the levels of anti-oxidative enzymes including superoxidedismutase (SOD) and glutathione peroxidase were observed with shikonin levels up to 34.7 μM. As the authors used PC12 cells that had been treated by NGF for differentiation, it is possible that NGF-induced PC12 cells (that halt proliferation) can tolerate higher doses of shikonin compared to undifferentiated and proliferating PC12 cells.

An increasing number of studies focus on the signaling pathways mediating the mechanisms by which shikonin reduces ROS level in non-cancer cells. Wang et al. showed that acetylshikonin exhibited the most potent anti-apoptotic activity through the inhibition of the generation of ROS [91]. They also showed that upregulation of heme oxygenase-1 (HO-1) by acetylshikonin is a key step mediating its anti-apoptotic activity from oxidative stress in SH-SY5Y cells. Thus, shikonin not only exerts anti-oxidant effects by itself, but also has profound effects on anti-oxidant pathways. Among such pathways, the nuclear factor E2 (erythroid-derived 2)-related factor 2 (Nrf2)/HO-1 signaling pathway has been considered an important one [92,93]. In the following, we briefly review on Nrf2 and the antagonism between Nrf2/HO-1 and NF-κB.

Nrf2 is a redox sensitive transcription factor that plays a pivotal role in cellular protection against cancer and diverse diseases where oxidative stress and inflammation are common conditions [94,95]. Nrf2 has been the focus of research as a pharmacological target as Nrf2 regulates the expression of detoxifying enzymes through a promoter sequence known as

the antioxidant response element (ARE) and orchestrates antioxidant and anti-inflammatory cellular responses [95]. Nrf2 regulates the drug resistance via expression of electrophile, oxidants detoxification enzymes and efflux mechanism, and therefore, blocking the Nrf2 pathway has been suggested as a practical way to eliminate chemoresistance [96]. In unstressed cells, Nrf2 is constitutively expressed and degraded directly by its antagonist Keap1. In the presence of ROS, Keap1 cysteine residues modified and Nrf2 degradation by proteasome is prevented [97]. Nrf2 increases expression of HO-1, an enzyme with potent anti-oxidant, anti-inflammatory, and anti-proliferative effects. HO-1 is the enzyme that metabolizes heme into CO, free iron, and bilirubin. Both CO and bilirubin have anti-inflammatory effects [93]. Elevation of HO-1 also leads to reduced NF-κB signaling. Yang et al. showed that HO-1 downregulates NF-κB activity, explaining the antagonism between two pathways [98]. Specifically, using siRNA system, the authors showed that HO-1 is necessary for the docosahexaenoic acid (DHA)-mediated inhibition of TNF-α-stimulated nuclear translocation of NF-κB subunit p65 in human endothelial cell line EA.hy926.

Beneficial effects of Nrf2 agonists on diverse diseases affecting liver, kidney, and lung, and neurodegenerative diseases have been discussed [99]. Simply stated, the Nrf2 and NF-κB pathways work against each other in the homeostasis of pro-inflammatory/anti-inflammatory signaling. Nrf2 protects cells from oxidative stress, while NF-κB is a key activator that triggers inflammation as well as oxidative stress. This antagonism has been discussed in [100,101]. In our view, it is of particular importance to note that low oxidative stress induces Nrf2 pathway, whereas an intermediate amount of ROS induces NF-κB [100].

The effect to inhibit the NF-κB (often MAPK/NF-κB) and activate the Nrf2/HO-1 pathways is a feature shared by many anti-inflammatory drugs, including isovitexin [102], morin [103], sulforaphane [104], quercetin [105], geranin [106], hesperetin [107], tenuigenin [108], ketamine [109], glaucocalyxin B [110] and rosmarinic acid [111].

For shikonin, several studies examined the effect on the Nrf2/HO-1 pathway. Huang et al. showed that shikonin induces expression of anti-oxidative proteins, such as HO-1, glutamate cysteine ligase modifier, catalase, and SOD1 in EA.hy926 endothelial cells and effectively protects the cells from oxidized LDL (oxLDL)-induced endothelial dysfunction including ROS increase [112]. In the authors' system, ROS served as a direct or indirect activator of NF-κB. They further showed that Nrf2 knockdown attenuated the ability of shikonin to inhibit the oxLDL-induced NF-κB activity. Although transient, increased phosphorylation of Akt, p38 and ERK1/2 phosphorylation, was observed in 30 min treatment of cell with 1 μM shikonin. Another suggestion on Nrf2-mediated shikonin effect was given by Tian et al. that showed that the protective effect of shikonin in the LPS/D-galactosamine liver injury model is associated with the ROS reduction via upregulation of HO-1, NAD(P)H:quinone oxidoreductase 1 (Nqo1), and glutamate-cysteine ligase catalytic subunit (Gclc) and modifier subunit (Gclm). The expression of these are known to be regulated by Nrf2 [49]. In a LPS-induced kidney injury model, shikonin showed a protective effect via Nrf2 activation [113]. Acetylshikonin downregulates pro-inflammatory mediators such as NO and PGE2 by suppressing PI3K/Akt-dependent NF-κB activity induced by ROS as well as inducing Nrf2-dependent HO-1 activity [114]. Overall, in these studies, the anti-inflammatory effects of shikonin were likely to be exerted because the Nrf2/HO-1 pathway was dominant compared to the NF-κB pathway upon shikonin treatment, so that ROS was suppressed by Nrf2/HO-1 pathway.

As we have seen above, ROS production is an important mechanism for the anticancer effect of shikonin. Then, a question arises as to why in cancer cells the produced ROS cannot be fully scavenged by Nrf2-mediated antioxidant system, which is also activated by shikonin. Here, it should be reminded that exposure of cancer cells to ROS-generating anticancer agents exhausts the cellular antioxidant capacity. Shikonin also directly inactivates TrxR1 [74]. Once the ROS level exceeds a threshold, apoptosis or other death processes can take place [115]. In relation to this issue, the Nrf2 *vs.* NF-κB balance is likely to work in different ways depending on the concentrations of ROS [100]. In the cases of shikonin-treated cancer cells, the NF-κB, which mediates the anti-apoptotic signaling as well, should become dysfunctional due to the inhibition by shikonin, and this dysfunction may contribute to the induction of the hyperphysiological levels of ROS in cancer cells. The response to high levels of ROS is likely to differ between Nrf2 and p53. In cancer cells, shikonin tends to enhance ROS levels, and once ROS reaches a certain level, Nrf2-mediated antioxidant system becomes inactive whereas p53 begins to act to raise ROS even further, thereby inducing apoptosis. The antagonism between Nrf2 and p53 is also discussed in the section entitled 'p53'.

In Ahmed et al., even low doses of shikonin were found to increase intracellular ROS [116]. In this study, shikonin showed a significant increase in the Nrf2-mediated oxidative stress response in a lymphoma cell line U937. The 30 min treatment with 0.1 μM shikonin did not show effects on cell survival, yet the intracellular level of peroxides increased. With this condition, heat shock protein 70 (HSP70) expression increased over the time period examined (24 h). Their gene chip analysis showed the increased expression of genes for HSPs such as HSP 70kD protein 1A and DNAJ (Hsp40) homologue, and Nrf2-target genes such as HO-1, Nqo1 and sequestosome 1. Thus, at least in the authors' system, even a non-lethal dose of shikonin acts to increase ROS in cells, but Nrf2-mediated homeostasis appears to be able to protect the cells from the damages by ROS. Given that, even at 0.1 μM, shikonin is accumulated in mitochondria [83], this may increase its local concentration, causing aberrant ROS generation.

The dysfunction of Nrf2 in the presence of higher levels of ROS was analyzed in Yang et al. [78]. After demonstrating that shikonin induces apoptosis in neuroblastoma via induction of ROS, Yang et al. analyzed the sources of shikonin-induced ROS in glioma cells. Analysis with inhibitors showed at least three sources of ROS, including mitochondria complex II, NADPH oxidase, and lipoxygenase

although differences in the sources and the induced species of ROS exist between U87MG cells (high grade glioma) and Hs683 (low grade glioma). Importantly, their data suggested that the a high dose of shikonin (8 μM) may produce very high ROS which not only outweighs the defensing capacity of Nrf2 system, but also causes dysfunction of the Nrf2 system, causing cell death at a later stage.

p53

p53 regulates a variety of processes including cell survival, proliferation and apoptosis. At physiological levels of p53 and non-excessive levels of ROS, p53 acts to maintain ROS levels at nontoxic levels through antioxidant genes [117]. So, p53 has generally been considered as a negative regulator of ROS via the expression of anti-oxidative stress proteins [118]. However, at hyperphysiological levels of p53 and ROS, p53 in turn acts toward ROS accumulation and induction of apoptosis [117].

As we discussed above, shikonin has the ability to induce ROS production from several sources [78]. This leads to antioxidant glutathione depletion. These events are considered to disrupt the key balance between the production of ROS and the protection via antioxidant, causing mitochondrial transmembrane potential disruption and apoptosis. ROS production was partly reliant on p53 upregulation with shikonin treatment [119]. Upregulation of p53 in U87MG glioma cells induced by a high dose of shikonin (7.5 μM) was a rather slow event (a slight increase observed at 6 h, and a 2.5-fold increase at 12 h) [119]. This is consistent with the view that, within a certain range of ROS level, p53 forms a positive feedback loop with ROS.

Yeh et al. showed that shikonin at lower doses (1.0 - 2.5 μg/mL) induced early apoptosis, while higher doses (5.0 - 10.0 μg/mL) induced late apoptosis and necrosis in A549 lung cancer cells. Analysis using pifithrin-α, a specific inhibitor of p53 showed a mediating role of p53 in shikonin-induced apoptosis of A549 cells [120]. This support the pole of p53 in the ROS enhancement.

The following study highlighted the antagonism between Nrf2 and p53. Ko et al. showed that shikonin induces apoptosis of human gastric cancer AGS cells via a caspase 3-dependent manner and in association with increased ROS levels in cells [121]. Intriguingly, the shikonin treatment induced p53 expression and accumulation of excessive ROS, while simultaneously leading to the inhibition of Nrf2 expression. At 62.5 nM of shikonin some nuclear translocation of Nrf2 was observed, but this was not observed at 250 nM shikonin. In Kato 3 (p53 null) cells, Nrf2 nuclear translocation was observed even at 250 nM shikonin. This supports the view that p53 negatively regulates the activity of Nrf2 [122], suggesting the antagonism between the Nrf2 pathway and the expression of p53. This study also demonstrated that the p53 and the JNK pathway, which is known to activates p53, play a key role in shikonin-induced apoptotic cell death. [123].

From the findings shown by Yang et al. [78] and Ko et al. [121], it is suggested that up to the break point Nrf2 counteracts the ROS induced by shikonin, but very high levels of ROS cause dysfunction of Nrf2. These highlight the contrast between Nrf2 and p53 in stability in the presence of hyperphysiological levels of ROS; while Nrf2 just breaks down at hyperphysiological levels of ROS, p53 remains functional and serves to enhance ROS levels further to induce apoptosis [117]. These studies may provide important clues for understanding the basis that causes apparently opposite effects of shikonin dependent on the setting.

HIF-1 and glycolysis

Hypoxia-inducible factor 1 (HIF-1) is a master regulator of transcriptional responses to hypoxia, and is important for transcription of many genes encoding for metabolic enzymes, growth factors, extracellular matrix, and thrombosis under hypoxic condition. Here we just mention that NF-κB directly activates transcription of HIF-1α (the component of HIF-1 controllable by oxygen level), and therefore NF-κB plays a key role in hypoxic response [124,125]. On the other hand, HIF promotes the bactericidal activities of phagocytic cells and supports the innate immune functions [126]. In this sense, these two pathways are interdependent. Thus, the NF-κB/HIF-1α axis has come to be considered the primary target for the anti-inflammatory effect of shikonin [127]. HIF-1α facilitates the vascularization of tumors. Shikonin inhibits lymphangiogenesis in vitro by interfering the NF-κB/HIF-1α pathway [128]. As vascularization plays a key role in cancer survival, shikonin-mediated inhibition of the NF-κB/HIF-1α pathway has important perspectives.

Many cancer cells exhibit increased glycolysis and lactate production and decreased O_2 consumption, rather than oxidative phosphorylation for energy production, compared to normal cells. This is a phenomenon known as the Warburg effect [129]. As a master regulator of transcriptional responses to hypoxia, HIF-1 promotes the expression of VEGF-A, thereby facilitating the vascularization of tumors. HIF-1 controls energy metabolism as well. As a transcription factor, the target genes of HIF-1 include those which support the Warburg effect such as *GLUT1, LDHA, PDK1* that inactivates pyruvate dehydrogenase, thereby keeping pyruvate from entering into the TCA cycle [130]. Despite the presence of O_2, cancer cells exhibits the Warburg effect that normally occurs in the absence of O_2. Specifically, HIF-1α prolyl hydroxylation is inhibited, thereby stabilizing HIF-1α protein [131]. Of note, mammalian target of rapamycin (mTOR) positively enhances the activity of HIF-1 [132]. Given that PI3K/Akt activation is implicated as a causative factor for many cancers [133,134] and that the activation of HIF-1 is associated with drug resistance, the inhibition of aerobic glycolysis and the suppression of the activity of Akt/mTOR/HIF-1α axis has become an important goal in cancer therapeutics [e.g., 135,136].

Recently, the importance of pyruvate kinase (PK) in expression of HIF-1 gene has been recognized. In tumor tissues, total expression of the alternative splice forms PKM1 and PKM2 of PK is high, reaching ~3-fold compared to normal tissues [137]. The activity of PKM2, but not PKM1, showed clear associations with tumor growth and the Warburg effect of cancer cells [138,139]. PKM2 promotes the Warburg

effect and tumorigenesis and this effect involves enhancement of HIF-1 function [131]. Despite that PK serves for glycolysis that is a cytoplasmic process, a recent study by Luo et al. showed that PKM2 interacts with HIF-1 and promotes the Warburg effect by serving as a transcriptional coactivator for HIF-1 (i.e., by enhancing HIF-1 binding to hypoxia response elements) in cancer cells [131]. This and other findings support the view that there is a positive feedback loop between PKM2 and HIF-1 and that this loop promotes HIF-1 transactivation and reprograms glucose metabolism in cancer cells [140,141].

Of importance to cancer therapeutics, shikonin and derivatives have been shown to inhibit the activation of HIF-1α [142,143]. The mechanism for this inhibition remains to be determined; the inhibitory effect of shikonin against NF-κB appears to be involved as discussed in [128]. Tang et al. showed that, in esophagus cancer EC109 and EC9706 cells shikonin induced cell cycle arrest and apoptosis in association with decreased EGFR, PI3K, and p-Akt expression, along with decreased HIF-1α and PKM2 expression [144], suggesting involvement of these pathways.

PKM2 and HIF-1 play important roles not only in cancer therapeutics but also in inflammation [145]. Yang et al. showed that PKM2 activity increases in peritoneal macrophages in both LPS mice and cecal ligation and puncture (CLP) mice models, but this increase can be completely inhibited by shikonin [146].

Recently, the regulation of metabolism, and in particular, the balance between glycolysis and fatty acid oxidation has attracted much interest of immunologists [147]. Early important studies in this regard include Vats et al. that showed that IL-4 induced anti-inflammatory program of macrophage activation is linked to PPARγ-coactivator-1β (PGC-1β)-mediated induction fatty acid oxidation [148]. Such findings led to a consensus that, as opposed to M1 macrophages that rely on glycolysis for energy source, M2 macrophages utilize fatty acid oxidation. Intriguingly, shikonin increased mRNA expression of the β-oxidation genes PPAR-α, PPARγ-coactivator-1α(PGC-1α), and acyl CoA oxidase 1 (ACOX1) in liver and skeletal muscle [149]. This finding points to a view that the mechanisms for anti-inflammatory effects of shikonin involve the metabolic reprogramming from glycolysis to fatty acid oxidation.

Akt/GSK-3β/Nrf2

We discussed above Nrf2 and its interplay with NF-κB and p53. Here we discuss the signaling pathway regulating Nrf2. Akt is a family of serine/threonine kinase, whose members are broadly expressed in most organs and tissues [150]. Akt is one main effector of PI3K, and mTOR is one main effector of Akt. The PI3K/Akt pathway regulates broad aspects of cellular functions including metabolism, cell growth, proliferation, cell motility and migration, apoptosis and survival. Akt plays an important role in glucose metabolism, with glycogen synthesis kinase-3 (GSK-3) being a well known target of Akt. GSK-3 is constitutively active and inactivated via signaling of a variety of agonists. A well-known example is insulin; in response to insulin, Akt inhibits GSK-3, thereby increasing glycogen synthesis [151]. Intriguingly, in the regulation of the balance between pro-inflammatory and anti-inflammatory pathways, GSK-3 serves as a switching node between NF-κB (pro-inflammatory) and IL-10-producing CREB (anti-inflammatory). Active GSK-3 promotes NF-κB/CBP complex formation that enhances transcription of pro-inflammatory cytokines, whereas inactive GSK-3 acts to CREB/CBP complex formation leading to IL-10 expression [152]. Accordingly, GSK-3 acts to inhibit IL-10 production in macrophages [153], whereas inhibition of GSK-3 leads to increase in anti-inflammatory cytokine expression [154].

The PI3K/Akt pathway has been long known to be important in adaptive immunity, but, it is now recognized that the pathway has important roles in innate immunity as well [155-157]. In particular, the PI3K/Akt pathway has been considered to exert a self-limiting mechanism. In accord with this view, stimulation of human monocytes with LPS reduces the activity of GSK-3β by phosphorylation in a manner dependent on the PI3K/Akt pathway, and this inactivation of GSK-3β is likely to be involved in the increase of IL-10 expression [158-160].

Recently, several studies demonstrated the involvement of the GSK-3 pathway in the protective effects of shikonin on normal cells. Huang et al. systematically analyzed the effects of shikonin on gene expression levels in rat hepatocytes. Shikonin treatment increased transcripts of CYP isozymes and detoxification enzymes such as glutathione S-transferases (GST), Nqo1 and UDP glucuronosyltransferase 1A1. Shikonin also increased expression of drug transporters in rat hepatocytes. These regulations were mediated by the arylhydrocarbon receptor (AhR) and Nfr2 [161]. Notably, Nrf2 stability is partly regulated by GSK-3. GSK-3/β-TrCP (β-transducin repeat-containing protein) leads to ubiquitin-proteasome degradation of Nrf2 [162-164]. Recently, shikonin was shown to prevent acetaminophen hepatotoxicity by upregulation of Nrf2 via the PI3K/Akt/GSK-3β pathway [165]. In addition, Wang et al. showed that, in a myocardial ischemia/reperfusion injury (IRI) model, shikonin increased the phosphorylation of Akt and GSK-3β in association with the protective effect of shikonin [166]. These findings implicate the PI3K/Akt/GSK-3 pathway and its interrelationship with the Nrf2 pathway in the cytoprotective effects of shikonin on normal cells.

Akt: to activate or to inhibit ?

Currently available data show that the effect of shikonin on the Akt pathway is different between cancer cells and normal (but stressed) cells. From this observation and the early findings showing redox biology related to PI3K/Akt, we also consider the possible of ROS in this section.

Components of the PI3K/Akt pathway is usually overexpressed or activated excessively in numerous types of cancer [167,168]. In general, shikonin inhibits the PI3K/Akt pathway in cancer cells [169]. In Chen et al., shikonin inhibited Akt phosphorylation in prostate cancer cells [56]. Ahn et al. showed that shikonin-induced apoptosis in HeLa cells is associated with inhibition of Akt and activation of p38

through excessive ROS accumulation [170]. PI3K/Akt pathway inhibition by shikonin (or its derivatives) was also reported for leukemia [50], melanoma [171] and glioblastoma cells [172]. Thus, in general, shikonin exhibits anticancer effects in association with its effect to downregulate p-Akt.

The anti-inflammatory effect of shikonin may also be associated with Akt inhibition in some cases. In the system using LPS-stimulated microglial cells, Nam et al. showed that shikonins attenuated the inflammatory responses by inhibition of ERK, Akt, and NF-κB [47]. A similar observation was reported from Jayasooriya et al. [114]. However, the number of studies that have addressed the shikonin effect on Akt in immune cells is limited.

Intriguingly, in an analysis with in vitro culture of murine lung fibroblasts, Nie et al. showed that shikonin downregulated p-Akt and inhibited fibroblasts proliferation and migration. That is, the effects of shikonin on Akt phosphorylation in this fibroblast system showed similarity with the effects in cancer cells. [11]. The IC_{50} at 48 h of this effect was 0.87 μM, which was as low as the values observed for cancer cells. This finding supports our notion of the similarity of murine lung fibroblasts with cancer cells. Compared with normal cells, cancer cells are considered metabolically active and require a high level of ATP supply to maintain cell proliferation [51]. This feature is in association with the high levels of ROS production in cancer cells [51]. It is interesting that fibroblasts that can proliferate in primary culture show shikonin sensitivity similar to cancer cells. Given that proliferating normal cells, such as hematopoietic stem cells, are generally sensitive to chemotherapy, such cells might have high basal levels of ROS or high potential to generate ROS, which increase their sensitivity to anti-cancer drugs. It may be that such hyperphysiological levels of ROS inhibit the Akt pathway in the presence of shikonin.

In contrast to such cancer treatment cases, cytoprotective effects of shikonin against cell stress are typically associated with upregulation of the Akt pathway. Here are a couple of examples. Shikonin *upregulated* Akt/GSK and Nrf2 and attenuated hepatotoxicity in an acetaminophen model used by Li et al. [165]. In a hepatic IRI model, shikonin attenuated injury by inhibiting apoptosis and autophagy through a mechanism involving the *activation* of PI3K/Akt signaling [89]. In renal tubular epithelial cells (NRK-52E), shikonin showed renoprotective activity against high-glucose induced cytotoxicity [173]. In this system, shikonin upregulated the anti-oxidant system (SOD and catalase) and decreased caspase-3, Bax and p-GSK-3β, and *increased* p-AKT, although relatively high concentrations of shikonin were necessary. In the case of the aforementioned in EA.hy926 endothelial cells system, the shikonin-induced *increas*e in p-Akt appeared to mediate the protective effects of shikonin (1 μM) against oxLDL-induced expression of anti-oxidant proteins [112]. In a rat model of osteroarthritis used in Fu et al., shikonin showed protective effects and alleviated the suppression of p-Akt level [174]. Therefore, for standard tissue injury models, shikonin-mediated upregulation of p-Akt is likely to mediate the cell protection by shikonin (Figure 1).

Overall, the PI3K/Akt pathway in cancer cells tends to be inhibited by shikonin whereas the same pathway in the normal cells placed under stress tends to be upregulated by shikonin (Figure 1). At least this does not support an idea that any of PI3K and Akt is a direct target of shikonin with high efficacy. Rather, it may be important to recall the early finding that purified human PTEN is inactivated by H2O2 through disulfide bond formation between Cys71 and Cys124, which can be reversed by thioredoxin activity [175]. PTEN inactivation causes its substrate PtdIns(3,4,5)P3 to increase, thereby activating PI3K and Akt. Leslie et al. showed that, in macrophages stimulated by LPS and PMA in a condition that produces ROS, PTEN was oxidatively inactivated, and a concomitant oxidant-dependent activation of Akt was observed [176]. Thus, ROS is a physiologically important player that mediates Akt activation upon stimulation of cells. So, regarding the issue of Akt activation or inhibition, ROS are likely to control this switch. However, the oxidative inactivation of PTEN does not explain why in cancer cells shikonin enhances ROS and inhibits Akt. It is possible that ROS accumulation induced by a prolonged treatment with shikonin inactivates Akt (Figure 1). Further studies are necessary to figure out the mechanisms underlying such a cell-dependent difference in shikonin effect on PI3K/Akt pathway.

Other Akt-related pathways

Apoptosis signal-regulating kinase 1 (ASK_1) is a kinase regulated by Akt and belong to ASKs, a family of kinases belonging to a mammalian mitogen-activated protein kinase kinase (MKK) kinase. Activated (dephosphorylated) ASK_1 activates JNK and p38 MAPK pathways, promoting apoptosis. $p21^{cip1}$ can associate with ASK_1 and block apoptosis [177]. However, ROS can trigger proteasome-mediated degradation of $p21^{cip1}$. Importantly, shikonin-mediated ROS enhancement caused Akt inactivation that led to $p21^{cip1}$ degradation [170]. Interestingly, Akt was initially activated but gradually inactivated by prolonged exposure of shikonin, likely due to the effect of ROS accumulation. This Akt inactivation, in turn, led to ASK_1 activation (dephosphorylation), leading to p38 MAPK activation and apoptosis (Figure 1, right). Interestingly, a similar example of oxidation-mediated deblocking of ASK_1 had been reported; ROS act on thioredoxin and glutaredoxin, to dissociate from ASK_1 for its activation, resulting in the activation of JNK [178]. Technically, these studies suggest that careful analyses using various inhibitors and ROS scavengers can allow us to infer intricate crosstalks between ROS on signaling pathways. The study by Ahn et al. is also intriguing in showing that a prolonged treatment with shikonin reverses Akt activation to inactivation. Simply stated, it appears that, when the Akt activity is gone (by ROS), the cancer cell dies (via apoptosis).

The authors of [170] later focused on forkhead box (FOX)O transcription factors, other downstream targets of Akt. FOXOs is normally inactivated by phosphorylation by Akt and are localized in cytosol.

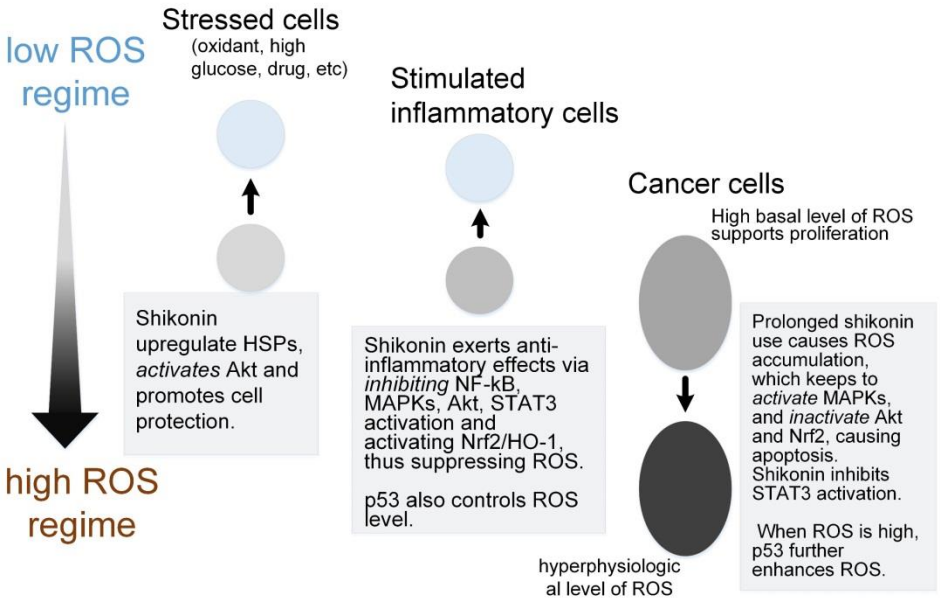

Figure 1: Schematic presentation of shikonin effect on ROS level and signaling pathways. The vertical position in this figure represents the ROS level with the low and high ROS levels corresponding to the top and bottom of the figure, respectively. For stressed cells (left), stimulated inflammatory cells (center) and cancer cells (right), the cell states before and after shikonin treatment are represented by circles or ovals and connected by arrows that represent the shikonin effects. It should be noted that this presentation has over-simplification; it is possible, for example, that for stressed cells a short-term shikonin treatment activates Akt, but a prolonged treatment of the same cells may inactivate Akt [170].

Dephosphorylated (activated) FOXO proteins translocate to the nucleus and induce the transcription of genes regulating cell cycle, apoptosis and other functions. In addition to Akt-mediated regulation, acetylation/deacetylation also regulates FOXOs activity. In lung carcinoma A549 and NCI-H1437 cells, shikonin induced Akt inactivation in association with FOXO3a dephosphorylation (activation) as well as the activation of early growth response protein (EGR)1, which induced apoptosis [179].

Perspectives

In 2002, Chen et al. hypothesized that shikonin is an effective inhibitor of protein-protein interactions with multiple targets in both intracellular and extracellular compartments, and this feature confers it a pleiotropic pharmacological capability [6]. A growing number of studies lend support to this view, as we have seen above. When the shikonin dose is low, only a limited set of targets would be affected. It seems fortuitous that IKK-β is inhibited by 0.25 − 0.5 μM shikonin [42], as, at this low dose, NF-κB mediated processes can be the inhibited. It is important to note, however, that even 0.1 μM shikonin can increase intracellular level of peroxides [116] likely mediated by shikonin accumulation in mitochondria. With higher doses of shikonin, many targets are likely to be modulated all at once, jointly forming some robust features that lead to decision making regarding the cellular outcome, such as ROS generation and induction of apoptosis.

NF-κB is an important target of shikonin. Although the role of NF-κB in innate immunity is well known, NF-κB also contributes to induction of proliferative genes, regulates anti-apoptotic molecules and drives many processes including cellular differentiation [180]. So, it is likely that the suppression of NF-κB activity plays a pivotal role not only in anti-inflammatory effect, but also in the anti-tumorigenic effect of shikonin. On the other hand, it should be kept in mind that an anti-tumorigenic effect of NF-κB has also been reported for several cancers [180]. This may partly explain the difference in shikonin efficacy between cancer cells.

Information about the target molecules of a given drug would provide clues if a list of the indications in treatment has to be prioritized. For instance, in a recent study by Tang et al., the expression level of PKM2 in the cancer cells provided the basis for the choice of shikonin [181]. Gefitinib is known to be effective in the treatment of mutant EGFR non-small cell lung cancer (NSCLC), but has a limited effect for wild-type EGFR lung cancer. In their study, as the expression level of PKM2 was found to increase in wild-type EGFR NSCLC after gefitinib treatment, the authors examined the effect of shikonin (as a PKM2 inhibitor) and found that shikonin enhanced the antitumor effect of gefitinib, inducing cell cycle arrest and apoptosis, in association with inhibition of PKM2/STAT3/cyclinD1 pathway [181]. This example illustrates the importance of subtyping of cancers based on molecular etiology as well as characterization of the targets of each drug.

Regarding the shikonin effects, several essential questions remain unanswered. How does shikonin protect normal cells from ROS whereas it enhance ROS in cancer cells and induce cell cycle arrest and apoptosis? Given the profound impacts of ROS on signaling pathways [79], it is reasonable to consider first the overall features of cellular outcome using the ROS level as an important axis (Figure 1). Cancer cells normally produce more ROS than do normal cells and are under increased oxidative stress compared to normal cells [51,182,183]. The idea that cancer cells are more vulnerable to oxidant stress because they function with a heightened basal level of ROS-mediated signaling was supported by the findings in Trachootham et al. [182,183]. It is likely that many chemotherapeutic agents show cancer cell-selective cytotoxicity since they enhance ROS beyond their limit in these originally stressed cells [51,182]. However, it is largely unknown why shikonin sensitivity shows profound differences even among cancer cells. In Duan et al., HL60 cells showed high sensitivity to shikonin with IC_{50} of ~0.8 μM (72 h), in association with high ROS production and glutathione depletion [74], whereas some other cancer cells showed sensitivities with IC_{50} of 2−3 μM. Careful comparison among different types of pro-oxidative agents might elucidate whether, for example, the expression level of the NADPH oxidase complex (the source of shikonin-induced ROS) is diverse among cancers and whether or not among the shikonin target genes, the genes for NADPH oxidase NOX2 ($gp91^{phox}$), COX-2 and a few cytochrome p450 enzymes, might be activated by such drugs to the extent that differs among cancers. However, the molecular basis causing such between-cell difference in the state of redox system require further analyses.

Related to the above question, also unknown is the mechanism by which shikonin activates the PI3K/Akt pathway in stressed normal cells (such as those in oxLDL-mediated stress), but inhibits it in cancer cells (Figure 1). As we discussed above, the activation of PI3K/Akt in stressed cells may be mediated by the oxidative inactivation of PTEN. However, this mechanism does not explain the shikonin-induced PI3K/Akt inactivation in cancer cells, prompting us to take into account the effects of accumulating ROS. Notably, there is a precedent to such reversal of activity depending on ROS concentration. Liu et al. pointed out that hyperphysiological and physiological levels of p53 have quite distinct effects [117]. Specifically, hyperphysiological levels of p53 activate pro-oxidant genes and suppress (or induce imbalance) of antioxidant genes whereas physiological levels of p53 maintain cellular redox state via sustained expression of antioxidant genes. Similarly, it seems possible that a hyperphysiological level of ROS may rather inactivate PI3K/Akt via a mechanism that is independent from PTEN whereas. Further analysis of shikonin and PI3K/Akt may help our understanding of the roles of ROS in the cell fate decision.

Although our interpretation remains highly speculative, Figure 1 summarizes the overall feature of shikonin effects on stressed cells, stimulated pro-inflammatory cells, and cancer cells. HSP70 is considered an intracellular and extracellular cytoprotective mediator [184], and therefore both HSP70 and Akt are cytoprotective factors. Intriguingly, the findings in Shiota et al. [185] and Lee et al. [186] suggest that there appears to be a positive feedback loop between HSP70 and Akt. In such cases, p38 MAPK is likely to be involved in the cell protection.

Finally, we acknowledge that in this article we did not exhaust the target molecules and pathways of shikonin. For instance, we did not detail Huang et al. that showed that shikonin induces necroptosis in glioma cell lines C6 and U87, in association with the increase in the expression of RIP-1 [187]. Intriguingly, this is in contrast with the report from Gong and Li that showed that, in hepatoma BEL7402 and Hu7 cells, the shikonin treatment caused decreases in the expression level of RIP-1. As RIP-1 is considered important for cell's decision to live or die, further analyses are hoped to elucidate its role in various cell contexts [188].

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Conflict of Interest

Authors declare that they have no conflict of interest.

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