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Review

Molecular mechanism of shikonin inhibiting tumor growth and potential application in cancer treatment

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Abstract

Shikonin is one of the major bioactive components of *Lithospermum erythrorhizon*. It has a good killing effect in a variety of tumor cells. Its antitumor effect involves multiple targets and pathways and has received extensive attention and study in recent years. In this review, we systematically review recent progress in determining the antitumor mechanism of shikonin and its derivatives, specifically their induction of reactive oxygen species production, inhibition of EGFR and PI3K/AKT signaling pathway activation, inhibition of angiogenesis and induction of apoptosis and necroptosis. We also discuss the application of nanoparticles loaded with shikonin in the targeted therapy of various cancers. Finally, we suggest new strategies for the clinical application of shikonin and its derivatives.

Keywords: shikonin, antitumor, apoptosis, reactive oxygen species, nanoparticles

Introduction

Cancer is one of the major killers in the world, and its high morbidity and mortality seriously threaten human health. Because of the infinite proliferation and metastasis of cancer cells, cancer treatment is difficult [1, 2]. Currently, the main methods of cancer treatment are surgery and chemotherapy, but they have certain limitations. Surgery is suitable for tumors that are easy to remove. For tumors that are difficult to remove, chemotherapy and immunotherapy are alternative treatments [3]. Although high-dose chemotherapy drugs are sufficient to kill tumor cells, the side effects of chemotherapy drugs and drug resistance have limited the application of chemotherapy drugs [4–7]. The latest immunotherapy is not suitable for all patients because of its specificity, and the success rate is very low [8]. Therefore, there is an urgent need to design and discover new drugs with better pharmacological properties for cancer treatment. Currently, many anticancer drugs are extracted from botanicals and structurally modified to be used in the clinical treatment of cancer [9, 10].

Lithospermum erythrorhizon is a perennial herbaceous plant, also known as "Zicao", and is a component in traditional Chinese medicine. It is mainly distributed in Inner Mongolia, Xinjiang, Gansu, Western Tibet, and northeast China [11].. Shikonin is the main bioactive component of *L. erythrorhizon* extracts. Studies have shown that shikonin promotes wound healing and has antibacterial, anti-inflammatory and antitumor activity [1, 12–14]. In particular, the antitumor effect has attracted extensive attention from researchers. Research in the past 30 years has shown that shikonin is a potential anticancer drug. Numerous studies have confirmed that its antitumor mechanism involves multiple targets. These targets include inducing apoptosis, regulating signaling pathways and inducing the production of reactive oxygen species (ROS) and antitumor vascular regeneration (Figure 1). Therefore, shikonin is currently one of the research hotspots of antitumor therapy in traditional Chinese medicine [15–17].

Structural Analysis of Shikonin

The main components of *L. erythrorhizon* are naphthoquinones, alkaloids, quinones, phenolic acids, polysaccharides and other chemical components, with fat-soluble naphthoquinones and water-soluble polysaccharides being the main antitumor substances [18, 19]. Among these components, naphthoquinone compounds, including shikonin, isobutyrylshikonin, deoxyshikonin and acetylshikonin, have been the most extensively studied [20]. The antitumor activity of naphthoquinone compounds is mainly related to the structure of the α-1,4 naphthoquinone core. The naphthoquinone scaffold can induce the generation of ROS and indirectly affect

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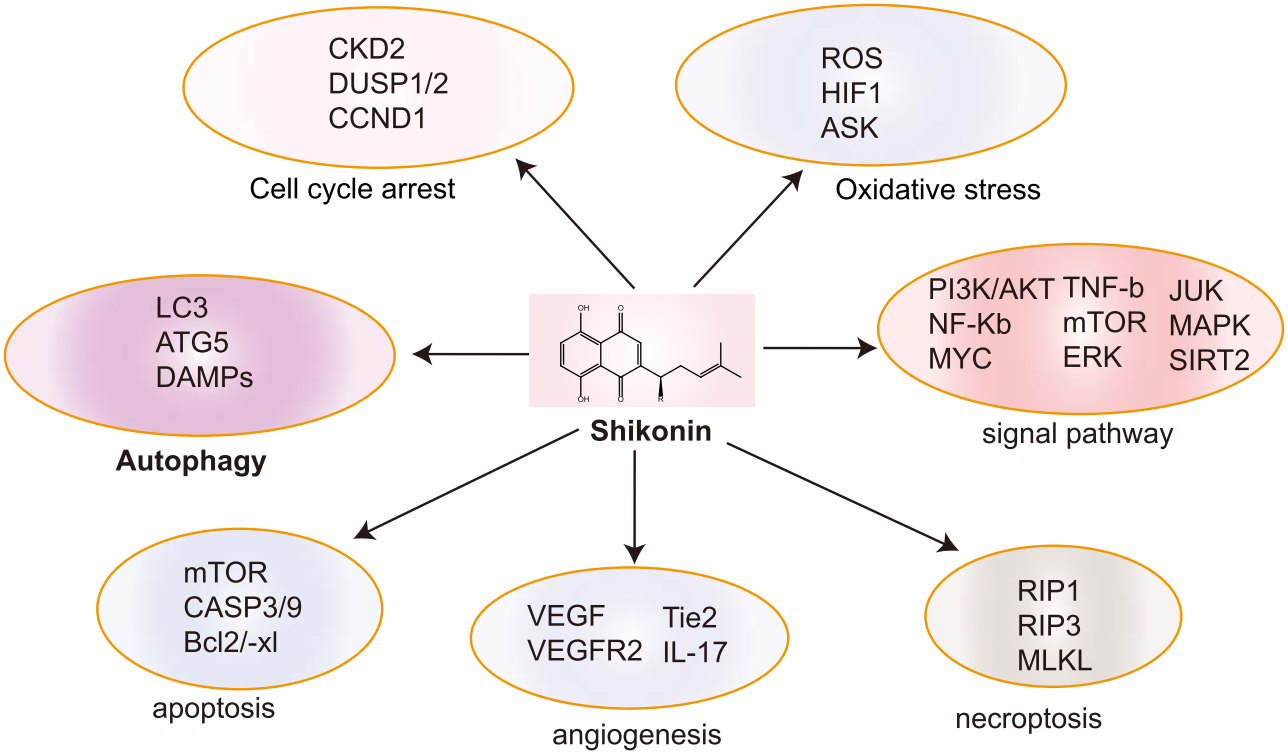

Figure 1. Multiple targets involved in the anti-tumor mechanism of shikonin.

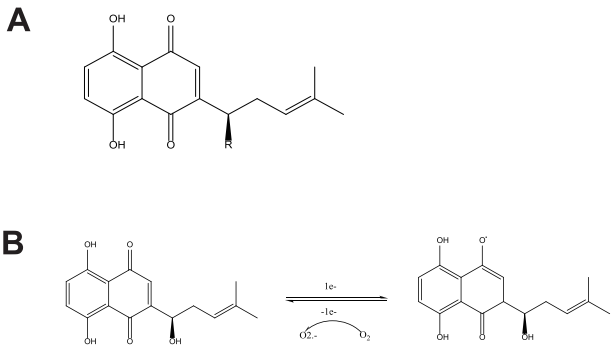

Figure 2. A. Parent nuclear structure of shikonin compounds. B. Mechanism of shikonin inducing reactive oxygen species (ROS) production.

oxidative stress in cells (Figure 2A) [21]. The semiquinone group of the naphthoquinone ring is reduced by single electron transfer and then oxidized back to quinone under the action of oxygen molecules. In this redox process, a large amount of ROS is simultaneously produced to affect the membrane potential of cells and mitochondria (Figure 2B) [22, 23].

Antitumor Mechanism of Shikonin

Induction of ROS production

Many studies have shown that ROS can induce cell death of different types of cancer cells after treatment with anticancer drugs [24]. DCF-DA staining is used to detect the generation of ROS, and ROS were thus found to be produced by shikonin and its derivatives, leading to the increased consumption of reduced glutathione (GSH) and increased Ca^{2+} concentration in the cells and destroying the mitochondrial membrane potential. The accumulation of ROS causes oxidative stress in cells, leading to the death of gastric cancer (GC) cells [25–27]. Treatment with shikonin for 6 h resulted in necroptosis due to changes in mitochondrial permeability, but the effect was reversed by Nec-1. However, after 24 h of treatment with shikonin, ERK 1/2 and AKT activities were significantly inhibited, and p38 activity was upregulated, which ultimately led to pro-caspase-3 cleavage and triggered the apoptosis of GC cells. This process was suppressed by NAC, but not by Nec-1 [28–30].

Similarly, shikonin-induced apoptosis of hepatoma cells is associated with increased levels of ROS. The production of ROS in Huh 7 and BEL 7402 cells increased in a time-dependent manner after shikonin treatment. When hepatoma cells were pretreated with the ROS scavengers NAC and GSH before treatment with shikonin, the production of ROS was significantly inhibited, the cytotoxicity of shikonin was attenuated, and cell viability was rescued [31]. In glioma cells, RIP1 binds to NADPH oxidase-1 (NOX1) and the small GTPase RAC1 to induce ROS production. RIP3 upregulates ROS levels through its physical interaction and subsequent activation with GLUD1. MLKL, the functional substrate of RIP3, can also cause excessive production of mitochondrial superoxide dismutase by binding to mitochondrial proteins, thus participating in shikonin-induced DNA double-strand breaks (DSBs). It was also shown that shikonin can promote the production of ROS, reduce the concentration of glutathione and destroy the mitochondrial membrane potential, thus leading to apoptosis of glioma cells [32].

Inhibition of the epidermal growth factor receptor (EGFR) signaling pathway

EGFR is a transmembrane glycoprotein in the receptor tyrosine kinase (RTk) superfamily and contains an extracellular ligand binding domain, a single transmembrane domain and an intracellular domain with an ATP-binding site [33, 34]. DEGFR is a mutant of EGFR in glioma that plays a key role in glioma cell migration, invasion, and drug resistance due to the lack of an extracellular binding domain [35, 36]. Studies have found that the combined use of shikonin and erlotinib to treat glioma cells. These two compounds compete for the ATP-binding site in the phosphorylated TK domain of EGFR, inhibiting the EGFR signaling pathway. In DEGFR U87MG cells, shikonin and erlotinib synergistically inhibited the phosphorylation of DEGFR and regulated the phosphorylation level of downstream molecules such as AKT, P44/42, MAPK and PLCγ1 to kill glioma cells. Studies have shown that shikonin blocks EGFR signaling and reduces ERK activity, thereby reducing the proliferation of epidermoid cancer cells [37–39].

Inhibition of the PI3K/AKT signaling pathway

Shikonin can inhibit the phosphorylation of AKT by inhibiting the PI3K/AKT signaling pathway to prevent activated AKT from expressing its downstream target proteins Bad and caspase and to upregulate the level of Bax protein [40, 41]. Shikonin can also prevent the activation of NF-κB by AKT and then downregulate the expression of Bcl-xl, a member of the Bcl-2 family, and plays a role in promoting apoptosis in endometrial carcinoma [42, 43]. The protein encoded by the PTEN gene in cervical cancer cells has phosphatase activity and plays a key negative regulatory role in the PI3K/AKT signaling pathway. It can reduce PIP3 activation of AKT and its downstream molecules, inhibit cell growth and promote apoptosis [44, 45]. H1650/R and H1975/R non-small cell lung cancer (NSCLC) cells are resistant to afatinib. Shikonin negatively regulates the PI3K/AKT signaling pathway, increases caspase-3 cleavage and reduces Bax expression, thereby inducing apoptosis in these cells [46].

Antitumor angiogenesis

Tumor blood vessels can provide the necessary nutrition and oxygen for tumor development. Tumor cells have a function in inducing angiogenesis in vitro. Vascular endothelial growth factor (VEGF), as the most important factor for promoting angiogenesis, plays a key role in tumor angiogenesis, leading to tumor cell survival and migration [47–50]. It has been found that shikonin not only directly promotes the death of tumor cells but also inhibits the regeneration of tumor blood vessels and reduces the supply of nutrients to tumor cells [51]. Shikonin and b-HIVS can inhibit angiogenesis by inhibiting the phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2) and Tie2, thereby reducing the expression of VEGFR2 and Tie2 [52]. Other studies have shown that shikonin can inhibit the expression of VEGF mediated by IL-17, and its mechanism may be related to the JAK2 and STAT3 pathways [53, 54].

Induced apoptosis

Apoptosis is the type of programmed cell death critical for cell clearance in normal tissue. It is a basic life process in multicellular organisms, and it depends on caspases, with the cleavage of PARP and pro-caspase-3 being two important events in apoptosis [29, 55–57]. Shikonin has been found to induce apoptosis through a caspase-dependent pathway in HL-60 human promyelocytic leukemia, A375-S2 melanoma cells, HeLa human cervical cancer cells, and Colo-250 and human colorectal cancer cells [58–60] (Table 1). PARP cleavage and caspase-3 expression increased when AGS cells were treated with shikonin. Studies have shown that, in addition to the effective induction of the cleavage of caspase-8, caspase-9 and PARP and the inhibition of PI3K/AKT and NF-κB signaling pathways, shikonin increases intracellular ROS production, thereby inducing the apoptosis of liver cancer cells [26, 40]. It was also found that shikonin can reduce the expression of Bcl-2 protein and increase the level of Bax protein in A549 cells. Shikonin can also reduce the mitochondrial membrane potential of A549 cells and destroy the integrity of the mitochondrial membrane. Then, shikonin promotes the release of proapoptotic proteins and thereby activates downstream signaling pathways (Figure 3) [61–63]. Recently, it was reported that shikonin induces apoptosis and attenuates epithelial-mesenchymal transition in human colon carcinoma [64].

Induction of necroptosis

Necroptosis is a type of programmed cell death that is independent of caspase activation. Necrotic cells generally have two significant characteristics. One is that they are morphologically different from apoptotic cells. The other is that they are dependent on the formation of necrotic bodies mainly composed of RIP1 and RIP3 [65, 66]. Coimmunoprecipitation of RIP1 or RIP3 revealed that shikonin can induce the aggregation of necrosis factors, such as RIP1 and RIP3, which are involved in the regulation of shikonin-induced ROS production [67, 68]. It has been shown that the shikonin-induced DNA DSBs in SHG-44 and U87 glioma cells are caused by ROS, and ROS are involved in necrotic body formation and the upregulation of RIP-1 and RIP-3. On the one hand, RIP-1 and RIP-3 complexes directly increase intracellular ROS levels and are involved in shikonin-induced DNA DSBs. On the other hand, RIP1 and RIP3 complexes phosphorylate MLKL to activate MLKL, which interferes with mitochondrial function, increases ROS levels and promotes glioma cell necroptosis by promoting nuclear translocation of AIF and the formation of γ-H2AX [69–71]. The RIP1 inhibitor Nec-1 and the RIP3 inhibitor GSK 872 significantly block cell death induced by shikonin

Table 1. Shikonin induces tumor cell apoptosis

Cancer types	Cell types	Molecular mechanism	References
Glioma	U251 and U87MG cells	Downregulation of CD147	[87]
Non-small cell lung cancer	A549 cells	Inhibited the STAT3 and AKT pathways, downregulated the expression of Bcl-2	[88]
Breast cancer	4 T1 and MDA-MB-231 cells	Regulated p38 signaling pathways	[15]
Hpatocellular cancer	huh7 and BEL7402 cells	Induced reactive oxygen species (ROS), downregulation of AKT and RIP1/NF-κB activity	[24]
Gastric cancer	AGS、AZ521 and SCM-1 cells	Upregulated p38 activities,inhibited ERK1/2 and AKT activities	[17]
Glioma	GBM、U87MG、DK-MG cells	Inhibited the epidermal growth factor receptor signaling pathway	[52]
Melanoma	A357 cells	Activated ROS-mediated ER stress and p38 pathways	[74, 89]
Non-small cell lung cancer	A549 and NCI-H1437 cells	Activated FOXO3a/EGR1/SIRT1 signaling	[90]
Gastric carcinoma	AGS cells	Regulated p53 and Nrf2 signaling pathways	[91]
Thyroid carcinoma	TT cells	Through the mitochondrial signaling pathway	[16]
Gastric cancer	NCI-N87 cells	Inhibition of PI3K/AKT signal pathway	[40]
Leukemia	K562 cells	Increased the PTEN level and inactivated the PI3K/AKT signaling pathway	[44]

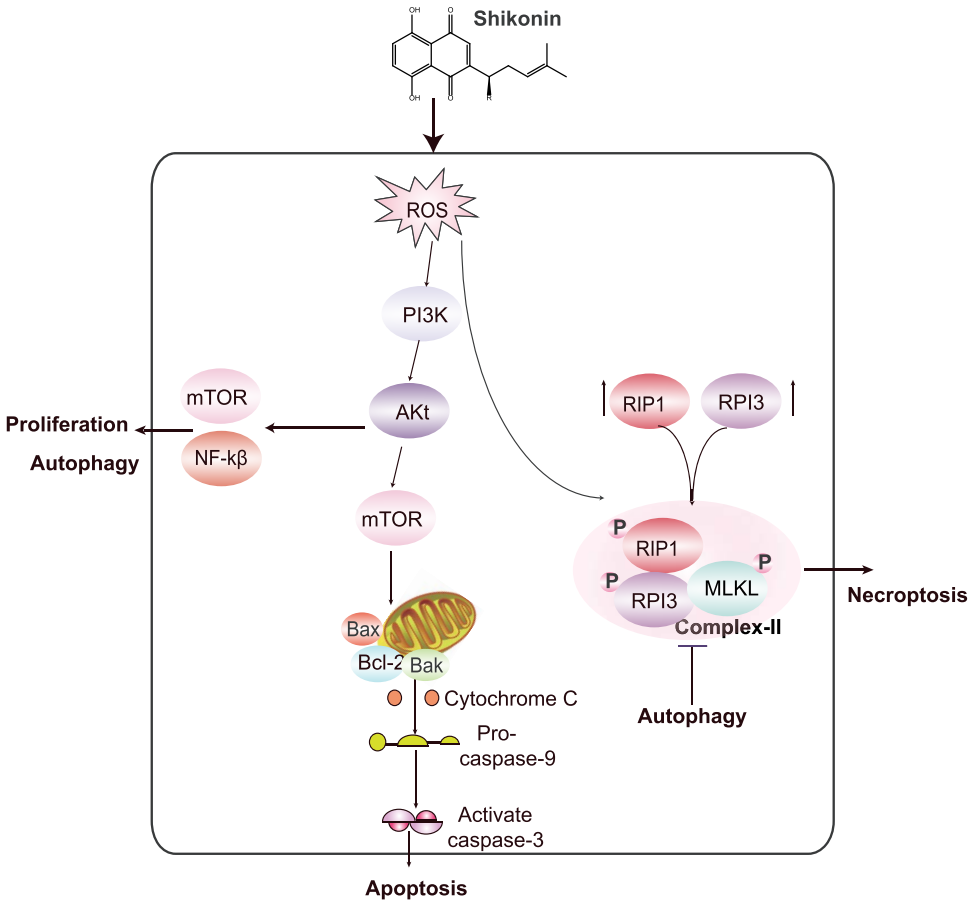

Figure 3. Cell death pathways involved in the anti-cancer mechanism of Shikonin.

but also block ROS production [69, 72]. Similarly, shikonin induced RIP1- and RIP3-dependent necrosis in the mouse osteosarcoma cell lines K7, K12 and K7 M3 (Table 2) [73]. These results indicate that ROS promote the formation of necrotic cells by enhancing the interaction between RIP1 and RIP3 and are involved in the killing effect of shikonin on glioma cells (Figure 3).

Induction of autophagy and cell cycle arrest

Shikonin can also induce autophagy in several types of cancer cells through different mechanisms (Table 3). In melanoma cells, shikonin induces apoptosis and autophagy via ROS-mediated ER stress and p38 pathways [74]. Shikonin can also induce autophagy in colorectal carcinoma cells by targeting galectin-1/JNK

Table 2. Shikonin induces tumor cell necroptosis

Cancer types	Cell types	Molecular mechanism	References
Glioma	SHG-44、U87、U251 cells	Upregulation of RIP1 and RIP3, induced DNA DSBs	[67]
Multiple myeloma	K7、K12 and K7M3 cells KMS-12-PE and U266 cells	Upregulation of RIP1 and RIP3	[68, 92]
Gastric cancer	AGS、AZ521 and SCM-1 cells	Induced reactive oxygen species (ROS)	[17, 93]
Non-small cell lung cancer and glioma	A549 cells Rat C6 glioma cells	Increased the levels of the RIP1	[62, 94]
Histiocytic lymphoma	U937 cells	Up-regulation of TNF expression	[95]
Glioma	SHG-44 and U251 cells	Inducesd MLKL activation and chromatinolysis	[96]
Nasopharynx cancer	5-8F cells	Increased ROS production and the upregulation of RIPK1/RIPK3/MLKL expression	[97]
Prostate Cancer	PC3, DU145, LNCaP, and 22Rv1	cell cycle arrest	[98]

Table 3. Shikonin induces autophagy and inhibit cell proliferation

Cancer types	Cell types	Molecular mechanism	References
Pncreatic cancer	BXPC-3, A375	Asociated with the PI3K/Akt signaling pathway, p38 pathways	[74, 99]
Hpatocellular carcinoma	BEL7402 and Huh7	Ativation of ERK	[100]
Colon cancer	HCT116 and SW620	Inhibiting es-associated protein, targeting galectin-1/JNK signaling axis	[15, 101]
Non-small cell lung cancer	A549	Regulated necroptosis	[75]
Skin Carcinogenesis	JB6 Cl-41	Suppressed the ATF2,and Cdk4,	[76]
Esophageal cancer	EC109 and EC9706	Decreased EGFR, PI3K, p-AKT, HIF1α and PKM2	[78]
Esophageal cancer	KYSE150	inhibited cell proliferation by suppressing PKM2	[102]
Lung cancer	A549 and H446,	downregulating PFKFB2 expression	[103]

signaling pathway [15]. In non-small cell lung cancer cells, shikonin-induced necroptosis is enhanced by the inhibition of autophagy [75].

Shikonin can also induce cell cycle arrest and proliferation in different types of cancer cells (Table 3). In human skin cancers, shikonin suppresses the ATF2 pathway in skin carcinogenesis. Furthermore, inhibition of ATF2 expression also decreased the expression levels of Cdk4 and Fra-1 [76]. RNA-seq transcriptome analysis indicated that shikonin induces the expression of dual specificity phosphatase (DUSP)-1 and DUSP2 and causes cell cycle arrest and apoptosis in breast cancer cells [77]. In esophageal cancer, shikonin decreased EGFR, PI3K, p-AKT, HIF1α and PKM2 expression by regulating HIF1α/PKM2 signal pathway [78].

Nanoparticles composed of shikonin

Studies have shown that shikonin is a potential anti-glioma drug. However, due to the limitations caused by its poor water solubility, it has a short half-life and non-selective biological distribution. Therefore, an effective route of administration is urgently needed to improve shikonin bioavailability and safety [79–81]. Nanoparticles have valuable pharmacokinetic properties, a large surface mass ratio, high drug solubility and adjustable controlled release of cargo [82, 83]. The lactoferrin receptor is highly expressed in glioma cells, and lactoferrin functionalized shikonin PEG-PLGA nanoparticles can be effectively routed upon administration. Because of their unique functions in targeting glioma cells and crossing the blood–brain barrier, these nanoparticles have important application prospects for the targeted therapy of glioma [84, 85].

Studies have shown that shikonin-loaded polylactic acid (PLGA) biodegradable nanoparticles killed only epithelial ovarian cancer cells in the treatment of ovarian cancer but did not induce strong cytotoxicity in normal ovarian cells, endothelial MS1 cells or lymphocytes. Therefore, these nanoparticles can be used as new drug treatments targeted to solid tumors [9, 86].

Conclusion

Although great progress has been made in the antitumor research of shikonin, there are still many questions to be solved. Firstly, shikonin and its derivatives have certain effects on many kinds of tumor cells. However, there are some problems such as selectivity and cytotoxicity. Secondly, the anti-tumor effect of shikonin and its derivatives has a wide range of mechanisms, involving many targets, such as inducing apoptosis, necrosis, anti-tumor angiogenesis, regulating signal pathway and so on. The interaction between them needs to be further determined. Finally, the naphthoquinone core structure of shikonin and its derivatives can produce superoxide radicals and potential bioalkylation. Although it has significant antitumor activity, this structure is also a source of side effects.

In general, the anti-tumor effect of shikonin and its derivatives has attracted wide attention, but its anti-tumor mechanism research is not deep enough. At present, the therapeutic effects of shikonin and its derivatives are based on cells and animal studies, but clinical trials are rarely reported. Therefore, more clinical trials must be carried out to verify the anti-tumor potential of shikonin. In addition, we can further improve the structure and functional group modification of shikonin from the perspective of chemical modification. We can also screen out derivatives with high activity, high selectivity and low cytotoxicity to minimize side effects. In addition, improving the way of administration is the focus of future research.

Author contributions

Qiang Wang, Xiaoli Ju and Jiayou Wang wrote the manuscript, Jing Wang and Heng Zhang revised the manuscript. All authors read and approved this manuscript.

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Declaration of conflict of interest

None.

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