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Inflammation Research

# Shikonin inhibits IgE-mediated histamine release by human basophils and Syk kinase activity

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**Abstract.** *Objective:* Shikonin, a component of the herbal medicine "Shikon", is known to suppress inflammatory reactions, but its molecular targets are not identified. This study examines the effect of shikonin on human basophil degranulation response and aims to identify its targets.
*Materials:* Human basophils in isolated leukocytes from healthy volunteers' peripheral blood; recombinant human Syk and Lyn tyrosine kinases.
*Methods:* Histamine release from basophils stimulated with anti-IgE antibody was analyzed fluorimetrically. Syk and Lyn kinase activities were tested *in vitro* with recombinant proteins and analyzed by off-chip mobility shift assay.
*Results:* Shikonin dose-dependently inhibited the histamine release from basophils induced by anti-IgE antibody ($IC_{50}$ = 2.6 ± 1.0 μM; mean ± SEM). A search for the target(s) of shikonin in the signal cascade of IgE-mediated activation showed that it strongly inhibits Syk ($IC_{50}$ = 7.8 μM, in the recombinant kinase assay), which plays a pivotal role in the degranulation response. A less significant inhibition was found for Lyn, which phosphorylates FcεRI-βγ subunits and also Syk.
*Conclusions:* These results indicate that the inhibition of Syk-dependent phosphorylation events might underlie the blocked histamine release from human basophils, thus contributing to the anti-inflammatory effects of shikonin.

**Key words:** Shikonin – Syk – Lyn – IgE-mediated histamine release – Human basophils – FcεRI – Off-chip mobility shift assay

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## Introduction

Shikonin (β-alkannin) is a purple naphthoquinone dye extracted from *Lithospermum erythrorhizon*, a medicinal herb called "Shikon", which has been used in ointments for treating wounds and burns in China since the 5th century. It has been shown to have many pharmacological effects both *in vitro* and *in vivo* (for a review, see reference [1]), such as inhibition of tumor progression [2], anti-fungal activity [3], inhibition of angiogenesis [4], induction of apoptosis [5], accelerated granuloma-formation in rats [6] and anti-inflammatory activity [7, 8]. Wang et al. [8] have shown that hind-paw edema and plasma exudation in polymyxin B-injected mice could be prevented by acetylshikonin administration. Numerous studies have suggested different mechanisms for the anti-inflammatory effects of shikonin and related naphthoquinones such as acetylshikonin (herein denominated 'shikonins'). One is based on its radical-scavenging properties proven for reactive oxygen species like singlet oxygen, superoxide anion and *t*-butylperoxyl radical [9, 10]. Chen et al. showed another possibility, that shikonin blocks chemokine binding to CC chemokine receptor-1 [11]. Other effects that might contribute for the anti-inflammatory activity of shikonins are the prevention of leukotriene B4 biosynthesis in leukocytes [12], inhibition of superoxide-generating activity [13, 14] and impairment of phosphatidylinositol signaling in neutrophils [15]. Acetylshikonin was found to suppress histamine release from rat peritoneal mast cells stimulated with compound 48/80 [8], a classical mast cell secretagogue which directly activates GTP-binding protein and induces phospholipase C-related cell signaling [16]. The molecular mechanisms involved in the suppression of IgE-mediated degranulation by shikonins remain, however, unknown.

We have been interested in knowing whether shikonin could elicit a similar effect on basophils. It is now definitively proven that basophils play an active role in IgE-mediated development of chronic allergic inflammation and are regarded

as markers of allergy and primary allergic effector cells [17]. Therefore, we aimed herein to verify the effect of shikonin on histamine release from human basophils and identify its target(s) in the degranulation response downstream of the high-affinity IgE receptor (FcεRI). We show that shikonin prevents histamine release via inhibition of key molecules in the basophil FcεRI-mediated signaling pathway, most likely Syk.

## Materials and methods

### *Reagents*

Shikonin was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and dissolved in dimethylsulfoxide (DMSO). Goat anti-human IgE antibody was from Medical & Biological Labs (Nagoya, Japan), and all other reagents were from Nacalai Tesque (Kyoto, Japan). Purified glutathione S-transferase(GST)-tagged recombinant human proteins used in the cell-free kinase assays described below were from Carna Biosciences, Inc. (Kobe, Japan).

### *Cell preparation*

Heparinized blood was drawn from normal volunteers who gave their informed consents. Human leukocytes were isolated by dextran sedimentation as described previously [18], washed and suspended in 25 mM piperazine-*N,N'*-bis-(1-ethanesulfonic acid) (PIPES) buffer (pH 7.4) supplemented with 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.1 % (w/v) bovine serum albumin, 1 mM $CaCl_2$, and 0.4 mM $MgCl_2$. Basophils in the leukocyte fraction were used without further purification to maintain a good releasing ability, as described previously [18].

### *Histamine release*

Histamine release from human basophils was assayed essentially as described previously [19]. In brief, human leukocytes prepared as described above and suspended in PIPES buffer were incubated with various concentrations of shikonin for 10 min at 37 °C, followed by challenge with human anti-IgE antibody (1:2000 dilution) for 45 min to induce histamine release. The DMSO background was 0.1 % (v/v) in control and shikonin-containing assays, and presented no effect on the cell histamine release. Then, the supernatant was collected and analyzed for histamine content by an automated *o*-phthalaldehyde fluorometric technique. The inhibition of histamine release is expressed as percent (%) [20], using the following formula:

% inhibition = $[1 - (F_{IgE\ with\ shikonin} - F_{spontaneous})/(F_{IgE} - F_{spontaneous})] \times 100$, where $F_{IgE\ with\ shikonin}$ = fluorescence intensity of histamine released upon anti-IgE stimulation in the presence of shikonin; $F_{IgE}$ = fluorescence intensity of histamine released with anti-IgE alone (which corresponds to release of 35.6 ± 10.5 % of the total cellular histamine, mean ± SEM of 6 assays); and $F_{spontaneous}$ = fluorescence intensity of histamine released without any stimulant (which corresponds to 4.0 ± 0.4 % of the total cellular histamine). Experiments were repeated four times in duplicate and data are shown as mean ± SEM.

### *Cell-free inhibition assays with purified protein tyrosine kinases*

Inhibition by shikonin of key protein tyrosine kinases in the signal cascade downstream of FcεRI, namely Lyn (V-yes-1 Yamaguchi sarcoma viral related oncogene homolog) and Syk (spleen tyrosine kinase), was tested in *in vitro* kinase assays using recombinant proteins. The full-length human recombinant kinases (Lyn and Syk accession numbers: NP_002341 and NP_003168, respectively) were expressed using baculovirus expression system as N-terminal GST-fusion proteins and purified by glutathione Sepharose chromatography. The kinase activities were evaluated by off-chip mobility shift assay after reacting 1 μM of the indicated substrate with GST-Lyn a (Srctide: GEEPLYWSFPAKKK-$NH_2$) or GST-Syk (Blk/Lyntide: EFPIYDFLPAKKK-$NH_2$) in a reaction buffer consisted of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) containing 5 mM $MgCl_2$ plus an optimum amount of ATP (10 μM for Lyn and 25 μM for Syk assay). The final DMSO concentration in all the assays was 1 % (v/v), and had no influence in the reactions. After incubation for one hour at room temperature, the reaction was stopped by adding HEPES termination buffer containing EDTA and coating-3 reagent, and analyzed by electrophoretic separation of substrate and products [21] using a Caliper LC3000 platform (Caliper Life Sciences, Mountain View, CA). The phosphorylated product (P)/(P + substrate) ratio was calculated at each concentration of shikonin and the percent inhibition was expressed relative to the control assay in the absence of shikonin. Staurosporine was used as a reference inhibitor of Syk and Lyn kinase assays. All kinase assays were carried out at Carna Biosciences Inc. (Kobe, Japan).

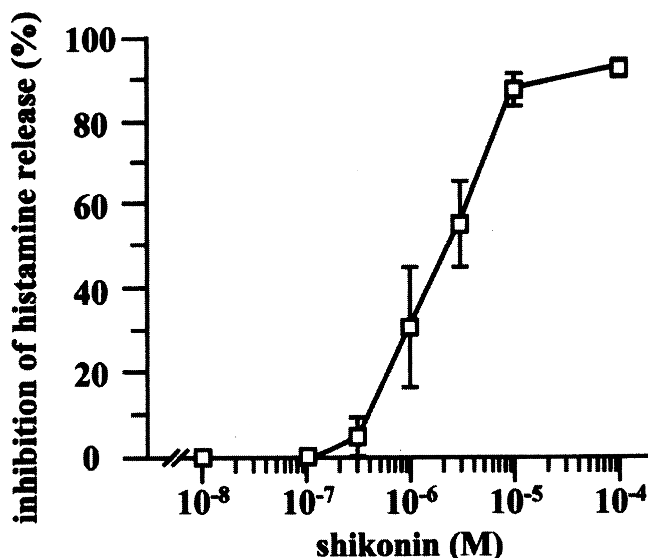


**Fig. 1.** Inhibition of histamine release by shikonin. Histamine release from human leukocytes ($1 \times 10^6$ cells/assay) was analyzed as described in Materials and Methods, after pre-incubation for 10 min at 37 °C with or without shikonin followed by challenge with human anti-IgE antibody (1:2000 dilution) for 45 min. The means ± SEM of four experiments performed in duplicate are shown.

## Results and discussion

### *Effect of shikonin on IgE-mediated histamine release from human basophils*

Like mast cells, basophils express a specific, high affinity IgE receptor (FcεRI), on the cell surface. The FcεRI is a multimeric protein composed of a unique α-subunit, one β- and two γ-subunits. When FcεRI becomes bridged upon association of receptor-bound IgE with allergens, the mediators like histamine and leukotriene $C_4$ are released and cytokines are synthesized [22, 23].

When basophils were treated with shikonin prior to challenge with anti-IgE antibody, histamine release decreased

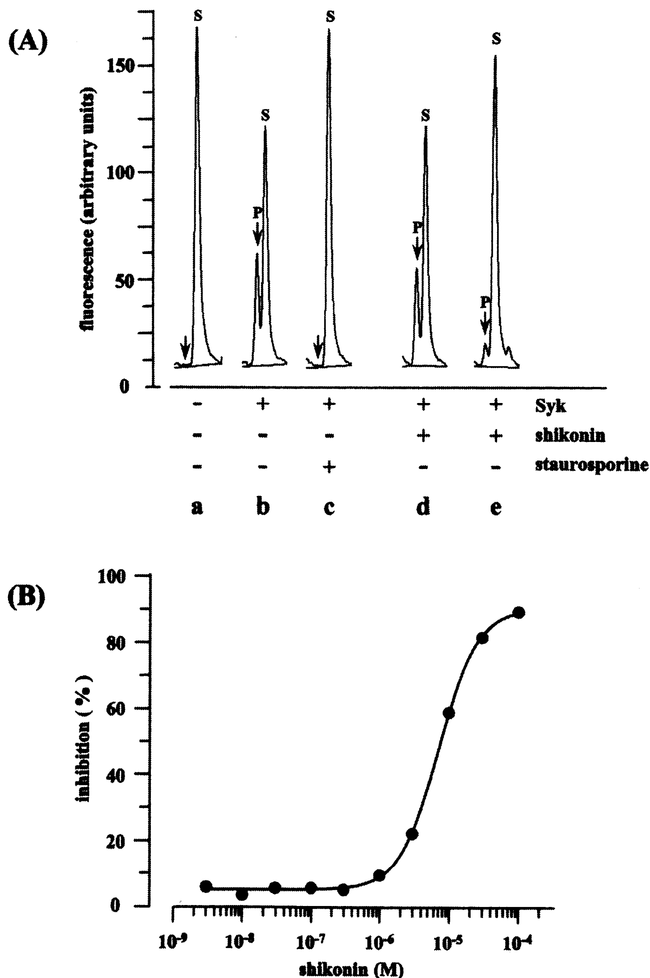


**Fig. 2.** Shikonin inhibition of Syk kinase activity as determined by off-chip mobility shift assay. Recombinant Syk was incubated with fluorescence-labeled substrate, Blk/Lyntide, in the presence of $Mg^{++}$ and ATP and incubated for 1 h at room temperature. The kinase reaction was stopped as described in Materials and Methods and the quenched reaction products were serially slipped into sipper capillary connection of Caliper LC3000. The phosphorylated product (P) and the remaining non-phosphorylated substrate (S) were then separated into two peaks by a high electric field and directly detected using fluorescence. **(A)** The panel shows representative charts of a reaction without Syk (a), plus Syk but without any inhibitor (b), or with 30 nM staurosporine (c), or with 30 nM (d) or 30 μM (e) shikonin. The P/(P + S) ratio in the absence of shikonin was 0.317. **(B)** Inhibition of Syk kinase activity by shikonin, assayed as described above: percent inhibition is expressed relative to the control assay in the absence of shikonin. Staurosporine was used as a reference inhibitor of the assay and presented an $IC_{50}$ of 0.18 nM. The curve is representative of two independent experiments, and points are the means of duplicate assays.

dose-dependently. As shown in Fig. 1, shikonin inhibited the IgE-mediated histamine release with an $IC_{50}$ of 2.6 ± 1.0 μM. About 90% of inhibition occurred with 10 μM shikonin, which alone was unable to cause histamine release from human basophils. The strong inhibition of IgE-mediated basophil degranulation response by shikonin agrees with a previous report that acetylshikonin suppresses histamine and β-glucuronidase release from rat peritoneal mast cells stimulated with compound 48/80 ($IC_{50}$ of ~11 μM) [8].

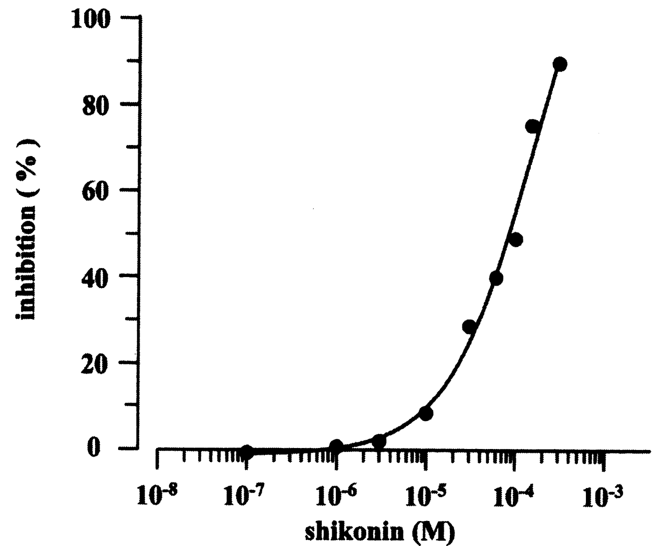


**Fig. 3.** Inhibition of Lyn kinase activity by shikonin. Lyn kinase activity was assayed *in vitro* with recombinant Lyn and Srctide as substrate, similarly to the assay described in Fig. 2A. Percent inhibition is expressed relative to the control assay in the absence of shikonin [P/(P + S) ratio = 0.435]. Staurosporine was used as a reference inhibitor of the Lyn kinase assay and presented an $IC_{50}$ of 1.5 nM. The curve is representative of two independent experiments, and points are the means of duplicate assays.

*Shikonin-targeted molecules as determined by off-chip mobility shift assay*

Next, we investigated which pathway(s) of the FcεRI-mediated degranulation response is targeted by shikonin. FcεRI-crosslinking activates the receptor-associated Src family protein kinase, Lyn, which phosphorylates tandem tyrosines of the immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic domains of the β- and γ-subunits of FcεRI [24]. These ITAM phosphotyrosines provide docking sites for the SH2 domains of Syk. Upon binding with phosphorylated ITAMs, Syk becomes activated through its auto- and also Lyn-dependent phosphorylations (reviews [23, 25]). Activated Syk then phosphorylates downstream proteins including linker for activation of T cells, SH2-containing leukocyte-specific protein of 76 kDa (SLP-76), Vav, phospholipase C (PLC)-γ1 and PLC-γ2 [23].

Since Lyn- and Syk-catalyzed phosphorylation events are at the almost top site in the FcεRI-signaling, we tested whether their kinase activities are affected by shikonin in a cell-free assay using recombinant proteins followed by off-chip mobility shift assay. The off-chip mobility shift assay explores the electrophoretic mobility of labeled substrates upon enzymatic conversion to product [21]. A preliminary test performed with addition of 10 μM shikonin to the assay resulted in a strong inhibition of Syk activity by 88%, suggesting that Syk could be a major target of shikonin in the IgE-mediated FcεRI response. A mild inhibition of Lyn was also observed.

Then, the effect of shikonin on the Syk and Lyn kinase activities *in vitro* were further investigated in the recombinant assay. The resulting $IC_{50}$ for Syk was 7.85 μM (mean of two separate experiments, which gave $IC_{50}$s of 8.0 and 7.7 μM, re-

spectively; Fig. 2B), and that for Lyn, around ten-fold higher (70 μM; Fig. 3). These results indicate that shikonin affects Syk stronger than Lyn. The $IC_{50}$ for Syk, although being in the same order of magnitude, was three-fold higher than that of the cell-based assay using intact human basophils (Fig. 2B versus Fig. 1). Such a difference between the $IC_{50}$s might suggest that shikonin affects other signaling molecule(s) downstream of Syk and Lyn in the IgE-mediated response at the cellular level, a possibility that we do not exclude. In an attempt to verify effects of shikonin in downstream signaling pathways, its influence on $Ca^{2+}$ ionophore (A23187)-stimulated histamine release of basophils was checked: no inhibition was observed up to 3 μM, and higher concentrations still gave modest inhibition (data not shown). These results favor the view that downstream $Ca^{2+}$-dependent pathways in the degranulation response are less affected by shikonin. This interpretation was supported by previous findings that acetylshikonin was not inhibitory towards phorbol ester-stimulated protein kinase C of neutrophils ($Ca^{2+}$-dependent conventional PKC) [17]. However, further investigations about whether shikonins affect multiple components in the whole cell are required.

Another explanation for justifying the lower $IC_{50}$s in the cell-based assay might be synergistic effects of Syk and Lyn inibitions. Within the cell, Syk activation depends on its initial phosphorylation by Lyn. Therefore, even though being weaker, the inhibition of Lyn by shikonin might relay the effects to Syk activity at the cellular level, leading to a more drastic blockage of downstream signaling, thus lowering the $IC_{50}$ in intact basophils. In addition, there is possibility of shikonin affecting Syk's autophosphorylation ability. In the cell, autophosphorylation of Syk is proposed to occur by transphosphorylation of a Syk molecule bound to one receptor by another Syk bound to the adjacent receptor [26, 27]. Because the effect of shikonin in the autophosphorylation of Syk could not be checked in our recombinant kinase assay standardized with pre-phosphorylated Syk protein, this issue remains to be elucidated.

Nevertheless, Syk targeting at upstream level in the degranulation response might contribute considerably to prevent histamine release.

### *Implications of present findings with inhibitory effects of shikonin in inflammatory cells*

Besides basophils [28], Lyn is expressed in platelets, granulocytes, macrophages, mast cells, and B cells [29], and Syk is found in a wide variety of cells, including mast cells [30], macrophages [31], B-cells [32], neutrophils [33] and dendritic cells [34]. Consequently, shikonin effects based on Syk and Lyn inactivation would be extensive to cells where these kinases are expressed. This might be the case of a previously reported prevention by acetylshikonin of mast cell degranulation in the hind-paw edema model [8], as well as the inhibition of neutrophil respiratory burst caused by impaired tyrosine phosphorylation reactions [14]. Also, the previously reported inhibition of PLC activity in neutrophils by acetylshikonin [15] might involve targeting of Syk, because Syk is the upstream kinase that phosphorylates PLC leading to its activation [23].

In addition, the herein proposed suppression of FcεRI-mediated degranulation response by shikonin through Syk-targeting in basophils is in good agreement with the effects observed with other Syk inhibitors like piceatannol [35], flavonoids [36], R406 [37, 38], ER-27319 [39], and R112 [40]. All of them prevent degranulation of mast cells [35–40], and also the production of interleukins and tumor necrosis factor-α [37, 38] thus preventing allergy and inflammation of airways [37] and arthritis [38]. Among these Syk inhibitors, piceatannol and ER-27319, though presenting $IC_{50}$s of around 10 μM, had their clinical use hindered by non-specific effects or toxicity. In this respect, shikonin is likely a new promising lead molecule for anti-allergic and anti-inflammatory drug targeting Syk, since "Shikon" herb has been used safely in ointments from ancient times.

In conclusion, we propose that the inhibition of IgE-mediated basophil histamine release by shikonin might involve inactivation of Syk. Since Syk is a key molecule in the FcεRI-mediated inflammatory response which leads to production of leukotrienes, cytokines, chemokines and histamine, Syk is a promising target for development of an anti-inflammatory drug derived from shikonin.

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