Shikonin Suppresses NLRP3 and AIM2 Inflammasomes by Direct Inhibition of Caspase-1

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Abstract

Shikonin is a highly lipophilic naphtoquinone found in the roots of Lithospermum erythrorhizon used for its pleiotropic effects in traditional Chinese medicine. Based on its reported antipyretic and anti-inflammatory properties, we investigated whether shikonin suppresses the activation of NLRP3 inflammasome. Inflammasomes are cytosolic protein complexes that serve as scaffolds for recruitment and activation of caspase-1, which, in turn, results in cleavage and secretion of proinflammatory cytokines IL-1β and IL-18. NLRP3 inflammasome activation involves two steps: priming, i.e. the activation of NF-κB pathway, and inflammasome assembly. While shikonin has previously been reported to suppress the priming step, we demonstrated that shikonin also inhibits the second step of inflammasome activation induced by soluble and particulate NLRP3 instigators in primed immortalized murine bone marrow-derived macrophages. Shikonin decreased NLRP3 inflammasome activation in response to nigericin more potently than acetylshikonin. Our results showed that shikonin also inhibits AIM2 inflammasome activation by double stranded DNA. Shikonin inhibited ASC speck formation and caspase-1 activation in murine macrophages and suppressed the activity of isolated caspase-1, demonstrating that it directly targets caspase-1. Complexing shikonin with β-lactoglobulin reduced its toxicity while preserving the inhibitory effect on NLRP3 inflammasome activation, suggesting that shikonin with improved bioavailability might be interesting for therapeutic applications in inflammasome-mediated conditions.

Introduction

Shikonin is a naphthoquinone found in the roots of Lithospermum erythrorhizon Siebold & Zucc. and other plants of the Boraginaceae family. Shikonin and its derivatives are the main active ingredients of Zicao, a traditional Chinese medicine used for its antibacterial, antiviral, anti-inflammatory and antipyretic effects. The indications for the use of L. erythrorhizon roots include skin lesions, burns, dermatitis, eczema, bedsores, sore throat, macular eruptions and
measles [1, 2]. In recent decades, shikonin and its derivatives attracted large interest for their tumor-suppressing effects [3] and were shown to act on multiple cellular targets including pyruvate kinase-M2 (PKM2), transcription factor NF-κB, cyclooxygenase-2, TNF-α and STAT3 as reviewed by Andujar and coworkers [4]. In mouse models of lethal endotoxemia, shikonin reduced serum levels of an inflammatory mediator high-mobility group box 1 (HMGB1) and protected mice from LPS-induced death [5]. In normal conditions, HMGB1 acts as a transcriptional regulator, however when secreted from activated immune cells it mediates various inflammatory responses. HMGB1 is also secreted upon inflammasome activation [6].

Inflammasomes are cytoplasmic protein complexes that sense and are activated in response to pathogen- and danger-associated molecular patterns. They serve as scaffolds for recruitment and autoprteolytic activation of caspase-1, which cleaves the inactive precursor cytokines pro-IL-1β and pro-IL-18 into proinflammatory cytokines IL-1β and IL-18. The most studied NLRP3 inflammasome is composed of the sensor NLRP3, the adaptor protein ASC and procaspase-1. It is activated by a plethora of different stimuli ranging from small molecules such as ATP and microbial pore-forming toxins [7, 8] to aggregates including uric acid [9] and cholesterol crystals [10], silica [11, 12], asbestos fibers [11], amyloid β aggregates [13] and prion protein fibrils [14]. Inflammasome activation leads to efficient defense against pathogens, while missense mutations in the NLRP3 protein-encoding gene (Cias1) cause autoinflammatory disorders cryopyrinopathies (i.e. cryopyrin-associated periodic syndromes, CAPS), where IL-1β is constitutively processed [15–17]. Inappropriate activation of NLRP3 inflammasome is also implicated in common diseases including Alzheimer’s disease [18, 19] and diabetes [20]. The rapidly growing evidence for excessive inflammasome activation in a variety of common diseases highlights the need for small molecule inhibitors of NLRP3 inflammasome.

While NLRP3 inflammasome is controlled on transcriptional and posttranslational levels and by several endogenous inhibitors and modulators as reviewed by Rathinam and coworkers [21], current therapy of CAPS is based on the broad blockade of IL-1β or IL-1R signaling. No anti-IL-18 treatment is currently approved. Natural compounds parthenolide [22], artemisinin [23], scopolioside B [24] and curcumin [25, 26] have been shown to inhibit IL-1β secretion via NLRP3 inflammasome activation. Recently, one of the ketone bodies, β-hydroxybutyrate, was shown to specifically inhibit NLRP3 inflammasome [27] and synthetic compound MCC950 demonstrated effective and specific NLRP3 inflammasome inhibition with promising results in the mouse model of CAPS [28].

NLRP3 inflammasome activation requires prior expression of pro-IL-1β and NLRP3, which is achieved through activation of NF-κB. Versatile anti-inflammatory effects of shikonin have previously been observed both in cell culture and animal studies. Shikonin was shown to reduce edema in TPA-induced mouse ear edema model and effectively inhibited COX-2 and iNOS, all of which was attributed to decreased activation of NF-κB [29]. Inhibition of NF-κB by shikonin [29, 30] or shikonin/alkannin mixture [31] was confirmed in macrophage cell line RAW 264.7 stimulated by LPS.

In the present study we showed that shikonin inhibits NLRP3 inflammasome activation by acting on both signals. Previous studies demonstrated that it effectively inhibits NF-κB activation (i.e. the priming signal). We show that shikonin also dampens the maturation of pro-IL-1β by NLRP3 inflammasome in response to soluble and particulate NLRP3 instigators. We further demonstrated that shikonin also inhibits AIM2 inflammasome and that its effect on inflammasomes can be ascribed to both its inhibition of ASC oligomerization as well as its direct inhibition of caspase-1. Our results show that in addition to its previously reported targets, the observed anti-inflammatory effects of shikonin might also originate from its action on inflammasomes and caspase-1.
Materials and Methods

Materials

Shikonin (>98%, HPLC) was purchased from Enzo, acetylshikonin (>95%, HPLC) was from Biopurify Phytochemicals. Cell culture media, FBS and other cell culture supplies were from Gibco, Alum was from Thermo Scientific, ultrapure LPS from Escherichia Coli O111:B4, silica (SiO₂), LyoVec polydA:dT, imiquimod were from Invivogen. Nigericin, ATP and all other chemicals if not specified otherwise were from Sigma. Ready-SET-go ELISA kits (eBioscience) were used for determination of mouse and human IL-1β. Concentration of mouse IL-18 was determined by IL-18 Platinum ELISA (eBioscience).

Cell Cultures

Immortalized BMDMs from C57BL/6 mice described in [12] were a kind gift of K. A. Fitzgerald (University of Massachusetts Medical school, Worcester, MA, USA). Immortalized BMDMs were cultured in DMEM supplemented with 10% FBS. THP-1 cells (ECCAC 88081201) were cultured in RPMI 1640 supplemented with 10% FBS.

mRNA Isolation and qPCR

The messenger RNA (mRNA) was isolated using the RNasy Mini Kit (Qiagen), transcribed to cDNA with High Capacity cDNA Transcription Kit (Applied Biosystems). qPCR was performed using the LightCycler 480 SYBR Green I Master mix on LightCycler 480 instrument (Roche).

XTT and LDH Assays

Cells were cultured and treated as in experiments for IL-1β ELISA. Inflammasome stimulation was done in DMEM without phenol red. For XTT assay, cells in negative control were subjected to 0.1% Triton X-100 for 15 min. After supernatants were collected for IL-1β quantification, cells were cultured in DMEM without phenol red. A solution of XTT and phenazine methosulphate was prepared in DMEM without phenol red and added to the cells. After 9 h, absorbance at 490 nm (ref. 650 nm) was measured using multiplate reader SynergyMx (Biotek).

For LDH assay, supernatants were analyzed for the presence of LDH activity. Sample was mixed with lactate in Tris buffer, pH 8.2, and the mixture of phenazine methosulphate, NAD and iodonitrotetrazolium chloride. Supernatant from 0.1% Triton X-100 treated cells was used as positive control. After 15–30 min, absorbance at 490 nm was measured using multiplate reader SynergyMx (Biotek). LDH release in % was calculated using the supernatant of untreated cells as negative control and Triton X-100 treated supernatant as 100% LDH release.

ELISA Assays

All experiments were performed in serum-free DMEM. Cells were seeded at 1.5 x 10⁵ cells per well of 96 well plate and primed with ultra-pure LPS (100 ng/mL) overnight for IL-1β or 8 h for IL-18. The growth medium was removed and potential inhibitors were added 30 min before the addition of stimulators. Further, different activators in DMEM were added for 1 h (nigericin, ATP), 6 h (silica), 12 h (alum) or 24 h (imiquimod). The concentration of secreted IL-1β and IL-18 was measured by ELISA (e-Bioscience) according to manufacturer’s instructions.

ASC Speck Formation by Immunofluorescence

Endogenous ASC was labelled as previously described [32]. Cells were seeded into μ-slides (Ibidi). Priming, shikonin addition and nigericin treatment were done as described above.
After 45 min of nigericin treatment, cells were fixed with paraformaldehyde (4%, Electron Microscopy Sciences) for 15 min, permeabilized by 0.2% saponin, 1% BSA in PBS for 30 min. Further, cells were incubated with primary antibody against ASC (Biolegend) for 1 h at room temperature and after washing in secondary Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) in permeabilization/ blocking solution. After washing, Prolong Diamond Antifade solution with DAPI (Invitrogen) was added to wells. A Leica TCS SP5 laser scanning microscope mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems, Germany) with an HCX plan apo 63× (NA 1.4) oil immersion objective was used for imaging. A 405 nm laser line of 20 mW diode laser was used for DAPI excitation and emitted light was detected between 415 and 450 nm. A 488 nm laser line of 100 mW argon laser with 10% laser power was used for detection of Alexa 488 conjugate, where emitted light was detected between 500 and 600 nm. For acquisition and image processing, Leica LAS AF software was used.

Western Blotting

Experiments were performed as for measuring IL-1β ELISA, with exception that stimulation was done in 24-well plate format. Methanol precipitation was used to precipitate proteins from cell culture media. Cells were washed twice with cold PBS and lysed. Protein concentration in the cell lysate was measured with BCA. Proteins were separated on 15% SDS-PAGE gels, blotted onto the nitrocellulose membrane (GE Healthcare) and detected with appropriate primary and secondary antibodies for detection of caspase-1 p10: M-20 (Santa Cruz) and for the detection of caspase-1 p20 (Casper-1, Adipogen) followed by HRP-conjugated goat polyclonals to rabbit IgG (Abcam). NLRP3 was detected using Cryo-2 (Adipogen) as primary antibody and goat anti-mouse HRP conjugated antibodies (Jackson ImmunoResearch). SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) was used for detection of HRP-labeled bands.

Caspase-1 Activation

Activation of caspase-1 was measured by flow cytometer Cyflow (Partec) using Fluorochrome Inhibitor of Caspase 1 kit (Immunochemistry Technologies). Briefly, cells were seeded at 1.5 x 10^5 cells per well of 24-well plate and primed with ultra-pure LPS (500 ng/mL) for 2 h. Potential caspase-1 inhibitors were added 30 min before activation with 10 μM nigericin. After 30 min FLICA reagent was added and incubated for 30 min. On ice, cells were washed twice, detached in PBS and analyzed by flow cytometer Cyflow (Partec).

Caspase-1 Activity Assay

Potential inhibitors of caspase-1 were analyzed using Caspase-1 Inhibitor Drug Screening Kit (Fluorimetric) from BioVision. Shikonin and positive inhibition control (Z-VAD-FMK) were prepared in PBS and applied to black 96-well fluorescence plate (Corning). Active caspase-1 was added, followed by caspase-1 substrate YVAD-AFC. After 45 min incubation at 37°C, fluorescence of samples was measured using SinergyMx plate reader (Biotek).

IC50 Calculation

The data were fitted using the 4 parameter logistic (4PL) nonlinear regression model and IC50 was calculated as the concentration of the inhibitor yielding half-maximal IL-1β release.

Statistical Analysis

Unpaired two-tailed t-test was used for pairwise comparison.
Results and Discussion

Shikonin Inhibits the Maturation of IL-1β and LDH Release Induced by Nigericin, an Activator of the NLRP3 Inflammasome

The activation of NLRP3 inflammasome is a two-step process. In the first, so called ‘priming step’, NLRP3 and pro-IL-1β are expressed upon activation of NF-κB. Since shikonin is a known NF-κB inhibitor [29, 30], it is expected that the priming step of inflammasome activation is affected. However, previously identified NF-κB inhibitors Bay 11-7082 and parthenolide were also shown to inhibit the second step of NLRP3 inflammasome activation [22]. We were interested whether shikonin was also able to inhibit IL-1β maturation. We followed the activation of inflammasome with nigericin in LPS-primed immortalized bone-marrow-derived macrophages from C57BL/6 mice (iBMDMs) [12]. When shikonin was added prior LPS stimulation, concentration-dependent inhibition of IL-1β maturation was observed (Fig 1A, left; IC50 range 0.3–0.6 μM). Interestingly, at slightly higher concentrations (IC50 range 1.4–2 μM), shikonin was also effective when added after the priming step, implying that it also inhibits the second step of NLRP3 inflammasome activation (Fig 1B, left). To support this finding, we also followed the effect of shikonin on transcription of pro-IL-1β by qPCR (S1 Fig). When shikonin was added prior to LPS, the expression of pro-IL-1β induced by LPS was reduced as expected since shikonin is known to suppress NF-κB activation (S1A Fig). However, when shikonin was added after LPS priming, there was no inhibition of pro-IL-1β expression (S1B Fig), demonstrating that the observed effect of shikonin on IL-1β maturation (Fig 1B) was not due to decreased expression of pro-IL-1β. Interestingly, the expression of NLRP3 on the protein level was unaffected by shikonin added either prior or after LPS stimulation (S1C Fig). Shikonin is able to kill tumor cells via either apoptosis (below 2.5 μM) or necroptosis (above 10 μM) [33]. As demonstrated by XTT proliferation assay, shikonin induced cell death, but at concentrations significantly higher than those required to inhibit IL-1β release (Fig 1A, right and Fig 1B, right), suggesting that inflammasome inhibition and induction of cell death are two separate effects of shikonin. Further, inhibition of IL-1β release was also achieved in the presence of Nec-1s, a necroptosis inhibitor (S2 Fig), which, however, did not inhibit cell death induced by shikonin in our experiments (not shown). One of the hallmarks of inflammasome activation includes necrotic cell death called pyroptosis. We showed that shikonin is able to dose-dependently inhibit LDH release induced by nigericin (Fig 1C). Shikonin also inhibited IL-18 release from LPS-primed mouse iBMDMs (S3 Fig). The concentration of shikonin required for this effect was higher than for inhibition of IL-1β maturation and approached cytotoxic levels of shikonin (Fig 1B, right). Furthermore, shikonin inhibited IL-1β release from PMA-differentiated and nigericin-stimulated human monocytic cell line THP-1 (Fig 1D). We demonstrated that shikonin at non-toxic concentrations inhibits NLRP3 inflammasome activation by nigericin.

Shikonin is More Potent NLRP3 Inflammasome Inhibitor than Acetylsikokin

L. erythrorhizon root preparations contain several naphthoquinones, which were previously shown to exhibit anti-inflammatory effects as reviewed by Chen et al. [1]. Although the content of specific derivatives varies depending on the plant source and extraction procedure [1], acetylsikokin is more abundant in the roots of L. erythrorhizon than shikonin [1, 34]. We were interested whether acetylsikokin inhibits NLRP3 inflammasome similarly to shikonin. Shikokin and acetylsikokin were added either before priming (LPS) (Fig 2A) or before the addition of inflammasome instigator nigericin (Fig 2B, S4 Fig). Acetylsikokin concentration-
Fig 1. Shikonin Inhibits IL-1β Maturation and LDH release. (A, B) Shikonin inhibits IL-1β release from iBMDMs. Cells were primed with 100 ng/mL LPS for 12 h or left untreated (ctrl). Shikonin (5–0 μM) or vehicle (DMSO) was applied to cells 30 min before priming (A, left) or 30 min before activation with 10 μM nigericin (B, left) (nigericin is present in all samples but ctrl and LPS). Supernatants were collected 1 h after activation and assayed using IL-1β ELISA. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. Metabolic activity of the remaining cells was determined with XTT assay (A right, B right). Cell death control was induced by 0.1% Triton X-100 (dead). (C) Shikonin inhibits the release of LDH into the culture medium of LPS-primed and nigericin-stimulated iBMDMs. (D) Shikonin inhibits human IL-1β release from PMA-differentiated and nigericin-stimulated THP-1 cells. THP-1 cells were differentiated into adherent macrophages with 500 ng/mL PMA overnight. Cells were activated as in (B). Representative of 3 (A–E) independent experiments is shown. Error bars represent SD of triplicate wells.

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dependently inhibited IL-1β release, particularly when added before the priming step. However, the inhibition achieved by acetylshikonin (IC\textsubscript{50} = 3 μM) was weaker compared to shikonin (IC\textsubscript{50} range 0.3–0.6 μM). The difference was even more evident at the second step of NLRP3 inflammasome activation, where the inhibitory effects of shikonin and acetylshikonin had estimated IC\textsubscript{50} values of 2 μM and 21 μM, respectively (Fig 2B left and S4 Fig). Previous studies demonstrated several anti-inflammatory effects of both compounds. Acetylshikonin was more potent inhibitor of neutrophil respiratory burst than shikonin [35]. On the other hand, Cheng and coworkers showed that shikonin/alkannin was superior to other derivatives including acetylshikonin in downregulating MAPK/NF-κB signaling [31], which agrees well with our results regarding the priming (NF-κB-dependent) step of inflammasome activation (Fig 2A). Our results show that shikonin is superior to acetylshikonin in suppression of the priming as well as the second step of NLRP3 inflammasome activation.

Fig 2. Shikonin is More Potent Inhibitor of IL-1β Maturation than Acetylshikonin. Shikonin (left) or acetylshikonin (right) was applied to cells 30 min before priming with 100 ng/mL LPS (A) or 30 min before activation with 10 μM nigericin (B). Supernatants were collected 1 h after activation with nigericin and assayed using IL-1β ELISA. Representative of three experiments is shown. Error bars represent SD of triplicate wells. Molecular structures of shikonin (left) and acetylshikonin (right) are shown above the corresponding results.

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Shikonin Inhibits NLRP3 Inflammasome Activation with Particulate and Soluble NLRP3 Instigators and by AIM2 Activator dAdT

NLRP3 inflammasome is unique in its activation by a wide variety of chemically and morphologically distinct stimuli. Several mechanisms of their action were proposed, for example decreased intracellular K⁺ [36, 37] or increased cytosolic Ca²⁺ [38–40]. In contrast to soluble activators, the action of particulate activators requires their phagocytosis and subsequent lysosomal destabilization [12]. To determine whether shikonin specifically inhibits NLRP3 inflammasome in response to nigericin or has broader action, we tested its inhibitory capacity in treatment with various NLRP3 inflammasome activators. While nigericin is a soluble toxin, we showed that shikonin also inhibits inflammasome activation with particulate triggers silica (Fig 3A) and alum (Fig 3B). Further, shikonin inhibited IL-1β secretion induced by soluble triggers imiquimod (Fig 3C) and ATP (Fig 3D). Our results demonstrate that shikonin inhibits NLRP3 inflammasome activation independently of the NLRP3 trigger used, suggesting that it acts at downstream stages of inflammasome activation. As the recruitment of adaptor protein ASC and activation of caspase-1 are common to several inflammasomes including the AIM2 inflammasome, we were interested whether shikonin specifically inhibits NLRP3 inflammasome. We showed that shikonin also inhibits IL-1β secretion induced by dAdT, an AIM2 inflammasome activator acting independently of NLRP3 (Fig 3E). Similar inflammasome inhibitory phenotype was previously observed for cysteinyl leukotriene receptor antagonist, which, however, had no effect on the priming step of inflammasome activation [41].

Our results demonstrated that shikonin interferes not with the action of different NLRP3 inflammasome instigators, but with the process of inflammasome assembly shared among several inflammasomes.

Shikonin Inhibits Formation of ASC Specks and Directly Targets Caspase-1

Upon activation, both NLRP3 and AIM2 recruit the adaptor protein ASC, which multimerizes and acts as a platform for caspase-1 activation. Shikonin inhibited the activation of both NLRP3 and AIM2 inflammasomes suggesting that it either affects ASC oligomerization, pro-caspase-1 recruitment or its autoactivation. To determine whether shikonin affects ASC oligomerization, immunofluorescence of endogenous ASC was used [32]. In unstimulated or primed only cells, diffuse cytosolic stain was observed (Fig 4). Upon nigericin stimulation, however, ASC forms large oligomers called specks, resulting in condense bright spots in the perinuclear region. We showed that shikonin is capable of concentration—dependent inhibition of ASC speck formation (Fig 4).

Further, we observed that shikonin concentration-dependently inhibited the secretion and processing of pro-caspase-1 from primed and nigericin-treated macrophages (Fig 5A). Decreased caspase-1 activation was also observed in shikonin-treated nigericin-stimulated murine macrophages using membrane-permeant fluorescent caspase-1 inhibitor as followed by flow cytometry (Fig 5B). While the effect on pro-caspase-1 and IL-1β processing could be the mere consequence of shikonin inhibiting ASC oligomerization, we were nevertheless interested whether shikonin has any effect on caspase-1 activity. In the in vitro assay using isolated caspase-1, we showed that shikonin directly inhibits caspase-1 in the same concentration range as the conventional caspase inhibitor Z-VAD-FMK (Fig 5C).

Previous studies showed that glutathione reacts with C2 position of naphthazarin (the ring skeleton of shikonin) [42] and shikonin was shown to react with cellular thiols [43]. It also reacted with the free thiol of cysteine residue in β-lactoglobulin, possibly via 1,4-reductive Michael addition [44]. As caspase-1 is a cysteine protease harboring cysteine in its active site, it
Fig 3. Shikonin Inhibits IL-1β Release from Macrophages Treated with Activators of NLRP3 Inflammasome and AIM2 Inflammasome. iBMDMs were primed with 100 ng/mL LPS for 12 h or left unprimed (ctrl). They were subjected to shikonin (2.5–0 μM) or DMSO (solvent control) 30 min before activation with 20 μg/mL silica (A) for 6 h, 0.5 mg/mL alum (B) for 12 h, 20 μg/mL imiquimod (C) for 24 h, 5 mM ATP (D) for 1 h or 1 μg/mL dAdT (E) for 1 h. Concentrations of mature IL-1β in culture media were determined using IL-1β ELISA. Representative of 3 (A to D) or 2 (E) independent experiments is shown. Error bars represent SD of triplicate wells.

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Fig 4. Shikonin Inhibits Formation of ASC Specks. iBMDMs were primed with 100 ng/mL LPS overnight or left untreated (ctrl). 30 min before stimulation with 10 μM nigericin iBMDMs were subjected to shikonin (0–5 μM). After 45 min, cells were fixed and labelled for endogenous ASC. At least five 250 μm x 250 μm fields were recorded for each condition and used for analysis (below). Bar represents 50 μm. Nuclei are depicted in blue (DAPI) and ASC in green.
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Fig 5. Shikonin Inhibits Caspase-1 Activation. (A) iBMDMs were primed with 100 ng/mL LPS overnight or were left untreated (ctrl). 30 min before stimulation with 10 μM nigericin, they were subjected to shikonin (5–0 μM) or solvent control (DMSO). Shikonin dose-dependently inhibited caspase-1 activation, which was followed by the presence of 20 kDa (upper blot) and 10 kDa (below) subunits of active caspase-1 in the supernatant. Pro-caspase-1 and β-actin in cell lysate are used as loading controls. Representative Western
is possible that shikonin reacts with this cysteine rendering the enzyme inactive. Collectively, our data demonstrated that shikonin concentration-dependently inhibits inflammasome activation by acting directly on caspase-1, however further studies are needed to elucidate the exact mechanism of shikonin inhibition of caspase-1 activity.

Complexing Shikonin with β-lactoglobulin Decreases Its Cytotoxicity while Preserving the Inhibitory Capacity

Shikonin is a highly lipophilic molecule. Since low aqueous solubility severely reduces its bioavailability, several approaches have been used to circumvent this problem. Increased aqueous solubility and decreased toxicity was, for example, achieved by complexing shikonin with hydroxypropyl-β-cyclodextrin [45] or β-lactoglobulin [44], encapsulation of shikonin in biocompatible materials [46, 47] or the use of biocompatible polymer electrospun fiber mats [48, 49]. We prepared a complex of shikonin with β-lactoglobulin as described by Albreht and coworkers [44]. β-lactoglobulin-complexed shikonin retained the ability to inhibit IL-1β maturation induced by nigericin, although higher concentrations of complexed shikonin were needed to achieve the same effect (Fig 6A). Importantly, complexed shikonin was less cytotoxic than free shikonin (Fig 6B). Decreased cell viability was observed at 30–40 μM β-lactoglobulin-complexed shikonin, while complete inhibition of NLRP3 inflammasome signaling was already evident at 10 μM. Our results corroborate the study of Xia and co-workers showing that shikonin-containing liposomes have lower toxicity compared to free shikonin [50] and encourage further studies in shikonin complexation aiming at reducing toxicity while preserving anti-inflammatory action.

With more than two millenia of use of shikonin-containing plant extracts in folk medicine, the molecular mechanisms underlying its effects were mostly discovered in the last three decades. In the present study, we corroborate previous reports documenting the inhibitory effect of shikonin on the NF-κB pathway. We further showed that shikonin suppresses NLRP3-dependent maturation of IL-1β induced by soluble as well as particulate triggers. How such diverse activators are able to induce the assembly of NLRP3 inflammasome is not known. One mechanism proposes the involvement of oxidative stress [51]. Shikonin was previously reported to be a reactive oxygen species (ROS) scavenger [52]. Recent studies, however, reported that shikonin is in fact a ROS producer that decreases tumor cell proliferation by ROS-induced apoptosis [53, 54], implying that inhibition of ROS is not the mechanism of action of shikonin on NLRP3 inflammasome. Shikonin was also reported to inhibit pyruvate kinase-M2 (PKM2) [55] thus reducing IL-1β and HMGB-1 release from LPS-challenged cells and protecting mice from lethal endotoxemia and sepsis [5]. Pyruvate kinase mediates the rate-limiting step of glycolysis. Aerobic glycolysis is important in tumor cells and in the activated immune cells [56]. Therefore, downregulation of PKM2 could account for the inhibitory action of shikonin on NLRP3 inflammasome. Our data, however, demonstrated that shikonin inhibits inflammasome activation by dampening the formation of ASC specks and by directly inhibiting caspase-1. The fact that the concentrations of shikonin required for caspase-1 inhibition in the \textit{in vitro} assay were higher compared to cell culture-based systems supports the notion that shikonin inhibits inflammasome activation by acting on multiple targets. The emerging role of
NLRP3 inflammasome and caspase-1 in diseases characterized by chronic inflammation (e.g., diabetes mellitus, Alzheimer’s disease, gout, multiple sclerosis, AIDS) provides the scope for development of novel approaches to previously incurable diseases. In this context, successful therapeutic implementation of small molecule inhibitors crucially relies on mechanistic understanding of their mode of action.

Supporting Information

**S1 Fig. Effect of Shikonin on Expression of pro-IL-1β mRNA and NLRP3 Protein.** Cells were primed with 100 ng/mL LPS for 12 h or left untreated (ctrl). Shikonin (1 μM) was applied to cells either 30 min before priming (A and C, before) or after priming for 30 min (B and C, after).
after). (A, B) Two biological replicates were subjected to qPCR analysis to determine the relative abundances of pro-IL-1β mRNA, which are expressed as fold increase compared to the mRNA amounts in untreated cells. GAPDH was used as the reference and ΔΔCT method was used for quantification. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. Representative of three independent experiments is shown. (C) Representative Western blot of two independent experiments is shown. (PDF)

S2 Fig. Necroptosis Inhibitor Nec-1s Does Not Affect Shikonin-Mediated Inhibition of IL-1β Release. iBMDM cells were primed with 100 ng/mL LPS overnight or left untreated (ctrl). (A) 2 μM Nec-1s was applied to cells 30 min before the addition of shikonin. (A, B) Shikonin (10–20 μM) or vehicle (DMSO) was applied to cells 30 min before activation with 10 μM nigericin (omitted in ctrl and LPS). Supernatants were collected 1 h after activation and assayed using IL-1β ELISA. Representative of two experiments is shown. Error bars represent SD of triplicate wells. (PDF)

S3 Fig. Relatively High Concentrations of Shikonin are needed for Inhibition of IL-18 release from iBMDMs. Cells were primed with 100 ng/mL LPS for 8 h or left untreated (ctrl). Shikonin (7.5–20 μM) or vehicle (DMSO) was applied to cells 30 min before activation with 10 μM nigericin (nigericin is present in all samples but ctrl and LPS). Supernatants were collected 1 h after activation and assayed using IL-1β ELISA. Representative of three experiments is shown. Error bars represent SD of triplicate wells. (PDF)

S4 Fig. High Concentrations of Acetylshikonin (>20 μM) Moderately Inhibit IL-1β Release. Acetylshikonin was applied to iBMDM cells 12 h after priming with 100 ng/mL LPS and 30 min before activation with 10 μM nigericin. Supernatants were collected 1 h after activation and analyzed by IL-1β ELISA. Representative of three experiments is shown. Error bars represent SD of triplicate wells. (PDF)

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Author Contributions

Conceived and designed the experiments: IHB JZ. Performed the experiments: JZ IHB PS. Analyzed the data: IHB JZ PS. Wrote the paper: IHB JZ.

References

1. Chen X, Yang L, Oppenheim JJ, Howard MZ. Cellular pharmacology studies of shikonin derivatives. Phytother Res. 2002; 16(3):199–209. doi:10.1002/ptr.1100 PMID: 12164262.
2. Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, Nicolaou KC. The Chemistry and Biology of Alkannin, Shikonin, and Related Naphthazarin Natural Products. Angew Chem Int Ed. 1999; 38:270–300.
3. Andujar I, Recio MC, Giner RM, Rios JL. Traditional chinese medicine remedy to jury: the pharmacological basis for the use of shikonin as an anticancer therapy. Curr Med Chem. 2013; 20(23):2892–8. PMID: 23651309.
4. Andujar I, Rios JL, Giner RM, Recio MC. Pharmacological properties of shikonin—a review of literature since 2002. Planta Med. 2013; 79(18):1685–97. doi: 10.1055/s-0033-1350934 PMID: 24155261.

5. Yang L, Xie M, Yang M, Yu Y, Zhu S, Hou W, et al. PKM2 regulates the Warburg effect and promotes HMGB1 release in sepsis. Nat Commun. 2014; 5:4436. doi: 10.1038/ncomms5436 PMID: 25019241; PubMed Central PMCID: PMC4104986.

6. Lamkanfi M, Sarkar A, Vande Walle L, Vitari AC, Amer AO, Wewers MD, et al. Inflammation-dependent release of the alarmin HMGB1 in endotoxemia. J Immunol. 2010; 185(7):4385–92. doi: 10.4049/jimmunol.1000803 PMID: 20802146; PubMed Central PMCID: PMC3428148.

7. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature. 2006; 440(7081):228–32. PMID: 16407890.

8. Gurcel L, Abrami L, Girardin S, Tschopp J, van der Goot FG. Caspase-1 activation of lipid metabolic pathways in response to bacterial pore-forming toxins promotes cell survival. Cell. 2006; 126(6):1135–45. PMID: 16990137.

9. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature. 2006; 440(7081):237–41. PMID: 16407889.

10. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature. 2010; 464(7293):1357–61. PMID: 20428172. doi: 10.1038/nature08938.

11. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. NALP3 inflammasome sensing of asbestos and silica. Science. 2008; 320(5876):674–7. PMID: 18403674. doi: 10.1126/science.1156995.

12. Hornung V, Bauerfeind FG, Halle A, Samstad EO, Kono H, Rock KL, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol. 2008; 9 (8):847–56. PMID: 18604214. doi: 10.1038/ni.1631.

13. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reineckel T, et al. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol. 2008; 9(8):857–65. doi: 10.1038/ni.1636. PMID: 18604209; PubMed Central PMCID: PMC3101478.

14. Hafner-Bratkovic I, Bencina M, Fitzgerald KA, Golenbock D, Jerala R. NLRP3 inflammasome activation through Nalp3 inflammasome sensing of asbestos and silica. Science. 2008; 320(5876):674–7. PMID: 18403674. doi: 10.1126/science.1156995.

15. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. Immunity. 2004; 20(3):319–25. PMID: 15030775.

16. Dowds TA, Masumoto J, Zhu L, Inohara N, Nunez G. Cryopyrin-induced interleukin 1beta secretion in macrophage cell lines by prion protein fibrils as the source of IL-1beta and neuronal toxicity. Cell Mol Life Sci. 2012; 69(24):4215–28. doi: 10.1007/s00018-012-1140-0 PMID: 22926439; PubMed Central PMCID: PMC3508391.

17. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. J Biol Chem. 2004; 279(21):21924–8. doi: 10.1016/S1440-2481(04)00518-8. PMID: 15026001.

18. Ting JP, Williams KL. The CATERPILLER family: an ancient family of immune/apoptotic proteins. Clin Immunol. 2005; 115(1):33–7. PMID: 15870018.

19. Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Vieira-Saecker A, et al. NLRP3 is activated and contributes to pathology in APP/PS1 mice. Nature. 2013; 493(7434):674–8. doi: 10.1038/nature11729 PMID: 23254930; PubMed Central PMCID: PMC3812809.

20. Heneka MT, Carson MJ, El Khoury J, Landreth GE, Broseron F, Feinstein DL, et al. Neuroinflammation in Alzheimer's disease. Lancet Neurol. 2015; 14(4):388–405. doi: 10.1016/S1474-4422(15)70016-5 PMID: 25792098.

21. Vandannagars B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, et al. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nat Med. 2011; 17(2):179–88. doi: 10.1038/nm.2279 PMID: 21217695; PubMed Central PMCID: PMC3076025.

22. Rathinam VA, Vanaja SK, Fitzgerald KA. Regulation of inflammasome signaling. Nat Immunol. 2012; 13(4):333–42. doi: 10.1038/ni.2237 PMID: 22437086; PubMed Central PMCID: PMC3523703.

23. Shi JQ, Zhang CC, Sun XL, Cheng XX, Wang JB, Zhang YD, et al. Antimalarial drug artemisinin exerts anti-amyloidogenesis and neuroinflammation in APPswe/PS1dE9 transgenic mice via inhibition of nuclear factor-kappaB and NLRP3 inflammasome activation. CNS Neurosci Ther. 2013; 19(4):262–8. doi: 10.1111/cns.12066 PMID: 23406388.
24. Zhu T, Zhang L, Ling S, Duan J, Qian F, Li Y, et al. Scopolinol B inhibits IL-1β and cytokines expression through NF-kappaB and inflammasome NLRP3 pathways. Mediators Inflamm. 2014; 2014:819053. doi: 10.1155/2014/819053 PMID: 25386048; PubMed Central PMCID: PMC4216717.

25. Gong Z, Zhou J, Li H, Gao Y, Xu C, Zhao S, et al. Curcumin suppresses NLRP3 inflammasome activation and protects against LPS-induced septic shock. Mol Nutr Food Res. 2015. doi: 10.1002/mnfr.201500316 PMID: 26250869.

26. Li Y, Li J, Li S, Li Y, Wang X, Liu B, et al. Curcumin attenuates glutamate neurotoxicity in the hippocampus by suppression of ER stress-associated TXNIP/NLRP3 inflammasome activation in a manner dependent on AMPK. Toxicol Appl Pharmacol. 2015; 286(1):53–63. doi: 10.1016/j.taap.2015.03.010 PMID: 25791922.

27. Youm YH, Nguyen KY, Grant RW, Goldberg EL, Bodogai M, Kim D, et al. The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. Nat Med. 2015; 21(3):263–9. doi: 10.1038/nm.3804 PMID: 25688106; PubMed Central PMCID: PMC4352123.

28. Coll RC, Robertson AA, Chae JJ, Higgins SC, Munoz-Planillo R, Inserra MC, et al. A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. Nat Med. 2015; 21(3):248–55. doi: 10.1038/nm.3806 PMID: 25688105; PubMed Central PMCID: PMC4392179.

29. Andujar I, Recio MC, Bacelli T, Giner RM, Rios JL. Shikonin reduces oedema induced by phorbol ester by interfering with IkappaBalpha degradation thus inhibiting translocation of NF-kappaB to the nucleus. Br J Pharmacol. 2010; 160(2):376–88. doi: 10.1111/j.1476-5381.2010.00696.x PMID: 20423347; PubMed Central PMCID: PMC2874859.

30. Yang Y, Wang J, Yang Q, Wu S, Yang Z, Zhu H, et al. Shikonin inhibits the lipopolysaccharide-induced release of HMGB1 in RAW264.7 cells via IFN and NF-kappaB signaling pathways. Int Immunopharmacol. 2014; 19(1):81–7. doi: 10.1016/j.intimp.2014.01.003 PMID: 24447680.

31. Cheng YW, Chang CY, Lin KL, Hu CM, Lin CH, Kang JJ. Shikonin derivatives inhibited LPS-induced NOS in RAW 264.7 cells via downregulation of MAPK/NF-kappaB signaling. J Ethnopharmacol. 2008; 120(2):264–71. doi: 10.1016/j.jep.2008.09.002 PMID: 18835347.

32. Yang L, Xie M, Yang M, Yu Y, Zhu S, Hou W, et al. PKM2 regulates the Warburg effect and promotes HMGB1 release in sepsis. Nat Commun. 2014; 5. doi: 10.1038/ncomms5436.

33. Han W, Xie J, Li L, Liu Z, Hu X. Necrostatin-1 reverts shikonin-induced necroptosis to apoptosis. Apoptosis. 2009; 14(5):674–86. doi: 10.1007/s10495-009-0334-x PMID: 19288276.

34. Gwon SY, Ahn JY, Chung CH, Moon B, Ha TY. Lithospermum erythrorhizon suppresses high-fat diet-induced obesity, and acetylshikonin, a main compound of Lithospermum erythrorhizon, inhibits adipocyte differentiation. J Agric Food Chem. 2012; 60(36):9089–95. doi: 10.1021/jf3017404 PMID: 22900585.

35. Yoshimi N, Wang A, Morishita Y, Tanaka T, Sugie S, Kawai K, et al. Modifying effects of fungal and herb metabolites on azoxymethane-induced intestinal carcinogenesis in rats. Jpn J Cancer Res. 1992; 83(12):1273–8. PMID: 1483942.

36. Petrelli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J. Activation of the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. Nat Med. 2015; 21(7):713–23. doi: 10.1016/j.nm.2015.03.010 PMID: 25686108; PubMed Central PMCID: PMC4355422.

37. Rossol M, Pierer M, Raulien N, Quandt D, Meusch U, Rothe K, et al. Extracellular Ca2+ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. Nat Commun. 2012; 3:1329. doi: 10.1038/ncomms2339 PMID: 23271661; PubMed Central PMCID: PMC3396518.

38. Ollinger K, Llopis J, Cadenas E. Study of the redox properties of naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) and its glutathionyl conjugate in biological reactions: one- and two-electron enzymatic reduction. Arch Biochem Biophys. 1989; 275(2):514–30. PMID: 2512857.
43. Gao D, Hiromura M, Yasui H, Sakurai H. Direct reaction between shikonin and thiols induces apoptosis in HL60 cells. Biol Pharm Bull. 2002; 25(7):827–32. PMID: 12132652.

44. Albreht A, Vovk I, Simonovska B. Addition of beta-lactoglobulin produces water-soluble shikonin. J Agric Food Chem. 2012; 60(43):10834–43. doi: 10.1021/jf303153d PMID: 22985886.

45. Assimopoulou AN, Papageorgiou VP. Encapsulation of isohexenylnaphthazarins in cyclodextrins. Biomed Chromatogr. 2004; 18(4):240–7. doi: 10.1002/bmc.310 PMID: 15162386.

46. Assimopoulou AN, Papageorgiou VP, Kiparissides C. Synthesis and release studies of shikonin-containing microcapsules prepared by the solvent evaporation method. J Microencapsul. 2003; 20(5):581–96. PMID: 12909543.

47. Huang YI, Cheng YH, Yu CC, Tsai TR, Cham TM. Microencapsulation of extract containing shikonin using gelatin-acacia coacervation method: a formaldehyde-free approach. Colloids Surf B Biointerfaces. 2007; 58(2):290–7. doi: 10.1016/j.colsurfb.2007.04.013 PMID: 17548186.

48. Han J, Chen TX, Branford-White CJ, Zhu LM. Electrospun shikonin-loaded PCL/PTMC composite fiber mats with potential biomedical applications. Int J Pharm. 2009; 382(1–2):215–21. doi: 10.1016/j.ijpharm.2009.07.027 PMID: 19660536.

49. Kontogiannopoulos KN, Assimopoulou AN, Tsivintzelis I, Panayiotou C, Papageorgiou VP. Electrospun fiber mats containing shikonin and derivatives with potential biomedical applications. Int J Pharm. 2011; 409(1–2):216–28. doi: 10.1016/j.ijpharm.2011.02.004 PMID: 21316431.

50. Xia H, Tang C, Gui H, Wang X, Qi J, Wang X, et al. Preparation, cellular uptake and angiogenic suppression of shikonin-containing liposomes in vitro and in vivo. Biosci Rep. 2013; 33(2):e00020. doi: 10.1042/BSR20120065 PMID: 23176403; PubMed Central PMCID: PMC3561918.

51. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. Nat Immunol. 2010; 11(2):136–40. doi: 10.1038/ni.1831 PMID: 20023662.

52. Gao D, Kakuma M, Oka S, Sugino K, Sakurai H. Reaction of beta-alkannin (shikonin) with reactive oxygen species: detection of beta-alkannin free radicals. Bioorg Med Chem. 2000; 8(11):2561–9. PMID: 11092541.

53. Yang JT, Li ZL, Wu JY, Lu FJ, Chen CH. An oxidative stress mechanism of shikonin in human glioma cells. PloS one. 2014; 9(4):e94180. doi: 10.1371/journal.pone.0094180 PMID: 24714453; PubMed Central PMCID: PMC3979747.

54. Huang WR, Zhang Y, Tang X. Shikonin inhibits the proliferation of human lens epithelial cells by inducing apoptosis through ROS and caspase-dependent pathway. Molecules. 2014; 19(6):7785–97. doi: 10.3390/molecules19067785 PMID: 24962386.

55. Chen J, Xie J, Jiang Z, Wang B, Wang Y, Hu X. Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. Oncogene. 2011; 30(42):4297–306. doi: 10.1038/onc.2011.137 PMID: 21516121.

56. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. Nature. 2013; 496(7444):238–42. doi: 10.1038/nature11986 PMID: 23535595; PubMed Central PMCID: PMC4031686.