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Shikonin derivatives for cancer prevention and therapy

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ABSTRACT

Phytochemicals gained considerable interest during the past years as source to develop new treatment options for chemoprevention and cancer therapy. Motivated by the fact that a majority of established anticancer drugs are derived in one way or another from natural resources, we focused on shikonin, a naphthoquinone with high potentials to be further developed as preventive or therapeutic drug to fight cancer. Shikonin is the major chemical component of *Lithospermum erythrorhizon* (Purple Cromwell) roots. Traditionally, the root extract has been applied to cure dermatitis, burns, and wounds. Over the past three decades, the anti-inflammatory and anticancer effects of root extracts, isolated shikonin as well as semi-synthetic and synthetic derivatives and nanoformulations have been described. *In vitro* and *in vivo* experiments were conducted to understand the effect of shikonin at cellular and molecular levels. Preliminary clinical trials indicate the potential of shikonin for translation into clinical oncology. Shikonin exerts additive and synergistic interactions in combination with established chemotherapeutics, immunotherapeutic approaches, radiotherapy and other treatment modalities, which further underscores the potential of this phytochemical to be integrated into standard treatment regimens.

1. Introduction

Current approaches to chemotherapy do not have sufficient clinical effectiveness, and as a result many patients cannot be cured and die from cancer. In many cases, this is due to the development of resistance to established anticancer drugs, as well as to the severe and partly life-threatening side-effects of chemotherapy. Both drug resistance and side-effects prevent the application of drug doses high enough to kill 100% of all tumor cells in the body. For this reason, there is an urgent need for novel drugs with improved pharmacological features for cancer treatment. Since the majority of the clinically established anticancer drugs were derived from natural products, the search for novel chemical structures from natural sources is straightforward and promising [1,2]. While numerous phytochemicals with cytotoxic activity have been reported in the literature, there are only a few for which there is detailed information available about their mechanisms of action and proven anticancer activity *in vivo*. Therefore, shikonin appears to be an attractive candidate for further consideration in anticancer drug development.

Lithospermum erythrorhizon Sieb. et Zucc. is a perennial herbaceous plant that belongs to the family Boraginaceae [3]. A natural naphthoquinone known as shikonin ((±)-5,8-dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthoquinone) is a major chemical component in the roots of *L. erythrorhizon*. Traditionally, this plant was used to treat several diseases, such as burns, carbuncles, macular eruption, sore throat, and measles [4]. Recently, several studies demonstrated the anticancer activity of shikonin *in vitro* and *in vivo* [5]. Shikonin induces signaling pathways that regulate oxidative stress responses, mitochondrial function, and cytoskeleton formation. This compound accumulates in the mitochondria, which leads to the generation of reactive oxygen species (ROS), and deregulates intracellular Ca^{2+} levels. As a consequence, the mitochondrial membrane potential and microtubules are distorted, and cell cycle arrest and apoptosis are induced. Hence, shikonin may serve as a source compound for the development of novel anticancer drugs [6]. In this review, we summarized the different signaling pathways leading to cell death induction with which shikonin may interact. Furthermore, we reported on the results of studies that used the combination of shikonin with clinically established anticancer drugs and found that this triggered increased or even synergistic inhibition of tumor growth. Finally, we reviewed the various nanotechnologic drug delivery approaches available for use with shikonin. The literature was systematically screened by searching the PubMed database using the search terms “shikonin” and “cancer”.

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Abbreviations

1,4-NQs	1,4 naphthoquinones
4-HBA	4-hydroxybenzoic acid
Ab	antibody
ABC	adenosine triphosphate-binding cassette
ATO	arsenic trioxide
AVO	acidic vesicular organelles
CPL	chorismate pyruvate-lyase
CDK4	cyclin dependent kinase 4
CRT	calreticulin
DAMP	damage-associated molecular patterns
DC	dendritic cell
DOX	doxorubicin
EGFR	epidermal growth factor receptor
GBA	3-geranyl-4-hydroxybenzoate
Glo I	glyoxalase I
GPP	geranyl diphosphate
HMGB1	high mobility group box 1
HMGR	Hydroxyl-3-methylglutaryl-coenzymeA reductase
hnRNPA1	ribonucleoprotein A1
HSP70	heat shock protein 70
IFN	interferon
IHN	isohexenyl naphthazarins
IR	ionizing radiation
JNK	c-jun-N-terminal kinase
LC3	1A/1B-light chain 3
MDR	multidrug resistance
miRNA	micro-RNA
MMP	matrix metalloprotein
mRNA	messenger RNA
MVA	mevalonic acid pathway
NF-κB	nuclear factor kappa B
NK cells	natural killer cells
NP	nanoparticle
NSCLC	non-small cell lung cancer
PEG	polyethylene glycol
P-gp	P-glycoprotein
RIPK1/3	receptor-interacting serine-threonine kinase 1/3
PKC	protein kinase C
PKM2	pyruvate kinase M2
PLGA	poly (lactic-co-glycolic acid
lncRNA	long non-coding RNA
RGD	peptide motif
RGD-SSLs-SHK	RGD-modified shikonin-loaded liposomes
ROS	reactive oxygen species
TKI	thymidine kinase inhibitor
TNF	tumor necrosis factor
SLN	solid lipid nanoparticle
STAT3	signal transducer and activator of transcription 3
STP-NG	sarcoma-targeting peptide-decorated disulfide-crosslinked polypeptide nanogel
TCA	tricarboxylic acid
TCL	tumor cell lysate
TEM1	tumor endothelial marker 1
TNF	tumor necrosis factor
UTR	untranslated region

2. Chemistry and biosynthesis of shikonin

Chemistry: Shikonin is a type of 1,4-naphthoquinone (1,4-NQ) that consists of a benzene moiety (ring A) linearly fused with a fully conjugated cyclic diketone (ring B), in which the carbonyl groups are arranged in para-orientation (Fig. 1a), connected to a chiral six-carbon side-chain. Shikonin belongs to the class of polyphenols that have a C6–C4 skeleton (with 10 carbon atoms). These compounds are widespread in nature as secondary metabolites of fungi and microorganisms, as well as of plants. Naphthoquinones, including shikonin, alkannin, and their derivatives, belong to the largest group of quinone pigments (Fig. 1).

L. erythrorhizon is the main source of shikonin, which is easily extracted as a deep-red pigment from the roots of this plant [7] (Fig. 2). Brockmann [8] first successfully identified alkannin and shikonin as having different optical features from each other. The ratio of the content of alkannin to shikonin enantiomers varies from plant to plant, but is generally around 1:2 [9].

Plants have to grow up for 7 years before the shikonin content reaches 1–2% in their roots. This difficulty led to the development of

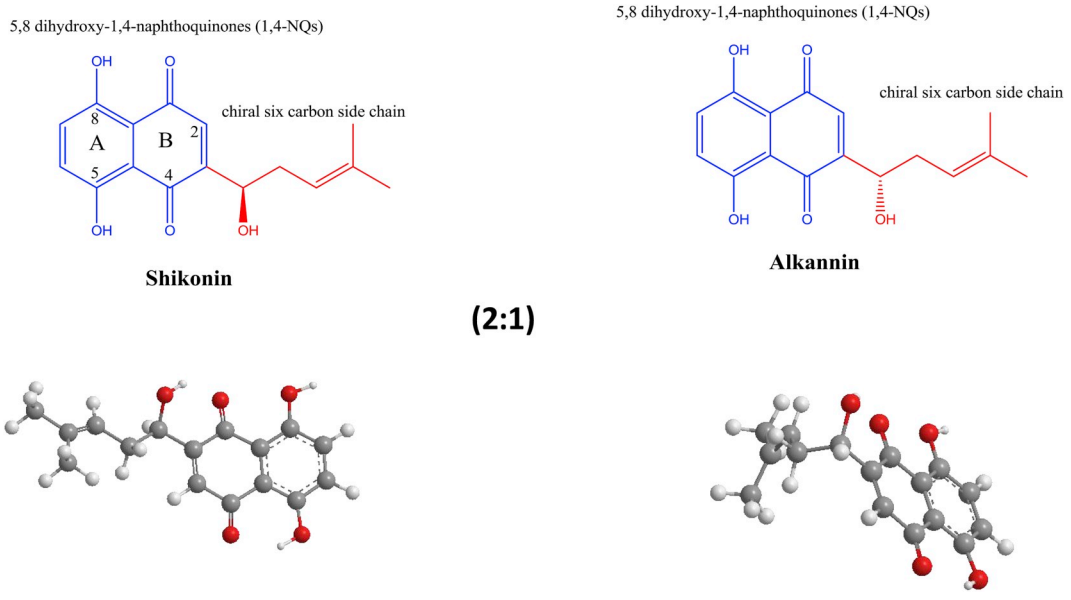

Fig. 1. Shikonin and alkannin enantiomer structures.

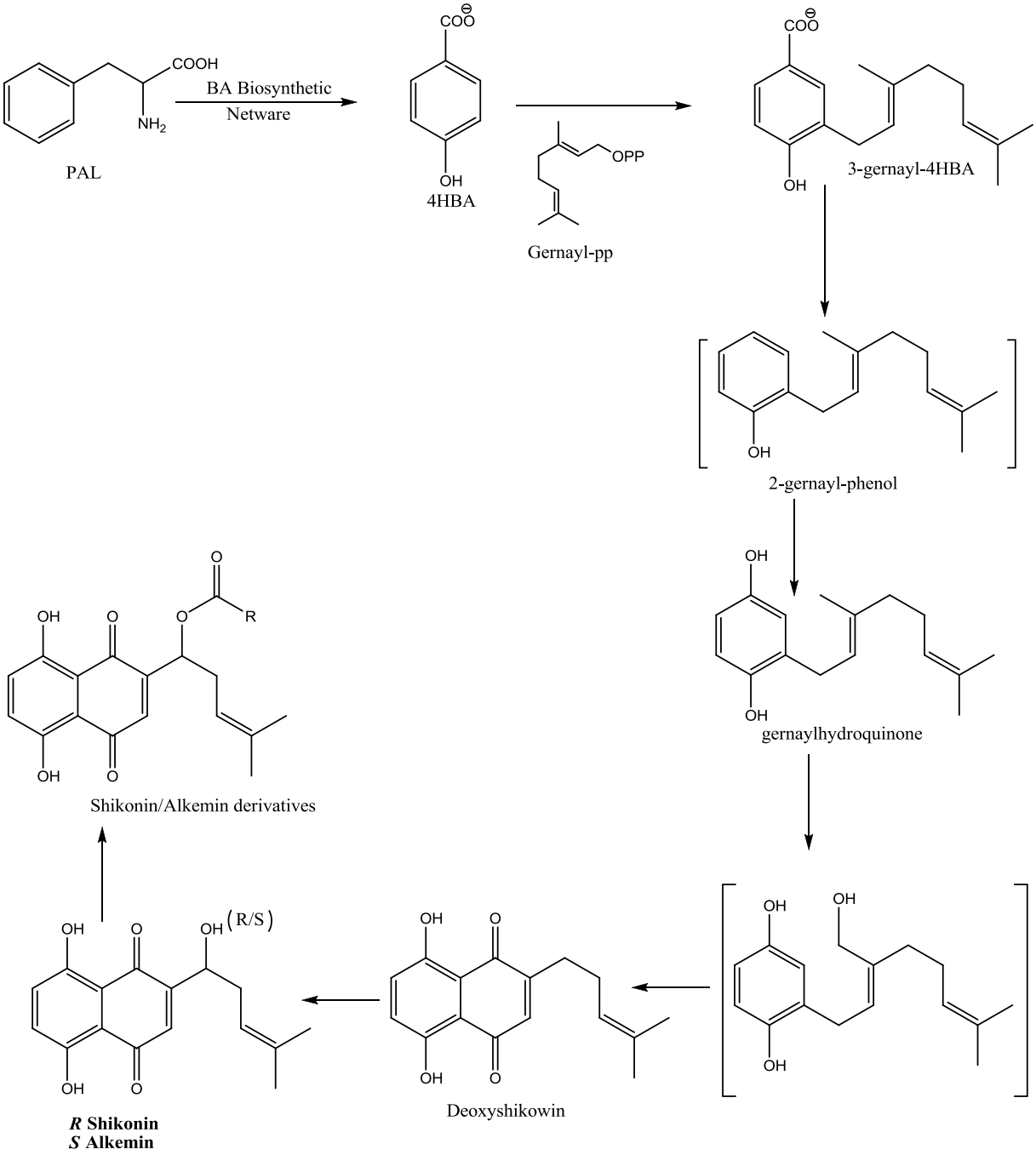

Fig. 2. Biosynthesis of shikonin.

biotechnological approaches to produce shikonin from plant cell cultures [10]. The production of natural shikonin metabolites represents one of the vital metabolic processes involved in the ecological adaptation of plants. Interestingly, several animal species also produce 1,4-NQs, such as those found in the secretions of tenebrionid beetles and in the scent-producing glands of certain arachnids [11,12]. Bacteria (*e.g., Actinomycetes*) produce numerous 1,4-NQs [13], as well as the C-5/C-8 dihydroxy scaffolds called naphthazarins that form core moieties in antimicrobial rubromycins [14]. Additionally, microorganisms have the ability to synthesize prenylated 1,4-NQs (*e.g.*, menaquinone), which were suggested to be ancestral quinones involved in electron transport chains used in anaerobic respiration [15]. Menaquinone was also found in some cyanobacteria and rhodophytes (red algae) [16], and in most diatoms (protists) [17].

Biosynthesis: Early tracer experiments demonstrated that phenylalanine (via cinnamic acid and 4-hydroxybenzoic acid (4-HBA)) and mevalonic acid are precursors for the benzene and quinone rings, respectively, of the alkannin produced in *Plagiobothrys arizonicus* [18]. This finding, in combination with the isolation of 3-geranyl-4HBA and geranyl hydroquinone from cell cultures of *L. erythrorhizon* [19,20], led to the hypothesis that alkannin and shikonin are likely synthesized by pathways analogous to ubiquinone biosynthesis with the addition of subsequent ring closure reactions (Fig. 2). Many boraginaceous species utilize the aromatic precursor 4-HBA and the mevalonic acid (MVA) pathway to synthesize a subclass of 1,4-NQs termed isohexenyl naphthazarins (IHNs) (Fig. 3).

The IHNs encompass red-pigmented compounds, such as shikonin, alkannin, and at least 40 other acylated derivatives synthesized in the

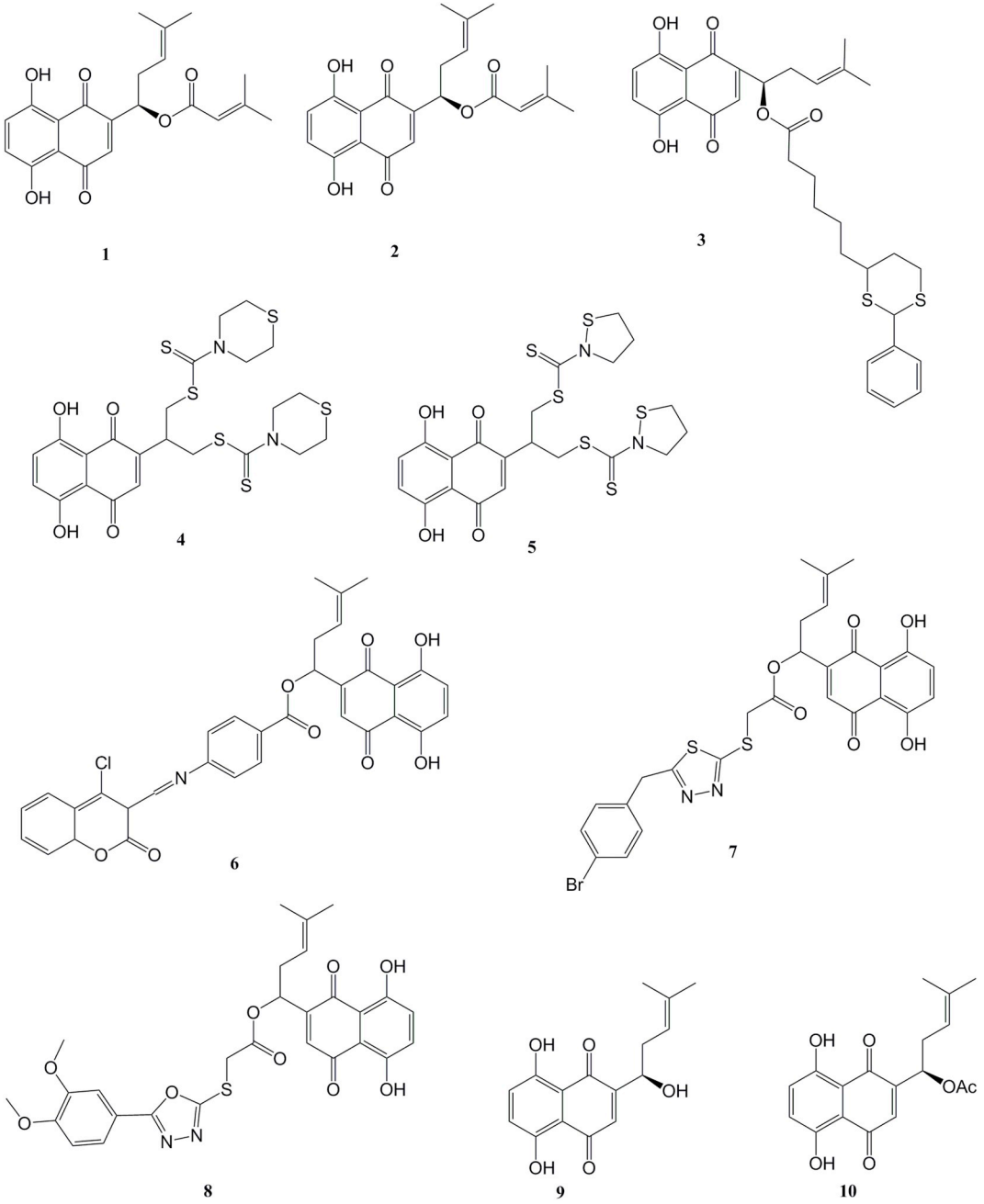

Fig. 3. Natural and synthetic shikonin derivatives.

roots of medicinal species (*e.g., L. erythrorhizon* and *Alkanna tinctoria*) [21]. In plants, the biosynthesis of benzoic acids from phenylalanine involves a complex network of metabolic routes branching off from the core phenylpropanoid pathway [22]. The precursor 4-HBA is formed *via* the shikimate and the phenyl-propanoid pathways, while the isoprenoid precursor, geranyl diphosphate (GPP), is derived from the MVA pathway [23].

The 4-HBA geranyl transferase reaction links the aromatic precursor 4-HBA to the isoprenoid precursor GPP and yields 3-geranyl-4-hydroxybenzoate (GBA), which is the first specific intermediate in shikonin biosynthesis [24]. Hydroxyl-3-methylglutaryl-coenzymeA reductase (HMGR) and 4-HBA geranyl transferase are important enzymes in the regulation of shikonin biosynthesis [25–27]. As previously summarized [28–31], the production of shikonin and its derivatives is influenced by many external factors, and is largely modulated by the transcriptional regulation of metabolic genes [20,32].

3. Natural and synthetic shikonin derivatives

Several research groups have focused not only on shikonin itself, but also on its natural or synthetic derivatives in an endeavor to obtain compounds with improved pharmacological features in terms of their target specificity, reduced toxicity toward normal tissues, better water solubility, and so on (Fig. 1).

Molecular dynamics calculations and structure-based drug design have been performed to investigate acetylshikonin and propionylshikonin as possible inhibitors of the bromodomain of CREB (cAMP response element-binding protein)-binding protein (CREBBP), which is a protein involved in the modification of histones and that is responsible for the reorganization of lysine residues in acetylated histones. According to quantum mechanical molecular dynamics energy calculations, propionylshikonin showed stronger binding and stability than acetylshikonin. The interactions between propionylshikonin and

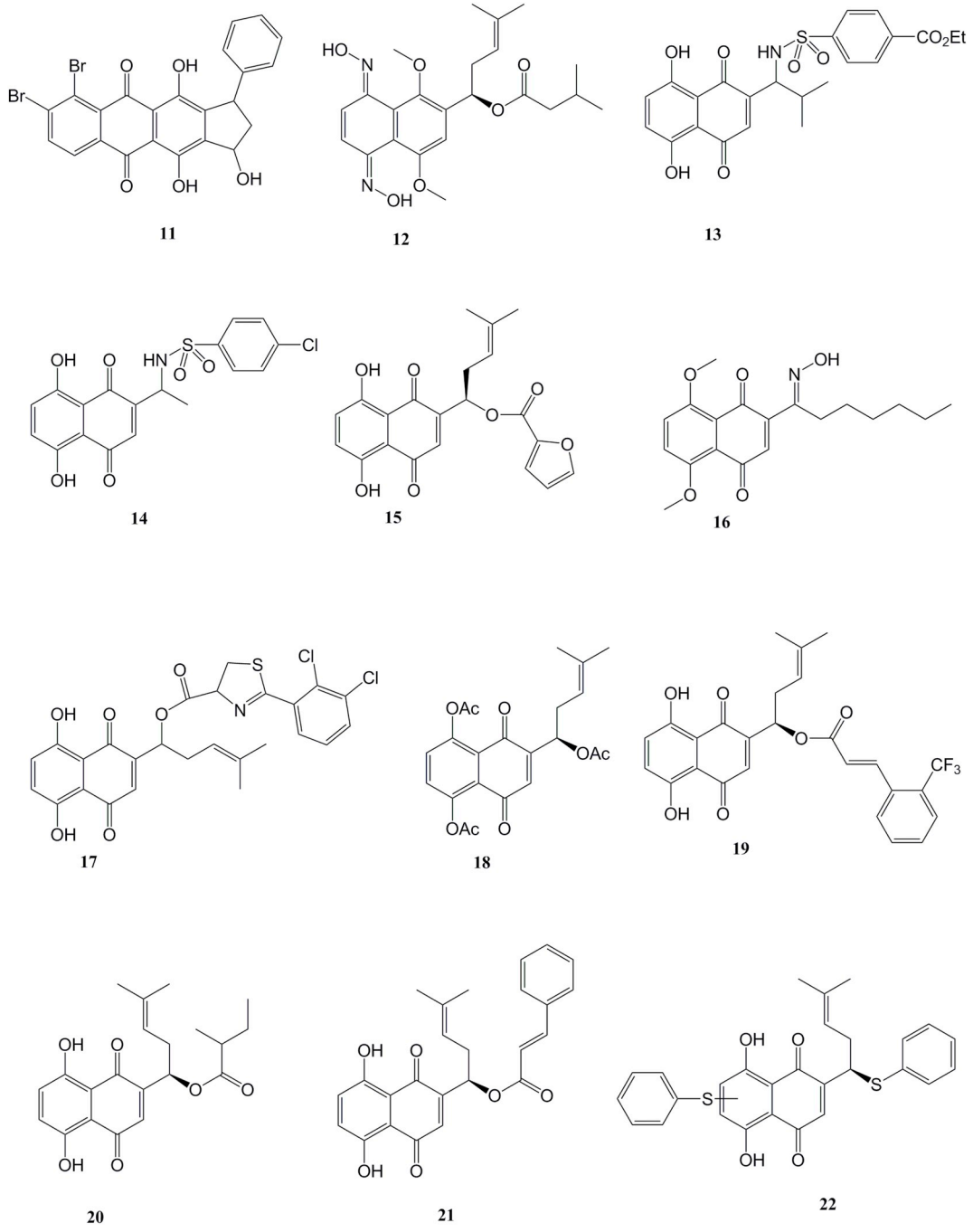

Fig. 3. (*continued*)

the CREBBP bromodomain were also considered to help design more potent and selective CREBBP bromodomain inhibitors [33].

Acetylshikonin (**2**), β,β-dimethylacryl shikonin (**1**), and several other shikonin derivatives from the root extracts of *Onosma paniculata* were used to treat metastasizing medullary thyroid carcinoma. Shikonin was the most effective compound to treat this cancer, as it significantly reduced tumor growth by inducing apoptosis and inhibiting cell proliferation [34].

Because the clinical introduction of shikonin as an anticancer agent is still limited by its strong toxicity and poor solubility, the concept of twin drugs has been applied to shikonin. For example, α-lipoic acid, which is a cofactor of pyruvate dehydrogenase (PD), was coupled to shikonin administration. Eighteen ester derivatives of α-lipoic acid-shikonin hybrids were designed and tested against various cancer cells lines. Among these, only one compound showed considerable cytotoxicity toward cervical cancer cells (HeLa), with considerable inhibitory activity against pyruvate dehydrogenase kinase 1 (PDK1). This derivative increased the aerobic metabolism in these cells, leading to inhibition of tubulin polymerization, G2/M cell cycle arrest, and ultimately apoptosis [35].

A series of 2,3-dithiocarbamate-substituted naphthoquinones were tested for their inhibitory activity toward the M2 isoform of pyruvate kinase (PKM2). Two derivatives were found to have better PKM2 inhibition activity than shikonin itself. Most of the compounds had half maximal inhibitory concentration (IC_{50}) values in the nanomolar range against B16, MCF7, H1299, HeLa, and HCT116 cells [36]. Shikonin coumarin carboxylic acid may be considered another promising compound because it induced apoptosis by the downregulation of hypoxia-inducible factor 1-alpha (HIF-1α) expression in HeLa cells [37].

Structural modifications of shikonin based on computational

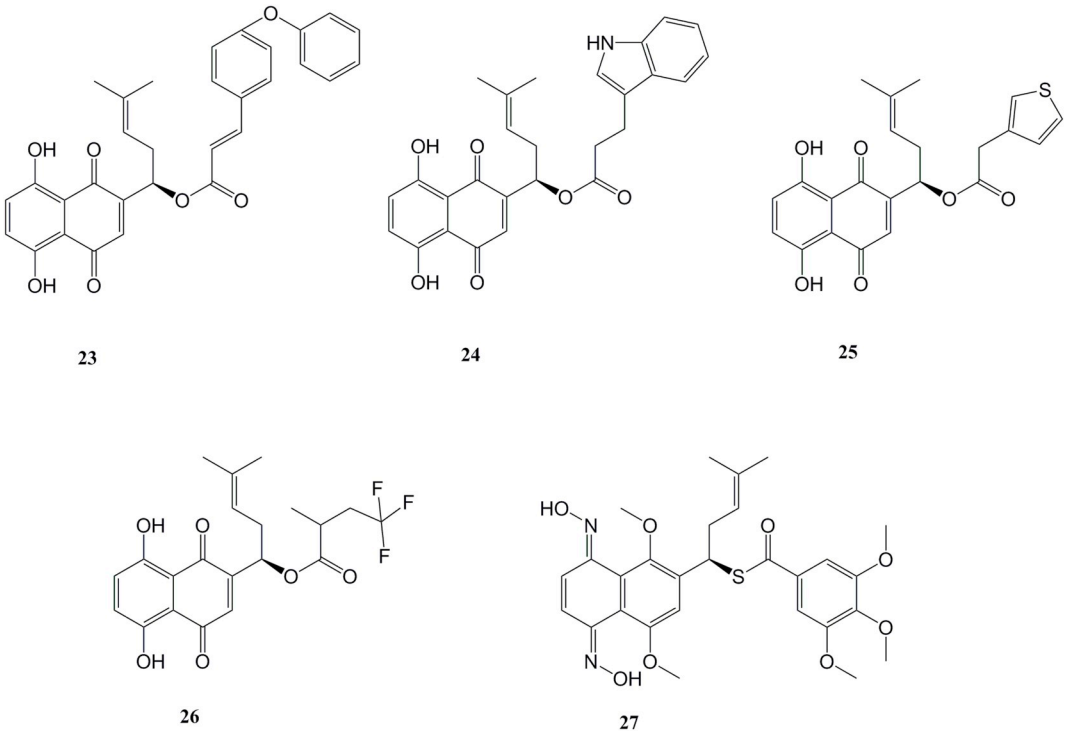

Fig. 3. (*continued*)

modeling led to the development of a new type of signal transducer and activator of transcription 3 (STAT3) inhibitor with inhibitory activity against a panel of breast cancer cell lines. This compound induced apoptosis and inhibited the nuclear translocation of STAT3 and the expression of genes downstream of STAT3. Interestingly, this effect was specific, as the compound was not observed to have activity against STAT1 and STAT5. Remarkably, this compound strongly suppressed the growth of MDA-MB-231 cells xenografted to nude mice. Another derivative also showed comparable activity and targeted the SH2 domain of STAT3 [38,39].

In human HeLa cervical cancer cells treated with one of the shikonin derivatives (β-HIVS), the expression levels of 70-kDa ribosomal protein S6 kinase, AKT, mTOR, and PI3K were downregulated and the cell cycle was arrested in the S phase. Moreover, the PI3K/AKT/mTOR signaling pathway was inhibited, leading to the induction of apoptosis [40].

The microarray-based investigation of a panel of natural or semi-synthetic derivatives of Japanese herbal medicines (Kampo) revealed that shikonin had the highest cytotoxic activity therein. The connection of specific microarray-based expression profiles in tumor cells to the responsiveness of these tumor cells to specific drugs or cytotoxic phytochemicals makes it possible to predict the responses of such cells to cytotoxic compounds in future approaches to personalized medicine [41].

The activity of newly synthesized naphthalene and naphthoquinone derivatives was tested against various human cancer cell lines in a previous study. The extent of their cytotoxic activities were significantly correlated with the inhibition of topoisomerase I by them and with the redox properties of the naphthazarin structure [42].

In oral squamous cell carcinoma cells treated with acetylshikonin, the phosphorylation of c-jun-N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (p38 MAPK), G2/M cell cycle arrest, and induction ROS-mediated apoptosis were observed. Importantly, these effects seemed to be tumor-specific to some extent, since the effects of this compound on normal HaCaT keratinocytes were only minimal [43]. Similarly, a novel tetracyclic 4b/4b′ anthraquinone was revealed to have potent cytotoxicity toward breast, cervical, and pancreatic cancer cell lines without having effects on the normal MCF-10 mammary epithelial cells used as a control, reflecting this compound's tumor selectivity [44].

A novel oxime shikonin derivative, DMAKO-05, inhibited murine B16F10 melanoma cells by inducing G1 arrest and AKT (protein kinase B) activation and triggering apoptosis through caspase-9/3 activation and poly ADP ribose polymerase (PARP) cleavage [45]. Various shikonin derivatives were isolated from the root extracts of *Onosma paniculata* and tested against MRC-5 lung fibroblasts and 8 other cancer cell lines. Among these, dimethylacrylshikonin exhibited the strongest cytotoxicity toward four melanoma cell lines (WM164, WM35, WM9, and SBcl2). Moreover, it induced apoptotic cell death by interfering with cell cycle progression, activating caspase-3/7, and inducing apoptosis [46].

Hydroxyanthraquinone, hydroxynaphthoquinone, and naphthoquinone derivatives were isolated from the roots of *Rheum palmatum, L. erythrorhizon*, and *Macrotomia euchroma*, respectively. Anthraquinone derivatives that had OH, CH_2OH, and COOH substitutions exhibited potent growth inhibitory activities against both multidrug-resistant P-glycoprotein-expressing and sensitive P-glycoprotein-sensitive cancer cells. Furthermore, all hydroxynaphthoquinone derivatives investigated showed very high growth inhibitory activities against both types of cancer cells [47]. Other novel analogues of shikonin with diverse side-chain variations showed cytotoxic activities toward several cancer cell lines. Under hypoxia (which is also a determinant of drug resistance), some of these analogues downregulated the expression of HIF-1α [48]. The novel naphthoquinone SH-7 inhibited DNA topoisomerases I and II more strongly than shikonin itself. Furthermore, this compound also increased the expression of phosphorylated γ-H2AX. Moreover, SH-7 displayed cytotoxicity toward three multidrug-resistant cell lines, with average resistance factors much lower than even those of reference drugs. This phenomenon of hypersensitivity was termed collateral sensitivity [2]. The anticancer effect of SH-7 was also shown *in vivo* in xenografts of PC-3 prostate cancer, BEL-7402 hepatocellular carcinoma, SMMC-7721, and syngeneic S-180 sarcoma tumor models implanted into mice. Considering its favorable effects against DNA topoisomerase II and multidrug-resistant tumors, SH-7 may be a promising antitumor drug candidate for further investigation [49]. Several naturally

occurring shikonin derivatives bypassed the drug resistance mediated by P-gp, BCRP1, Bcl-2, or Bcl-xL by inducing necroptosis [50].

Among the various derivatives of chemicals from *L. erythrorhizon* roots, 2-hyim-DMNQ-S33 revealed potent anticancer activity by inhibiting protein kinase C (PKC)-α and JNK expression and the phosphorylation of extracellular signal-regulated kinases (ERK) [51].

A synthetic aryl dihydrothiazol acyl shikonin ester derivative demonstrated better anti-proliferative activity than shikonin itself toward HeLa cervical carcinoma cells. Furthermore, it caused G2/M cell cycle arrest and induced apoptosis. Interestingly, it also caused disruption of tubulin polymerization, as shown by confocal microscopy, and binded to the paclitaxel-binding site in tubulin, as shown by molecular docking analyses [52].

Erlotinib is a small molecule thymidine kinase inhibitor that competes for the ATP-binding site in the thyrosine kinase domain of epidermal growth factor receptor (EGFR), leading to the inhibition of receptor phosphorylation and downstream signals. Due to the limited efficacy of erlotinib in glioblastoma therapy, shikonin and 14 shikonin derivatives were investigated in wild-type U87.MG and transfected U87.MGΔEGFR glioblastoma cells. Shikonin and its five derivatives showed synergistic cytotoxicity in combination with erlotinib toward U87MG.ΔEGFR cells. This result was confirmed using three other EGFR-expressing cell lines (DK-MG, A431, and BS153) [53].

The acetylshikonin derivative SK07 specifically activated the orphan nuclear receptor Nur77, which was then translocated from the nucleus to the mitochondria, bound to Bcl-2, and induced apoptosis. SK07 increased Nur77 protein expression by a posttranscriptional mechanism. In Nur77-knockout cells, the effect of SK07 was impaired and suppressed by leptomycin B, which is an inhibitor of the cytoplasmic localization of Nur77. Furthermore, apoptosis induction was accompanied by Bax activation [54].

Acetylshikonin was used to treat cells stably expressing hepatitis B virus X protein (HBX). As prerequisites for acetylshikonin-mediated apoptosis, it upregulated Nur77 expression and cytoplasmic export, as well as JNK activation. ROS-mediated endoplasmic reticulum stress and apoptosis were suppressed by the ROS scavenger N-acetylcysteine, which was associated with reduced Bip protein expression and ubiquitination. Conversely, salubrinal, a selective endoplasmic reticulum stress inhibitor, reactivated ROS generation and JNK expression and upregulated Nur77 expression and cytoplasmic translocation, leading to endoplasmic reticulum stress and apoptosis in HBX-expressing hepatocellular carcinoma cells [55].

A cinnamic acyl shikonin derivative was previously shown to be cytotoxic toward human SW872-s, A549, and A875 cell lines. It induced apoptosis by cleavage of caspase-3, caspase-7, caspase-9, and PARP [56]. In addition, 2-methyl-n-butyl shikonin induced apoptosis and inhibited cellular proliferation in human SGC-7901 gastric cancer cells. Different pathways were involved in this inhibition, including the ERK1/2 and JNK signaling pathways and mitochondrial apoptosis [57].

The shikonin analogue 93/637 is an analogue of β,β-dimethyl acryloyl shikonin derived from the roots of *Arnebia nobilis*. It was investigated for its effects on insulin-like growth factors in LNCaP, DU 145, and PC-3 prostate cancer cells. The compound strongly inhibited PC-3 cells in a dose- and time-dependent manner, but only slightly inhibited the other two cell lines. The mRNA expression of vascular endothelial growth factor (*VEGF*), insulin-like growth factor 2 (*ILGF2*), and insulin-like growth factor binding protein 3 (*IGFBP3*) was inhibited by this compound in PC-3 cells [58].

Pigment-LIII is a naphthoquinone extract taken from *Arnebia euchroma*, which was found to inhibit the proliferation of esophageal and stomach cancer cell lines. This effect was attributed to its roles in regulating RNA biosynthesis and changing the ultrastructure of cancer cells [59].

The shikonin derivative SYUNZ-7 was investigated against a panel of human cancer cell lines, including GLC-82 lung adenocarcinoma, CNE2 nasopharyngeal cancer, KB oral cavity cancer, human MGC-803 gastric cancer, and human HepG2 hepatocellular cancer cell lines. Its antitumor effect was validated *in vivo* using syngeneic Ehrlich ascites carcinoma (EAC) and CNE2 xenograft tumors. SYUNZ-7 not only arrested the transition of CNE2 cells from S to G2/M phase, but also inhibited angiogenesis by them in nude mice [37].

The investigation of two series of novel core-scaffold-modified alkannin and shikonin derivatives differing in their configurational and positional isomerism with high enantiomeric excesses showed that their dimethylated diacetyl derivatives had considerable antitumor activities without toxicity to normal tissues *in vivo*. Their low alkylating and ROS generation capacities supported the hypothesis that they may act as prodrugs. Their significant selectivity between tumor cells and normal tissues indicates that they represent promising candidates for drug development [60].

The new shikoninphenoxyacetic acid derivative **23** inhibited microtubule function, induced apoptosis, and inhibited cell growth in a concentration- and time-dependent manner. Confocal microscopy and molecular docking analyses revealed that the phenoxy moiety of compound **23** interacted with the hydrogen bond at the vinblastine-binding site of tubulin. Furthermore, it altered the architecture of microtubules and decreased their density. Compound **16** also arrested HepG2 cells in the G2/M cell cycle phase [61].

In another investigation, compounds **24** and **25** demonstrated good anti-proliferative activity against A875 and HeLa cancer cell lines. When compared to shikonin and colchicine, compounds **3** and **8** had better tubulin-inhibitory activities. Compound **3** in particular can be considered a promising drug candidate due to its low toxicity to healthy tissues [62].

A series of derivatives selectively acylated at the side-chain of the shikonin scaffold were designed and synthesized. Among these, compound **26** exhibited the most potent anticancer activity toward malignant B16–F10 melanoma, MG63 osteosarcoma, and A549 lung cancer cells. Molecular docking analysis results suggested that this compound could be a tubulin inhibitor [63].

The inhibitory effects of β,β-dimethylacryl shikonin (**1**) were investigated in the human colorectal cancer cell line HCT-116 *in vitro* and *in vivo*. This shikonin derivative inhibited tumor cell growth in a dose- and time-dependent manner, blocked the G0/G1 cell cycle phase, and induced apoptosis by the induction of the pro-apoptotic proteins Bid and Bax and repression of the anti-apoptotic proteins Bcl-2 and Bcl-xl. Nude mice treated with **1** showed significantly retarded growth of xenografted tumors in them [64].

A previous study of 5, 8-O-dimethyl acylshikonin derivatives found that they exerted selectivity against MDA-MB-231 and MCF-7 cancer cells, without toxicity to normal cells. Most of these 5, 8-O-dimethyl acylshikonin derivatives were more active against cancer cells than shikonin itself. Due to the differences in activity among the different isomers tested, the 5, 8-dimethoxy-1,4-naphthoquinone side-chain was suggested to be associated with these derivatives' cytotoxic activities against tumor cells [65].

Shikonin derivatives from *Arnebia euchroma* and *L. erythrorhizon* were tested to evaluate their immunomodulatory and antitumor effects in tumor-bearing mice. The median survival times of tumor-bearing mice were significantly increased by treatment with these shikonin derivatives. The growth of transplantable neoplasms was inhibited, and the number of $CD3^+$ and $CD19^+$ cells increased, as a result of these treatments. The shikonin derivatives increased natural killer (NK) cell activity and the transformation and production of interleukin-2. Hence, the immune systems of tumor-bearing mice were strengthened and their tumor loads were reduced by these derivatives [66].

A shikonin oxime derivative containing 5 sulfurs was evaluated for its cytotoxicity against a panel of human tumor cell lines (HCT-15, MGC-803, Bel7402, and MCF-7) and normal human HSF skin fibroblasts. This compound demonstrated slight toxicity toward normal HSF cells, but considerable cytotoxicity toward all the tested types of cancer cells. A structure-activity relationship study showed that this activity

was associated with the substituent group present in the side-chain of the molecule. Moreover, it induced G2/M cell cycle arrest and apoptosis in HCT-115 cells. This apoptotic response was correlated with the up-regulation of Bax, caspase 3, and caspase 9, and the downregulation of Bcl-2 [67].

4. Nanotechnological preparations of shikonin

Despite the impressive anticancer activity of shikonin and its derivatives *in vitro* and *in vivo*, further improvements in their solubility in aqueous solutions and tumor-specific accumulation are needed before they can be applied clinically. The incorporation of shikonin into thermosensitive nano-micelles was shown to enhance its cytotoxicity against breast cancer cells. These findings suggest that shikonin-loaded thermosensitive nano-micelles may be a promising formulation for clinical use [68].

Polyethylene glycolated (PEGylated) liposomes were also used as carriers of shikonin. Not only could the compound be successfully incorporated into liposomes, but its physicochemical characteristics, physical stability, entrapment efficacy, and release *in vitro* were also satisfactory. These results are promising, since shikonin-loaded PEGylated liposomes are more beneficial compared to the other common strategies used to incorporate such drugs [69].

The low solubility of shikonin in water and its low bioavailability also limit its clinical application. For this reason, RGD-modified shikonin-loaded liposomes (RGD-SSLs-SHK) were designed to improve the physical and chemical characteristics of shikonin. The use of RGD-SSLs-SHK resulted in higher apoptotic rates in $\alpha v\beta_3$-positive MDA-MB-231 breast cancer cells compared to shikonin alone by increasing the expression of the pro-apoptotic Bax protein and decreasing the expression of the anti-apoptotic Bcl2 protein. This treatment also inhibited metastasis by decreasing the expression of MMP-9 and NF-κB p65 without affecting MMP-2 expression. Consequently, the use of RGD-modified liposomes to deliver shikonin represents a potent strategy for therapy targeted against breast cancer [70].

An osteosarcoma is a malignancy that manifests in the bones, which frequently metastasizes into the lungs. Osteosarcomas are dangerous pediatric tumors, and effective delivery techniques for their treatment with shikonin are required. Shikonin combined with a sarcoma-targeting peptide-decorated disulfide cross-linked polypeptide nano-gel (STP-NG) enhanced the cytotoxicity of the treatment against cancer cells compared to that of shikonin alone and inhibited osteosarcoma by inducing necroptosis. However, a disadvantage of necroptosis induction therapies are their high toxicity to healthy tissues. Nonetheless, intravenously injected STP-NG/shikonin inhibited tumor growth *in vivo* and diminished lung cancer metastasis by enhancing necroptosis via the upregulation of receptor-interacting protein 1 (RIP1) and 3 (RIP3), but its cytotoxicity against normal cells was very low [71].

Shikonin was highly cytotoxic toward ID8 and OVCAR-5 ovarian epithelial carcinoma cells and IOSE-398 ovarian normal cells, but also toward MS1 endothelial cells and normal lymphocytes. To improve its tumor specificity, shikonin was engineered into polymeric biodegradable nanoparticles (NPs) consisting of poly(lactic-co-glycolic acid) (PLGA) loaded with shikonin that specifically targeted only the microvasculature of cancerous growths. Polyethylene glycol (PEG) and tumor endothelial marker 1 (TEM1) and endosialin-targeting antibodies were added to the surfaces of these NPs. Flow cytometry and fluorescence microscopy results revealed that antibody-armed NPs actively interacted with TEM1-positive MS1 cells, without having any effects on TEM1-negative MS1 cells. PEGylated NPs did not exhibit any cytotoxicity against lymphocytes that were exposed to PEGylated NPs for 2 h. The long exposure time of TEM1-positive MS1 cells and OVCAR-5 cells to antibody-armed and PEGylated NPs showed the greatest cytotoxicity of the latter. These findings suggest that SHK-loaded antibody-armed PEGylated PLGA NPs are promising nanomedicinal preparations to use to specifically target solid tumors [72].

To improve the cytotoxicity of shikonin, it was integrated into solid lipid nanoparticles (SLNs), which were stable for up to three months in storage. The cytotoxicity and anti-proliferative effects of this shikonin preparation *in vitro* were considerable better compared to those of free shikonin [73].

5. From traditional medicine to modern biotechnology

Traditional Chinese medicine used the root extract of *L. erythrorhizon* to treat dermatitis, carbuncles, burns, macular eruptions, sore throats, measles, and external wounds [5,43]. In particular, shikonin, the major chemical constituent of this medicinal plant, has

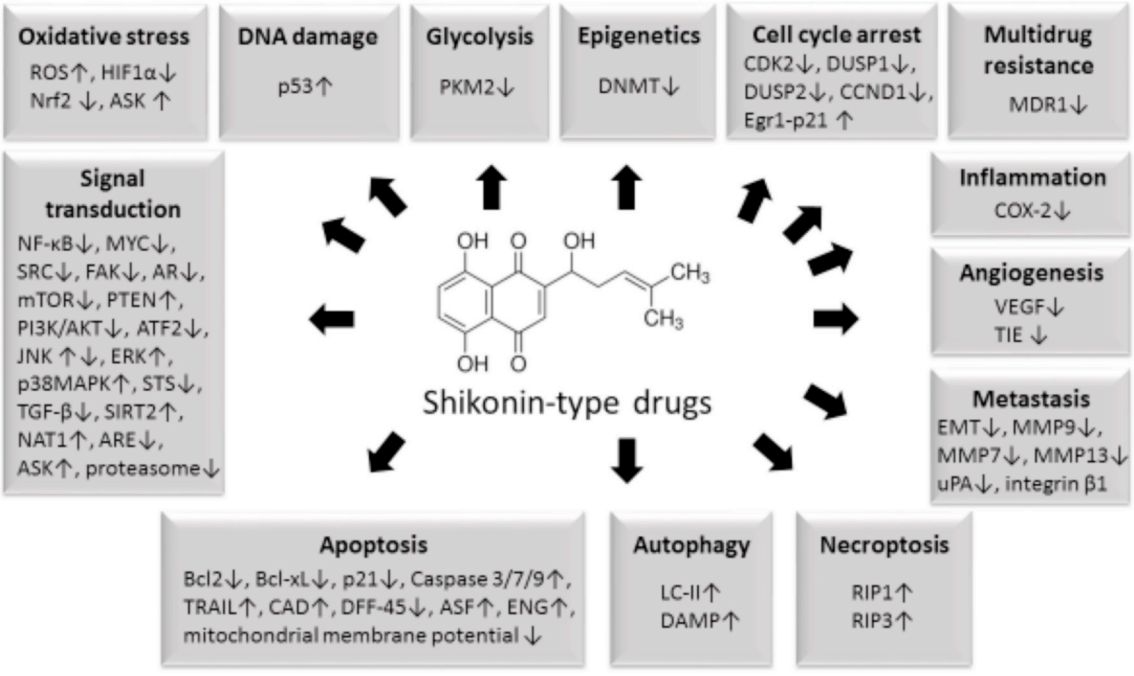

Fig. 4. Synopsis of the multi-factorial mode of action of shikonin-type drugs.

various pharmacological properties, such as anti-inflammatory, antioxidant, antimicrobial, anti-ulcer and anti-thrombotic, anti-gonadotropic, and anti-HIV1 activities [4,5]. Moreover, shikonin is used as an ointment for cuts, burns, and hemorrhoids. In European countries, shikonin and alkannin were mainly used as food pigments [74].

Considerable interest has been directed at synthetic and natural naphthoquinones owing to their profound anticancer activities. Among the most potent and intensively explored compounds with anticancer activity *in vitro* and *in vivo* were shikonin and its ramentaceone, plumbagin, from the rhinacanthin group. Thus, the production of these compounds in hairy root cultures for therapeutic purposes is a straightforward process [75].

The biosynthesis of shikonin is catalyzed by GPP:4-HBA 3-geranyltransferase, and the deregulation of this enzyme influences shikonin production because it is a key enzyme involved in shikonin biosynthesis in *L. erythrorhizon* cell cultures [76]. The introduction of the *ubiA* gene of *Escherichia coli* into *L. erythrorhizon* led to the identification of the biosynthetic pathway of shikonin in this plant. The *ubiA* gene encodes the enzyme 4-HBA 3-polyprenyltransferase, which catalyzes an important step in ubiquinone biosynthesis. If GPP serves as a substrate, it also catalyzes the formation of GBA, which is a key step in shikonin biosynthesis specifically. Vector constructs with various promoters were introduced into *L. erythrorhizon*, yielding hairy root lines with high enzymatic UbiA activity and 50-fold higher GBA accumulation

Table 1
Induction of apoptosis by shikonin in cancer cell lines *in vitro*.

Cell line	Cell type	Mode of action	References
Cell cycle:			
SMMC-7721	Hepatoma	Induction of apoptosis by inhibition of cell cycle progression, disruption of Ca^{2+}homeostasis, induction of oxidative stress, and mitochondrial dysfunction	[152]
MCF-7, SK-BR-3, MDA-MB-231	Breast cancer	Induction of cell cycle arrest and apoptosis by stimulating DUSP1 and DUSP2	[153]
M10	Mammary epithelial cells	expression that turns off JNK and p38 MAPK pathways	
EC109, EC9706	Esophageal cancer Esophageal epithelial cells	Induction of cell cycle arrest and apoptosis via the regulation of HIF1α/PKM2 signaling pathway	[154]
HCT116, SW620	Colon cancer	Cell cycle arrest by inhibiting HIF-1α signaling	[155]
A549	Lung adenocarcinoma	Inhibition of cell growth an cell cycle by regulating CCND1	[156]
AGS	Gastric cancer	Induction of cell cycle arrest through Egr1-p21 signaling pathway	[157]
8505C8305C, FTC133, BCPAP, TPC1C643IHH4, K	Thyroid cancer	Induction of cell cycle arrest	[158]
Htori	Immortalized thyroid epithelial cells		
Glycolysis:			
C6	Rat glioma	Activation of RIP1 and RIP3 leads to the suppression of glycolysis by increasing the levels of intracellular H_2O_2	[159]
SHG-44, U87, U251	Human glioma		
A2780	Cisplatin-sensitive and -resistant ovarian carcinoma	Attenuation of the epithelial-mesenchymal transition	[160]
SKOV3	Paclitaxel-sensitive and -resistant ovarian carcinoma		
MCF-7	Breast cancer	Inhibition of glycolytic enzymes	[95]
Huh7	Hepatoma	Growth inhibition by modifying cellular metabolism	[161]
JB6 Cl-41 (P+)	Murine skin epidermal cells	Suppression of the tumor promoter 12-O-tetradecanoylphorbol 13-acetate and activation of PKM2	[162]
MCF-7, MCF-7/Adr, MCF-7/Bcl-2, MCF-7/Bcl-xL, MCF-7/Neo	Breast cancer	Inhibition of glycolysis by inhibition of tumor-specific PKM2	[163]
A549	Lung cancer		
HeLa	Cervical cancer		
Metastasis:			
23, A549, HCC827	Non-small cell lung cancer	Inhibition of migration and invasion by inhibition of the epithelial-mesenchymal transition	[164]
U2OS	Osteosarcoma	Inhibition of invasion by decreasing MMP-13 levels and increasing TIPE2 levels	[165]
SKOV-3	Ovarian cancer	Inhibition of cell migration by inhibiting SRC and FAK	[166]
PC-3, DU145	Prostate cancer	ROS/ERK1/2- and AKT/mTOR-mediated inhibition of cell invasion by decreasing MMP-2/-9 expression	[61]
MCF-7, MDA-MB231	Breast cancer	Inhibition of invasion by inhibition of MMP-9 promoter activity and expression	[167]
	Breast cancer	Human class π-GST improved proliferation and migration by increased detoxification capacity	[168]
8505C8305C, FTC133, BCPAP, TPC1C643IHH4, K	Thyroid cancer	Inhibition of cell migration and invasion	[158]
Htori	Immortalized thyroid epithelial cells		
A549	Non-small cell lung cancer	Inhibition of invasion and metastasis by suppression of integrin β1 expression and ERK1/2 signaling	[169]
ACC-2, ACC-M	Adenoid cystic carcinoma	Inhibition of invasion by downregulating MMP-9 expression	[93]
DNA damage:			
A375-S2	Human malignant melanoma	Induction of apoptosis by inducing DNA damage and activating p53 and caspase 9	[170]
Inflammation:			
SW1353, 184B5/HER	Chondrosarcoma	Inhibition of COX-2	[171]
Angiogenesis:			
	Umbilical vein endothelial cells	Induction of angiogenesis by inhibiting VEGF and downregulation of uPA expression	[172]
Epigenetics:			
TPC-1	Papillary thyroid cancer	Suppression of DNMT1, demethylation of the *PTEN* gene and increasing	[173]
HTori-3	Normal thyroid cells	expression of PTEN protein	

compared to that in control cultures. However, the overall shikonin production was lower than in control cultures. Hence, *ubiA* requires the addition of further supplementary enzymes to increase shikonin production [77].

UbiC is another gene of *E. coli* that has been introduced into *L. erythrorhizon* hairy root cultures to modify the biosynthetic pathway of 4-HBA. *UbiC* encodes the enzyme chorismate pyruvate-lyase (CPL), which is normally not found in plant cells. This enzyme converts chorismate into 4-HBA. *UbiC*-containing vectors were introduced into *L. erythrorhizon,* which increased the CPL activity and 4-HBA production in hairy root cultures of this plant. However, *UbiC* transformation did not significantly increase shikonin production. Instead, it increased the accumulation of menisdaurin, which is involved in the metabolism of aromatic amino acids [78].

6. Molecular modes of action of shikonin in cancer cells

There is overwhelming evidence that the mode of action of shikonin-type drugs is multifactorial in nature (Fig. 4). Most, if not all, drugs from natural sources exert cytotoxic activity not by singular but by multiple mechanisms. During the evolution of life on Earth, multi-specific biomolecules seem to have been superior to mono-specific ones. Herbivores and microbial invaders may have developed resistance mechanisms more easily to mono-specific than to multi-specific secondary plant metabolites. Therefore, phytochemicals with multiple modes of action may have prevailed in the evolution of life [79,80]. It thus does not come as a surprise that shikonin-inhibited cancer proliferation also occurs by multiple mechanisms [3], for example by the induction of cell death (apoptosis, autophagy, necroptosis) and inhibition of glycolysis, carcinogenesis, and metastasis. The multi-specific mode of action of shikonin might help to avoid or at least delay the development of resistance of cancer cells against this compound.

6.1. Cell death modes

Apoptosis is achieved by the activation of caspases, and is characterized by the swelling of cell membranes, cell shrinkage, nuclear fragmentation, chromatin condensation, and DNA fragmentation. Shikonin induced apoptosis in cancer cells in a dose-dependent manner by increasing the intracellular levels of ROS, which are mainly generated by mitochondrial complex II, lipoxygenase, and NADPH oxidase [81]. In particular, low shikonin concentrations induced apoptosis, which was associated with the upregulated expression of apoptotic and cell cycle signaling regulatory proteins. Table 1 shows the various molecular mechanisms of shikonin-induced cell death.

In addition to apoptosis, autophagy represents another form of cell death, and has been termed “type II programmed cell death” [82]. Autophagy is a catabolic process in which cytoplasmic material is transported by double-membranous autophagosomes to lysosomes, where it is degraded. Autophagy is a mechanism for cellular survival under stressful conditions. However, if autophagy is over-activated, it leads to cell death [83–85].

Short-term treatment of human hepatocellular cancer cells with low shikonin concentrations promoted autophagy, as was demonstrated by the production of acidic vesicular organelles (AVOs), upregulation of microtubule-associated protein 1A/1B-light chain 3 (LC3)-II, and a punctuated fluorescence pattern of the GFP-LC3 protein during treatment [86]. Shikonin stimulated autophagy in pancreatic cancer cells, with the PI3K/AKT signaling pathway as the underlying mechanism behind this change [87]. Enhanced autophagic activity is frequently accompanied by necroptosis. The occurrence of shikonin-induced autophagy and receptor-interacting protein kinase 1 (RIPK1)- and 3 (RIPK3)-dependent necroptosis were highly correlated. Shikonin-induced autophagy also contributed to damage-associated molecular pattern (DAMP) upregulation [88].

In most published reports, shikonin induced apoptotic cell death (Table 1). However, at high concentrations shikonin induced necroptosis in various cancer cell lines. Necroptosis or “programmed necrosis” happens in a caspase-independent manner. It leads to the loss of plasma membrane integrity, swelling of organelles, and cell lysis. Numerous studies showed that necroptosis is triggered by members of the tumor necrosis factor (TNF) family. Necroptosis induces cell death mainly via RIPK1 and RIPK3. Stimulating necroptosis represents an approach to overcome apoptosis resistance of cancer cells [89].

Table 2
Induction of necroptosis by shikonin in cancer cell lines *in vitro*.

Cell line	Cell type	Mode of action	References
Necroptosis:			
MCF-7	Breast cancer	Induction of necroptosis and apoptosis by RIPK1-RIPK3	[174]
CNE-2Z	Nasopharyngeal carcinoma	ROS-mediated induction of necroptosis	[175]
C6	Rat glioma	ROS-mediated regulation of RIP1 and RIP3 expression, necrosome assembly and induction of necroptosis	[176]
SHG-44, U87, U251	Human glioma		
AsPC-1, SW1990, PANC-1, Capan-2	Pancreatic cancer	Induction of apoptosis and necroptosis by regulating the expression of RIP1 and RIP3.	[177]
A549	Non-small cell lung cancer	Induction of necroptosis and autophagy	[178]
KMS-12-PE, KMS-12-BM, RPMI-8226, KMM1,U266, KMS11	Myeloma	Induction of apoptosis at low shikonin doses. Induction of necroptosis at high concentrations	[134]
KMS11/BTZ	Bortezomib-resistant myeloma cells		
AGS, AZ521, SCM-1	Gastric cancer	Induction of apoptosis or necrosis by ROS generation	[179]
C6	Rat glioma	Oxidative-stress-mediated activation of RIP1 and induction of necroptosis	[180]
U87	Human glioma		
U937	Lymphoma	Induction of apoptosis and necroptosis	[133]
K7, K12, K7M3, U2OS, 143B	Osteosarcoma	Induction of necroptosis by RIP1 and RIP3	[181]
HL60, K562	Leukemia	Reversion of necroptosis to apoptosis in the presence of Nec-1	[182]
HL60/Adr, K562/Adr	Multidrug- resistant leukemia		
MCF-7	Breast cancer	Induction of necroptosis	[90]
HEK293	Embryonic kidney cells		
HeLa	Cervical cancer		
HL60	Promyelocytic leukemia		
Autophagy:			
A549	Non-small cell lung cancer	Induction of necroptosis and autophagy	[178]
Ferroxitosis:			
SK23, MEL501, MEL526, MEL624BJ, IMR90, MEL103, MEL187, RPMI 8322, VMM39, WM2664	Melanoma	Induction of ferroxitosis under hypoxic conditions	[183]

Shikonin bypassed the drug resistance mediated by Bcl-2 and Bcl-xL in this manner.

Furthermore, shikonin was also previously shown to circumvent the multidrug resistance (MDR) mediated by P-glycoprotein. The shikonin analogues α-methyl-n-butylshikonin, β,β-dimethylacrylshikonin, isovalerylshikonin, deoxyshikonin, isobutyrylshikonin, and acetylshikonin bypassed drug resistance mediated by the multidrug resistance-conferring ABC-transporters, MRP1 and BCRP1, via necroptosis [50]. Shikonin triggered necroptotic cell death in Bcl-2- or Bcl-xL-overexpressing breast cancer cells that were resistant to apoptosis-inducing drugs. It also stimulated necroptosis in breast cancer cells characterized by the overexpression of P-glycoprotein and elevated resistance to several anticancer drugs, such as *Vinca* alkaloids, taxanes, and anthracyclines [90]. Table 2 summarizes the findings of different studies on shikonin's function as a necroptosis inducer.

6.2. Signal transduction pathways

Another important aspect of shikonin is its ability to inhibit signal transduction pathways that regulate gene expression, cell cycle progression, glycolysis, metastasis, DNA damage repair, angiogenesis, inflammation, and epigenetics (Table 3).

Several of these signaling routes are regulated by TNF, transcription factors (*e.g.*, NF-κB), and protein kinases (*e.g.,* JNK, AKT, PI3K, p38MAPK, and ERK) [91]. The transcription factors NF-κB, MYC, and mTOR represent central players in the development of cancer. Their inhibition by shikonin may be a promising sign of this drug's likely utility in future treatment strategies [92].

6.3. Other mechanisms

Cell cycle arrest is a frequently observed mechanism by which cellular integrity is maintained after exposure to detrimental insults. Cell cycle arrest after exposure to shikonin has been associated with regulation by numerous proteins, such as DUSP1, DUSP2, HIF1α, CCND1, Egr1, p21, and others (Table 4).

Most cancer cells depend on aerobic glycolysis to produce the energy required for cellular processes. This phenomenon is known as the Warburg effect. Targeting glycolytic enzymes represents an attractive treatment approach in cancer therapy. Shikonin and its isomer alkannin inhibited PKM2, but did not affect the activities of PKM1 and pyruvate kinase-L, in previous studies [93,94]. Shikonin and other naphthoquinones decreased the rate of glycolysis in tumors by inhibiting the activities of pyruvate kinase and glycolytic enzymes [95,96]. PKM2 inhibition increased the chemosensitivity of bladder cancer cells to cisplatin and decreased tumor growth and progression by them [97]. Table 4 gives an overview of the mechanisms of glycolytic inhibition by shikonin.

The inhibition of metastasis represents an important treatment goal, since most cancer patients die not from their primary tumors, but from disseminated metastases. A central mechanism in metastasis is the epithelial-mesenchamyl transition (EMT), which is characterized by the loss of epithelial cell features by metastasizing cells, which then essentially become mesenchymal stem cells. Shikonin was shown to inhibit several major players in the metastatic process and the EMT, including MMP-2, -9, and -13, SRC, FAK, integrin β1, and so on (Table 4).

Other cancer-related proteins that are affected by shikonin include the tumor suppressor p53 that regulates DNA damage and apoptosis, COX-2, which is an activator of inflammation, the angiogenesis-regulating proteins VEGF and uPA, and DNMT1, which is a crucial component of epigenetic regulation (Table 4).

6.4. In vivo activity

A major problem with cytostatic phytochemicals is that their bioactivity has been abundantly shown in cell lines *in vitro*, but their anticancer activities *in vivo* (in living organisms) are much less well-known. As most natural products are efficiently metabolized in the liver and excreted from the body, demonstrating the activity of a compound *in vitro* is not sufficient to conclude its clinical utility, and convincing evidence of its activity *in vivo* is therefore indispensable.

In vivo experiments in xenografted, allografted, and orthotopic tumor models have revealed that shikonin initiates tumor cell-killing activity by multiple mechanisms, including by the stimulation of

Table 3
Effect of shikonin on signal transduction pathways.

Cell line	Cell type	Mode of action	References
Ishikawa, HEC-1A, KLE, RL95-2	Endometrial cancer cells	Induction of apoptosis by regulating miR-106b/PTEN/AKT/mTOR signaling	[184]
MCF-7, SK-BR-3, MDA-MB-231	Breast cancer	Induction of cell cycle arrest and apoptosis by stimulating DUSP1 and DUSP2 which shut down JNK and p38 MAPK	[153]
M10	Mammary epithelial cells		
	Gall bladder cancer	Induction of apoptosis by JNK signaling and cell cycle regulation	[185]
CCD19	Normal lung fibroblasts	ROS-mediated induction of apoptosis	[186]
HCC827, H1650, H197	Non-small cell lung cancer		
NB4	Promyelocytic leukemia	Induction of apoptosis by regulation of MAPKs and down-regulation of c-Myc	[187]
	Pulmonary fibroblasts	Inhibition of AKT signaling TGF-β expression induced extracellular matrix genes by regulating p38 MAPK and AKT	[188]
NCM460	Normal colon epithelial cells	Inhibition of *p*-ERK and up-regulation of SIRT2	[189]
HT29, HCT116	Well-differentiated colon carcinoma		
SW480	Poorly differentiated colon carcinoma		
MCF-7	Breast cancer	Inhibition of proliferation by reducing the level of miR-128 in exosomes	[190]
PC-3, DU-145	Prostate cancer	Increased shikonin sensitivity upon GRP78 knock-down	[191]
LNCaP, 22RV1	Prostate cancer	Transcriptional repression of androgen receptor (AR) and inhibition of its nuclear localization	[192]
HL-60	Promyelocytic leukemia	Nrf2/ARE pathway modulated intercellular redox homeostasis and cellular differentiation	[193]
MCF-7, T47D, MDA-MB-231	Breast cancer	Reversal of the inhibitory effect of estrogen-receptor signaling	[194]
MCF-7, SK-BR-3	Breast cancer	Down-regulation of STS expression	[195]
H22	Murine hepatoma	Inhibition of proteasome activity	[196]
P388	Murine leukemia		
PC-3	Human prostate cancer		
Tca-8113	Oral cancer	Induction of apoptosis by activation of caspases and inactivation of NF-κB	[197]
T24	Bladder cancer	Shikonin has an effect on NAT activity, *NAT1* mRNA gene expression, formation of AF-DNA adducts, and NAT Ag-Ab formation.	[198]
NCI–H522, DMS114	Lung cancer	Inhibition of protein tyrosine kinase	[199]

Table 4
Metabolic pathways targeted by shikonin.

Cell line	Cell type	Mode of action	References
Cell cycle:			
SMMC-7721	Hepatoma	Induction of apoptosis by inhibition of cell cycle progression, disruption of Ca^{2+}homeostasis, induction of oxidative stress, and mitochondrial dysfunction	[152]
MCF-7, SK-BR-3, MDA-MB-231	Breast cancer	Induction of cell cycle arrest and apoptosis by stimulating DUSP1 and DUSP2	[153]
M10	Mammary epithelial cells	expression that turns off JNK and p38 MAPK pathways	
EC109, EC9706	Esophageal cancer	Induction of cell cycle arrest and apoptosis via the regulation of HIF1α/PKM2	[154]
	Esophageal epithelial cells	signaling pathway	
HCT116, SW620	Colon cancer	Cell cycle arrest by inhibiting HIF-1α signaling	[155]
A549	Lung adenocarcinoma	Inhibition of cell growth an cell cycle by regulating CCND1	[156]
AGS	Gastric cancer	Induction of cell cycle arrest through Egr1-p21 signaling pathway	[157]
8505C8305C, FTC133, BCPAP, TPC1C643IHH4, K	Thyroid cancer	Induction of cell cycle arrest	[158]
Htori	Immortalized thyroid epithelial cells		
Glycolysis:			
C6	Rat glioma	Activation of RIP1 and RIP3 leads to the suppression of glycolysis by increasing	[159]
SHG-44, U87, U251	Human glioma	the levels of intracellular H_2O_2	
A2780	Cisplatin-sensitive and -resistant ovarian carcinoma	Attenuation of the epithelial-mesenchymal transition	[160]
SKOV3	Paclitaxel-sensitive and -resistant ovarian carcinoma		
MCF-7	Breast cancer	Inhibition of glycolytic enzymes	[95]
Huh7	Hepatoma	Growth inhibition by modifying cellular metabolism	[161]
JB6 Cl-41 (P+)	Murine skin epidermal cells	Suppression of the tumor promoter 12-O-tetradecanoylphorbol 13-acetate and activation of PKM2	[162]
MCF-7, MCF-7/Adr, MCF-7/Bcl-2, MCF-7/Bcl-xL, MCF-7/Neo	Breast cancer	Inhibition of glycolysis by inhibition of tumor-specific PKM2	[163]
A549	Lung cancer		
HeLa	Cervical cancer		
Metastasis:			
23, A549, HCC827	Non-small cell lung cancer	Inhibition of migration and invasion by inhibition of the epithelial-mesenchymal transition	[164]
U2OS	Osteosarcoma	Inhibition of invasion by decreasing MMP-13 levels and increasing TIPE2 levels	[165]
SKOV-3	Ovarian cancer	Inhibition of cell migration by inhibiting SRC and FAK	[166]
PC-3, DU145	Prostate cancer	ROS/ERK1/2- and AKT/mTOR-mediated inhibition of cell invasion by decreasing MMP-2/-9 expression	[61]
MCF-7, MDA-MB231	Breast cancer	Inhibition of invasion by inhibition of MMP-9 promoter activity and expression	[167]
	Breast cancer	Human class π-GST improved proliferation and migration by increased detoxification capacity	[168]
8505C8305C, FTC133, BCPAP, TPC1C643IHH4, K	Thyroid cancer	Inhibition of cell migration and invasion	[158]
Htori	Immortalized thyroid epithelial cells		
A549	Non-small cell lung cancer	Inhibition of invasion and metastasis by suppression of integrin β1 expression and ERK1/2 signaling	[169]
ACC-2, ACC-M	Adenoid cystic carcinoma	Inhibition of invasion by downregulating MMP-9 expression	[93]
DNA damage:			
A375-S2	Human malignant melanoma	Induction of apoptosis by inducing DNA damage and activating p53 and caspase 9	[170]
Inflammation:			
SW1353, 184B5/HER	Chondrosarcoma	Inhibition of COX-2	[171]
Angiogenesis:			
	Umbilical vein endothelial cells	Induction of angiogenesis by inhibiting VEGF and downregulation of uPA expression	[172]
Epigenetics:			
TPC-1	Papillary thyroid cancer	Suppression of DNMT1, demethylation of the *PTEN* gene and increasing	[173]
HTori-3	Normal thyroid cells	expression of PTEN protein	

apoptosis, autophagy, and necroptosis, and the inhibition of angiogenesis (Table 5).

7. Chemoprevention

Shikonin-type drugs have not only been considered for use in cancer therapy, but also as possible chemopreventive drugs owing to their considerable anti-proliferative activity *in vitro* and *in vivo* [96]. Shikonin acts as chemopreventive agent through its ability to suppress the disease conditions that favor carcinogenesis (*e.g.,* acute ulcerative colitis) (Table 5). An *in vivo* azoxymethane/dextran sulfate sodium model of colitis in mice showed that shikonin prevented the early phases of colorectal cancer development by inhibiting the pro-inflammatory environment produced during this disease [98]. Shikonin inhibited the formation of skin tumors in a chemically induced mouse skin carcinogenesis model. It also inhibited PKM2 and suppressed cell proliferation, but did not induce apoptosis. Microarray analysis revealed that shikonin suppressed the carcinogen-induced upregulation of activating transcription factor 2 (*ATF2*) and its downstream gene *CDK2* (cyclin-dependent kinase 4). Moreover, treatment of mouse skin with tumor-promoting agents increased nuclear ATF2 expression, but this was inhibited by shikonin. In ATF2-knockdown mutants, CDK2 expression was also decreased [99].

An important risk factor for the development of breast cancer is

Table 5
Anti-tumor effects of shikonin *in vivo*.

Tumor line	Tumor type	Mode of transplantation	Mode of action	References
Chemoprevention:				
Carcinogen induced colorectal cancer	Colorectal cancer	Carcinogen-induced	Prevention of ulcer formation by inhibition of pro-inflammatory factors	[98]
SW480	Colon cancer	Xenograft	Reduction of toxicity on non-neoplastic colon and liver tissues	[200]
Carcinogen-induced intestinal cancer	Intestinal carcinogenesis	Carcinogen-induced	Inhibition of intestinal neoplasms	[201]
Cell death:				
CNE-2Z	Nasopharyngeal carcinoma	Xenograft	Induction of necroptosis by ROS production	[175]
A549	Human non-small cell lung cancer	Xenograft and orthotopic	Induction of necroptosis and autophagy	[178]
Huh7	Human hepatocellular carcinoma	Xenograft	Induction of autophagy by accumulation of ROS and *p*-ERK	[86]
Huh7	Human hepatocellular carcinoma	Xenograft	Induction of apoptosis by ROS/AKT and RIP1/NF-κB pathways	[202]
Other mechanisms:				
Namalwa	human Burkitt's lymphoma	Xenograft	Inhibition of tumor growth	[103]
SW480	Colorectal cancer	Xenograft	Inhibition of tumor growth	[189]
HCT116	Human colon cancer	Xenograft	Inhibition of tumor growth	[155]
	Gall bladder cancer	Xenograft	Inhibition of tumor growth	[185]
FTC133	Thyroid cancer	Xenograft	Inhibition of tumor growth	[158]
Isolated primary cells	Macrophages	Isolated cells	Inhibition of inflammation by proteasome inhibition	[203]
Lewis cells	Lung carcinoma	Orthotopic	Inhibition of angiogenesis by inhibition of (1) phosphorylation and expression of VEGF and TIE2; (2) endothelial cell growth; (3) vessel remodeling.	[204]
H22	Murine hepatoma	Allografts	Inhibition of proteasome activity	[196]
PC-3	Human prostate cancer	Xenografts		
P388	Mouse leukemia			
Lewis cells	Lung carcinoma		Inhibition of tumor growth	[172]

estrogen, and a relevant defense mechanism against estrogen-associated carcinogenesis is the stimulation of detoxifying enzymes that eradicate toxic estrogens. The levels of detoxifying enzymes are regulated by the transcription factor Nrf2, which increases the expression of antioxidant enzymes. Therefore, one strategy of chemoprevention is to enhance Nrf2 function. Shikonin prevented breast cancer development by a dual mechanism of action. It inhibited estrogen signaling and induced Nrf2 expression, thereby inhibiting the transformation of normal breast epithelial cells to malignant states and preventing estrogen-dependent tumor development [100]. Tables 1–4 summarize different signaling pathways related to the antioxidative and anti-estrogenic effects of shikonin.

8. Modulation of drug resistance by shikonin

Many clinically established apoptosis-inducing anticancer drugs are substrates of drug efflux pumps of the ATP-binding cassette (ABC) transporter type. Both defects in the apoptotic pathways and the overexpression of ABC transporters lead to the development of drug resistance. As a result, tremendous efforts have been undertaken to overcome drug resistance by re-activating apoptotic pathways and inhibiting drug transporters.

As cell death is caused by several distinct molecular mechanisms (*e.g.,* autophagy, necroptosis, ferroptosis, mitoptosis, and others), one strategy that has been proposed to overcome apoptosis resistance is to activate other modes of cell death. Cancer cells developing resistance to apoptosis still showed sensitivity to the necroptotic pathway upon treatment with shikonin. Thus, the combination of drugs targeting different cell death pathways may be a promising approach to avoid drug resistance [101].

In vitro and *in vivo* studies showed that synergistic cytotoxicity resulted if shikonin was combined with an EGFR inhibitor, gefitinib, in wild-type EGFR non-small cell lung cancer. This effect was mainly due to the inhibition of PKM2, STAT3, and cyclinD1 [102].

The failure of chemotherapy in hepatocellular cancer is frequently due to MDR. Many factors lead to drug resistance, such as the increased efflux and decreased influx of anticancer agents by drug transporters, activation of detoxification systems, DNA repair, and blockage of apoptosis [103]. Even though drug resistance is multifactorial in nature, the efflux pump P-glycoprotein (P-gp) has been investigated in the greatest detail. P-gp is a member of the ABC superfamily of membrane transporter proteins. It is a 170 kD protein that is encoded by *ABCB1/MDR1*, the multidrug resistance 1 gene. Drug-resistant hepatocellular carcinoma cells (R-HepG2) overexpressed *MDR1*/P-gp compared to wild-type hepatocellular carcinoma cells (HepG2). Treatment of R-HepG2 with shikonin decreased cell viability, increased the apoptotic ratio, activated caspase 3, and reduced the expression of SIRT1 and *MDR1*/P-gp at both the mRNA and protein levels. These results suggested that shikonin bypassed drug resistance in these cases [104].

As drug resistance severely affects the final outcome of chemotherapy and the prognosis of the patient, novel drugs without vulnerability to MDR are urgently required. Shikonin not only destroyed tumors, but also evaded drug efflux and apoptotic failure, in previous research. A panel of cell lines was treated with shikonin for 18 months, and then their gene expression profiles and drug resistance were determined. These cells acquired only a two-fold increase in their resistance to shikonin. The fact that shikonin only induced resistance weakly makes it an interesting candidate for cancer therapy [105].

9. Synergistic interactions of shikonin with chemotherapy

Hepatocellular carcinomas are not only highly metastatic, but are also resistant to most of the commonly used chemotherapeutic drugs. Arsenic trioxide (ATO) is a novel drug that induces apoptosis in hepatocellular carcinoma cells *in vitro* and *in vivo*. Unfortunately, its activity was found to be poor in hepatocellular carcinoma patients. The combination of shikonin and ATO showed synergistic anticancer effects, in which ROS played an essential role. Furthermore, tumor growth was efficiently restrained if treatments with shikonin and ATO were combined *in vivo* [106].

The cytotoxicity of two shikonin derivatives (acetoxyisovaleryl shikonin and acetyl shikonin) alone and in combination with other chemotherapeutic drugs was tested against a panel of drug-sensitive

and drug-resistant cell lines. Both derivatives triggered the uptake of chemotherapeutic drugs and overcame ABC-transporter-mediated MDR [107].

Doxorubicin (DOX) is one of the anticancer drugs against which MDR often develops. However, the combination of DOX and shikonin induced apoptosis, damaged the mitochondrial membrane, lowered ATP levels, inhibited glycolysis, and inhibited ABC transporter expression in human A549 lung cancer cells [108].

Long non-coding RNAs (lncRNAs) are non-protein encoding transcripts that are made up of more than 200 nucleotides each. They are implicated in tumorigenesis because they regulate oncogenes and tumor suppressors at the post-transcriptional level. These lncRNAs also play a role in tamoxifen resistance. Several lncRNAs are preserved in humans, and have possible clinical functions in anticancer therapy. For example, uc.57 is an ultra-conserved breast cancer-related lncRNA. The anti-hormone tamoxifen is one of the standard treatments for breast cancer. A major disadvantage of tamoxifen is its tendency to stimulate drug resistance by lowering uc.57 expression and increasing BCL11A expression at both the mRNA and protein levels. The treatment of tamoxifen-resistant MCF-7R cells with shikonin increased the expression levels of uc.57 and decreased those of BCL11A. It also inhibited the PI3K/AKT and MAPK signaling pathways. Thus, shikonin may be an effective drug to combat tamoxifen resistance [109].

Osteosarcoma represents an aggressive bone malignancy that frequently develops DOX resistance. However, the activity of DOX was enhanced when administered in combination with shikonin by the activation of the pro-apoptotic caspases 3 and 8 [110].

The high mortality rates of patients suffering from advanced bladder cancer are mainly due to the development of cisplatin resistance. Recent studies revealed that the expression of the glycolytic enzyme PKM2 is greatly increased in bladder cancer cells. Shikonin was found to bind to PKM2 and induce cell death in bladder cancer cells in a dose-dependent manner. Downregulation of PKM2 by short hairpin RNA (shRNA) enhanced cisplatin and shikonin activity. Combining shikonin and cisplatin inhibited proliferation and induced apoptosis much more effectively than when either compound was administered alone. These results indicated that PKM2 induced cisplatin resistance, and that cisplatin-resistant bladder cancer cells were highly sensitive to shikonin. Moreover, the combination of cisplatin and shikonin decreased the tumor growth and metastasis of bladder cancers *in vivo*. Hence, PKM2 inhibition may be crucial to overcome cisplatin resistance and improve survival rates in patients with bladder cancer [97].

Chemotherapeutic regimens for many other tumor types contain cisplatin. The combination of shikonin with cisplatin induced synergistic anticancer effects and increased the selectivity between normal human colon mucosal epithelial cells and human colon cancer cells. Shikonin enhanced cisplatin-induced DNA damage and stimulated the mitochondrial pathway of apoptosis by inducing intracellular oxidative stress in cancer cells. Since inhibition of ROS generation also blocked shikonin- and cisplatin-induced apoptosis, ROS may play a decisive role in this drug synergism's effectiveness. An improved inhibition of colon tumors was also observed *in vivo* if cisplatin was combined with shikonin, as compared to that observed with cisplatin treatment alone [111].

The poor prognosis of gastric cancer patients is mainly due to drug resistance. Shikonin enhanced the activity of 5-fluorouracil and oxaliplatin, which are both clinically used for gastric cancer therapy [112].

Patients with non-small cell lung cancer (NSCLC) frequently experience tumor recurrence due to gefitinib resistance. Shikonin was tested in two gefitinib-resistant NSCLC cell lines, H1975 and H1650. Treatment with the addition of shikonin resulted in up to 10-fold increases in ROS levels and apoptosis rates, as well as higher PARP and caspase activities, compared to those observed in treatments with gefitinib alone. The ROS inhibitor N-acetylcysteine blocked shikonin-induced apoptosis, as well as PARP and caspase activation. The abnormal inhibition of EGFR phosphorylation led to EGFR degradation and the modulation of its downstream signaling pathways. Thus, EGFR contributes to the anticancer activity of shikonin in gefitinib-resistant NSCLC cell lines [113].

Micro-RNAs (miRNAs) are also involved in the response of tumor cells to chemotherapy, although their role in shikonin's effectiveness is not yet well-known. Previously, miR-143 was shown to be important in the cytotoxicity of shikonin against glioblastoma stem cells. Treatment with shikonin for 24 h decreased miR-143 expression levels. Additionally, BAG3, a regulator of apoptosis, was a target of miR-143. Shikonin treatment for 24 h increased BAG3 expression and downregulated miR-143 expression. However, ectopical miR-143 overexpression reduced BAG3 expression levels. High miR-143 levels enhanced shikonin's cytotoxicity *in vitro* and *in vivo*. However, this effect was reversed by BAG3 overexpression [114].

Gemcitabine is a clinically established drug used for treating pancreatic cancer, but the development of gemcitabine resistance limits its efficacy. The proliferation of PANC-1, BxPC-3, and AsPC-1 pancreatic cancer cells was inhibited by shikonin treatment, and shikonin enhanced the cytotoxic effect of gemcitabine. Shikonin also inhibited tumor growth and increased the anticancer effect of gemcitabine *in vivo*. These effects were associated with the lowered activity of NF-κB and expression of its downstream target genes, as well as increased apoptotic rates and decreased micro-vessel proliferation and density [115].

A clinical study was conducted with 30 patients between 34 and 72 years of age suffering from leukoplakia of the oral mucosa and erosive ulcerative lichen planus. These patients were treated with methylcellulose derivatives combined with shikonin and its esters. The topical application of the combined drugs caused prompt pain relief. The severity of lesions was reduced, and the foci in the oral mucosa damaged by inflammation were epithelized. Thus, this combined therapy improved the outcome for these patients compared to that of patients treated with methylcellulose derivatives alone [116].

10. Synergistic interactions of shikonin with immunotherapy

A recently thriving field in oncology has been the development of immunotherapies that block specific checkpoints in the immune system. Vaccination using dendritic cells (DCs), as well as the combination of therapeutic antibodies with small cytotoxic chemical molecules, have allowed numerous novel strategies for improving cancer therapy to be explored. Nevertheless, DC-based immunotherapies are still sub-optimal because DCs stimulate inhibitory immunological feedback loops in cancer, rendering immunological treatment approaches at least partly ineffective. For this reason, a major goal is to potentiate this immunogenicity through the use of small molecules. The use of phytochemicals and their derivatives represents an emerging approach within this field.

Shikonin was identified as one of the most powerful inducers of immunogenic cell death and DC-based immunotherapies [117]. The two phytochemicals hypericin and shikonin induced immunogenic cell death in certain cancer types, and thus increased the ability of the immune system to recognize cancer cells as foreign. In addition, the phytochemical derivative dihydrobenzofuranlignan (Q2-3) stimulated the release of the endogenous anticancer cytokine interleukin-25 from normal cells. These findings indicate that phytochemicals exert novel pharmacological activities that differ from those of the currently used cytotoxic anticancer agents. Therefore, phytochemicals warrant more detailed exploration to delineate novel strategies for improved immunotherapeutic approaches using them [118].

DC-based cancer vaccines are known to induce immunogenic cell death. Shikonin has been clinically applied as an adjuvant for this kind of vaccine. In fact, shikonin activated mitochondrial and receptor-mediated apoptosis and augmented DAMP expression when administered in this way. Combining DAMP with lipopolysaccharide (LPS) activated the maturation of DCs and strengthened the priming of Th1/

Th17 effector cells. When shikonin-treated tumor cell lysates were loaded into mature DCs, these expressed high levels of CD86 and MHC class II and stimulated Th1 cells. Vaccines containing these DCs strongly induced the cytotoxicity of splenocytes against specific cancer cells, retarded tumor growth, and increased the survival rates of mice. This is a promising approach for the future development of DC-based anticancer vaccines [119].

Shikonin possesses numerous other pharmacological activities. For instance, it accelerates granuloma formation, favors wound healing, and acts in an anti-inflammatory manner. Recently, the activity of shikonin as an immune-modifier for vaccines has been investigated. Transdermal gene-based vaccines are an interesting approach used to deliver DNA transgenes encoding tumor antigens directly into skin tissues. Skin DCs are powerful antigen-presenting cells that mediate and arrange tumor antigen-specific immunity against different cancer types. Shikonin strongly stimulated RANTES (chemokine (C–C motif) ligand 5, or CCL5) expression in normal human skin tissues. High expression levels of the murine Rantes protein were strongly induced by transfection of the murine *rantes* cDNA gene over a long period of time. However, this led to considerable skin damage. The number of skin DCs migrating to draining lymph glands was increased if shikonin was applied to the immunization area prior to gene gun-mediated vaccination. Vaccination using the *hgp100* cDNA gene combined with shikonin as the adjuvant *in vivo* elevated the cytotoxic activity of T-lymphocytes, splenocytes, and lymph gland cells toward sB16 melanoma. These results implied that shikonin successfully enhanced the anticancer activity of a gene-based cancer vaccine by stimulating the expression of RANTES at the site of skin immunization [120].

Another study done by Liu and Sun [108] demonstrated a role of shikonin in modulating the functioning of the immune system and its effect on NK cells. Shikonin stimulated NK cell proliferation and increased their cytotoxicity toward CaCo-2 colon cancer cells. It also enhanced the expression of the pro-apoptotic proteins GranB and perforin in a dose-dependent manner without affecting the expression of IFN-γ and TNF-α. Moreover, shikonin affected cellular signal transduction by increasing AKT and ERK1/2 phosphorylation [121].

Immunogenic cell death of cancer cells takes place by different pathways that stimulate the activity of immune cells against tumors. Shikonin induces immunogenic cell death and powerful immunogenicity against cancer cells. Ribonucleoprotein A1 (hnRNPA1) is a specific target of shikonin. *In vitro* studies done on the human breast cancer cells MDA-MB-231 and mouse mammary carcinoma 4T1 and 4T1-luc2 (*i.e.* 4T1 cells transfected by a luciferase cDNA gene) cell lines revealed that the binding of hnRNPA1 and shikonin induced immunogenic cell death, suppressed the processing of post-transcriptional mRNA, and inactivated the export of newly synthesized mRNAs from the nucleus. *In vivo* studies showed that this binding plays a major role in the anti-metastatic activity of tumor cell lysate-based vaccines. In addition, the clinical application of shikonin-induced immunogenicity may have considerable potential, since shikonin has been shown to precisely suppress specific types of post-transcriptional activities [122]. While the connection of hnRNPA1 to immunogenic cell death is novel, this protein has been previously described as a cell survival factor [123], and its inhibition by microRNA18a or the natural product quercetin led to apoptosis and cytotoxicity in cancer cells [124,125].

Necroptosis exerts strong immunogenic activity since it causes the liberation of DAMPs. Increased autophagic activity frequently occurs along with necroptosis. The comparative investigation of shikonin-induced autophagy and necroptosis showed that shikonin initiated necroptosis by affecting RIPK1 and RIPK3, and this mode of action coincided with increased autophagy. Moreover, shikonin-induced autophagy promoted the elevation of DAMP levels. For unknown reasons, only ectoDAMPs, but not the release of cellular DAMPs, was previously found to activate DCs. If autophagy was interrupted by chloroquine, the ectoDAMP and DC activities were upregulated more. Increased immunogenicity and vaccine efficiency was obtained by combining shikonin and chloroquine to treat cancer cells [126].

One disadvantage of cancer vaccines is their low ability to activate DCs for T-cell priming. Shikonin-induced immunogenic cell death and the resulting T-cell lines (TCL) enhanced the activity of TCL-pulsed DCs *in vivo*. Lin et al. [127] investigated the functions of DAMPs and their roles in DC activation. These authors focused on three DAMPs: HSP70, CRT, and HMGB1. Each of these proteins displayed a distinct mechanism by which it stimulated the expression of four of the main chemokines in DCs. HSP70 and CRT were shown to play an important role in TCL-induced DC immunity *in vitro* because they activated the proliferation of $CD8^+$ and $CD4^+$ T-cells. Mice transplanted with 4T1 breast tumors revealed that HSP70 was the main component facilitating their DC-based immunity's inhibition of metastasis and prolongation of survival, followed by CRT and then HMGB1. Moreover, the three main immunogenic cell death-associated receptors were only activated by HSP70 and HMGB1, but not by CRT. These findings indicated that immunogenic cell death components have roles in the activation of DC-based vaccines [127].

11. Synergistic interactions of shikonin with other treatment modalities

The control of the expression of miRNAs with particular functions represents an emerging field in cancer biology. The binding of miRNAs to the 3′-UTR mRNA sequences of target genes can inhibit their expression, cleave target mRNAs, or modulate their translation. Thus, miRNAs are important in many physiological processes, but abnormal miRNA expression is also implicated in some diseases. In cancer, miRNAs have dual roles as either tumor suppressors or oncogenes. Therefore, miRNAs can be used as biomarkers for the prognosis, diagnosis, and monitoring of diseases and treatment responses. The expression profiles of miRNAs can be altered by the effects of shikonin on cancer cell proliferation, the activation of cell death pathways, and improvements in the efficiency of combination therapies. Targeting miRNAs with shikonin may represent a novel approach to inhibit tumor growth and improve the survival rates of patients [128].

The Warburg effect is a process characterized by the ability of cancer cells to undergo metabolic reprogramming. This process relies on glycolysis to generate ATP, even under normal oxygen levels. A rate-limiting enzyme involved in glycolysis is glyoxalase I (GLO I), which detoxifies cytotoxic methyl glyoxal and is expressed at very high levels in many cancer types. Therefore, inhibiting glyoxalase I might be an efficient anticancer therapy. Recently a novel inhibitor of GLO I, known as TLSC702, has been discovered [129]. TLSC702 strongly inhibited the enzymatic activity of isolated GLO I. At the cellular level, however, higher amounts of TLSC702 were needed to induce apoptosis. This difference might be explained by the fact that cancers modify their metabolic pathways for energy production; for instance, they can rely on mitochondrial respiration rather than on glycolysis to escape apoptosis induction. Shikonin inhibits PKM2, which supplies pyruvate to the tricarboxylic acid cycle and which is expressed at particularly high levels in cancer cells. In a previous study based on this assumption, it was demonstrated that a combination therapy using TLSC702 and shikonin displayed high cytotoxicity against cancer cells [129].

NSCLC is a type of tumor that is resistant to most of the clinically established anticancer drugs. NSCLC is characterized by the expression of high levels of antioxidants, xenobiotic metabolism genes, and drug efflux proteins. Drug resistance in NSCLC is controlled by Nrf2, a redox-sensitive transcription factor, through the expression of efflux mechanisms, oxidant detoxification enzymes, and electrophiles. The expression of ABC transporters, antioxidants, and glutathione pathway genes is inhibited if Nrf2 expression is silenced by transfecting A549 cells with shRNA plasmids. Reduced Nrf2 expression in NSCLC mediated by RNAi stimulated ROS production, lowered glutathione levels, and thus inhibited cell proliferation. Hence, a practical way to restrain tumor growth and eradicate the drug resistance of NSCLC is to

target Nrf2 [130].

Kwak et al. [131] studied the effects of shikonin and its analogue, β,β-dimethylacryl shikonin (**1**), as radiosensitizers in HCT-116 colon cancer cells. Shikonin and β,β-dimethylacryl shikonin stimulated apoptosis in HCT-116 cells. The combination of these natural products with ionizing radiation synergistically enhanced the apoptosis of cancer cells. Shikonin stimulated ROS generation, and ionizing radiation induced DNA damage. If HCT-116 cells were pre-treated with the ROS scavenger N-acetylcysteine, the enhancement of ionizing radiation-induced apoptosis by shikonin was suppressed. Thus, shikonin acted as a radiosensitizer because of the high ROS production it induced. These *in vitro* results were confirmed *in vivo*. If ionizing radiation and dimethylacryl shikonin were combined to treat HCT-116 xenografted tumors, then tumor growth was suppressed.

Previous studies suggested that the ability to restore a wound healing microenvironment might be an efficient way to prevent cancer. Shikonin used alone did not significantly promote wound healing, but when it was applied in combination with two other natural products (aconitine and notoginsenoside R1) enhanced wound healing was observed. Moreover, when these natural products were combined together, they almost completely inhibited the incidence of urethane-induced lung cancer and tumor weight increases. Thus, restoring a wound healing microenvironment by combining natural products might be a potent strategy to prevent the development of cancer [132].

12. Conclusions and perspectives

The root extract of *L. erythrorhizon* not only has medicinal properties, as documented in traditional medicine, but also has considerable potential for use in the development of modern drugs. In this respect, shikonin, the active compound in the roots of *L. erythrorhizon*, has garnered much research interest. In fact, shikonin and its derivatives have been shown to suppress the growth of various types of tumors *in vitro* and *in vivo*. Preliminary clinical trials also provide favorable evidence that the translation of these effects into clinical practice might be possible. Shikonin induced apoptotic cell death at low concentrations and necroptosis at high concentrations [50,133–135]. Shikonin generated ROS and affected several cellular features that are characteristic of ROS activities, such as by causing alterations in the MAPK signaling pathway, mitochondrial dysfunction, and caspase activation downstream of ROS production [3]. Strategies developed from the fields of medicinal chemistry and nanotechnology have led to the creation of novel derivatives and nano-formulations based on shikonin with improved pharmacological features.

If shikonin-type drugs ever enter into clinical use, they will probably not be used as monotherapies, but rather as parts of combination therapies. Preliminary information is available showing that shikonin and its derivatives can be combined with standard anticancer drugs to enhance their therapeutic efficacy. As a next step, systematic investigations are required in the future to clarify how shikonin fits into existing and routinely applied combination therapy protocols for the treatment of specific tumor types to generate optimized treatments with high efficacy and tolerable side-effects.

Natural products are frequently seen as being relatively tolerable, with few side-effects. While “green medicine” is perceived as gentle by the general public, bioactive phytochemicals may exert similar unwanted toxic side-effects to those of synthetic drugs. Therefore, a stronger focus should be placed on the elucidation of the toxicity profiles of shikonin and its derivatives. A thorough safety assessment is a precondition for shikonin and its derivatives to be used in clinically approved treatments.

Another precondition for the clinical use of shikonin-type compounds to be established is the completion of clinical trials. A clinical trial conducted by Guo et al. [136] reported that when 19 patients suffering from late-stage lung cancer who were not subjected to surgery, chemotherapy, or radiotherapy were treated with shikonin, which inhibited tumor growth and improved the immune functions of these patients. Their tumor diameters were reduced by more than 25%, the remission rate was 37%, and the one year survival rate was 47%. The quality of life of the patients was also considerably improved. In fact, they regained appetite, as well as body weight. Moreover, shikonin reduced the severity and incidence of the chest pain, bloody mucus, and cough caused by lung carcinoma, and the expression levels of interleukin-2, a parameter representing immune function, were also increased. Shikonin did not have any adverse side-effects on the liver, heart, kidneys, and blood in this case [136]. Although this preliminary trial showed positive results, more information on the safety and efficacy of shikonin-based therapies in a clinical setting is still required. With this knowledge, drug production based on good manufacturing practices (GMP) can be initiated to perform clinical Phase III trials with the aim to achieve FDA approval can be enacted.

Shikonin represents a natural product on the verge of undergoing clinical testing for which there are sufficient amounts of data on its modes of action, *in vivo* activity, and even preliminary clinical data on its performance in cancer patients [136]. Shikonin appears to have several advantages over other phytochemicals. For example, the anticancer potential of curcumin from *Curcuma longa* L. has also been extensively investigated in recent years. Curcumin is the main ingredient in curcuma, which is a widespread spice used in India. While its health benefits are without doubt, it is questionable whether curcumin will find its way into use in clinical oncology due to the fact that its bioavailability is low. However, synthetic curcumin derivatives and nanotechnological preparations may yet advance curcumin's development as an anticancer drug [137,138].

A plethora of papers have been published on the polyphenol resveratrol, which can be found in red wine grapes and other plants. The idea that cancer can be fought with red wine is certainly appealing [139,140]. However, it is doubtful whether sufficient amounts of red wine could ever be consumed to reach the clinically relevant resveratrol concentrations in the body that would be needed to kill tumors. As a matter of fact, despite there being more than a thousand papers in the PubMed database on the experimental activity of resveratrol against cancer cells, there has not been a single randomized, placebo-controlled clinical trial on the anticancer activity of resveratrol in cancer patients.

The situation might be better for another phytochemical, artemisinin. This is the active constituent of *Artemisia annua* L, a medicinal plant used in traditional Chinese medicine. Artemisinin and its semisynthetic derivatives artemether and artemether are routinely used for the treatment of malaria, and have saved millions of lives over the past several decades. Youyou Tu obtained the Nobel Prize in Physiology or Medicine in 2015 for her achievements in the development and use of artemisinin as an anti-malaria drug [141]. Remarkably, it turned out that artemisinin and its derivatives are also active against other diseases, such as schistosomiasis, trypanosomiasis, viruses, and cancer [142–147]. Several clinical Phase I/II trials indicate that artesunate may be a useful drug for cancer therapy [148–151]. It can be expected that several phytochemicals, such as shikonin and other drugs, may be investigated in more detail in the future, and a level of scientific knowledge may be obtained that will justify their further development as novel anticancer drugs to fight tumors that remain unresponsive to current forms of chemotherapy.

Conflicts of interest

The authors declare that there is no conflict of interest.

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