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Research Article
Identification of Novel Targets for Shikonin as a Potent Drug for Inflammation and Cancer

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Abstract

Background and Objective: Shikonin is a natural medicinal substance with a wide spectrum of pharmacological activities. In last two decades shikonin has been extensively explored for its modulatory effect on diverse cellular signaling in multiple disease areas primarily in oncology. Shikonin was extensively evaluated in native mammalian cells and kinases in the area of oncology. Despite the fact of having enough scientific knowledge in this field, there are still opportunities to identify novel inflammatory and oncology targets that were not previously screened towards the effect of shikonin. The objective of this study is to examine the therapeutic potential of shikonin on disease specific targets, involved in cancer and inflammation pathogenesis. **Materials and Methods:** This study has evaluated the effect of shikonin on panel of GPCRs and recombinant enzymes. Human neutrophils were also tested to analyze the inhibitory role of shikonin on CXCR2 receptor. Cytotoxic effect of shikonin was studied in multiple cancerous mammalian cell lines at varying doses and incubation time. **Results:** Shikonin showed significant and differential inhibition of tested inflammatory and oncology targets. Moderate inhibition of adenosine 2B and histamine 1 receptors was observed post shikonin treatment with IC_{50} of 5.6 and 8 μM, respectively. Shikonin displayed strong anti-inflammatory and anticancerous effect on CXCR2 receptor by displacing IL-8 and showed IC_{50} of 1.3 and 1.4 μM on CXCR2 over expressed cells and human neutrophils, respectively. Shikonin also notably inhibited IL-8 induced neutrophil migration with IC_{50} of ~1 μM. The CRTh2 receptor and NRF2 protein were also found to be susceptible targets for shikonin. Shikonin exhibited marked effect on CRTh2 and NRF2 targets with IC_{50} of 1 μM and EC_{50} of 7 μM, respectively. Myelogenous leukemia and colon carcinoma cells were found to be most susceptible for shikonin in cell viability assay and showed LD_{50} of 12 and 40 nM at 72 h treatment. **Conclusion:** Shikonin has strong and promising anti-inflammatory potential via CXCR2, CRTh2 and NRF2 targets and can be developed as an anti-inflammatory drug in future through rational drug designing and structural activity relationship.

Key words: Shikonin, inflammation, cancer, CXCR2, CRTh2, NRF2

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The use of herbs and natural products is well known for the treatment of various ailments from prehistoric times. Traditional medicines have been practiced throughout the world well before the introduction of allopathic medicine. Alfalfa, aloe vera, asafoetida, chamomille, cinchona, digitalis, ginseng, opium and wheatgrass are the few examples of traditional medicines. Shikonin is a natural medicinal substance found in the roots of plants that belong to family Boraginaceae such as *Arnebia hispidissima*, *Arnebia euchroma*, *Lithospermum erythrorhizon* and *Onosma paniculatum*. *Lithospermum erythrorhizon* is a well known plant used in Chinese herbal medicine. The root of *Lithospermum erythrorhizon* has been utilized from early days as a drug for healing burns, inflammation and wounds[1]. *Arnebia* is a genus of hispid herbs, mostly confined to Asia with a few species occurring in drier parts of North Africa. Seven species of this plant are known to occur in India. These include *A. benthamii*, *A. euchroma*, *A. guttata*, *A. hispidissima* and *A. nobilis*[2]. Roots of various boraginaceous plant species especially *Arnebia* spp. is named as ratanjot and it is used as indigenous herbal medicine, coloring to foodstuff, oils and fats. Shikonin has wide applications as a colorant in food industries and as a dye for silk and cosmetic industries[3]. Shikonin is the first plant secondary metabolite to be commercially produced via plant cell culture. It is produced in small quantities in plant tissue culture as a secondary metabolite and to overcome this limitation, several techniques have been developed to enhance its production, induction of shikonin production in hairy root cultures of *Arnebia hispidissima* had been established through *Agrobacterium rhizogenes*-mediated genetic transformation[3,4].

Nutraceuticals are products that provide health and medicinal benefits including the prevention and treatment of diseases in addition to the basic nutritional value found in foodstuff[5]. Nutraceutical has advantage over the synthetic medicine because they have fewer side effects, used as naturally dietary supplement, easily available and economically affordable. In the recent time, herbal nutraceuticals are used as a powerful tool in maintaining health and to fight against diseases, enhancing longevity and quality of life. Major nutraceuticals include vitamins, PUFA, phytochemicals, prebiotics, minerals, amino acids, fibers, carotinoids, polyols and probiotics. Nutraceuticals are considered to be one of the fastest developing areas of interest for human health and disease prevention. The most rapidly growing nutraceuticals are dietary supplements (19.5% year^{-1}) and natural products[6] (11.6% year^{-1}). These nutraceuticals have proven their medicinal effect in different disease areas such as dyslipidaemia[7], cardiovascular disorders[8], cancer[9], inflammation[10], metabolic diseases[11] and many more.

Shikonin is an important component of the root of *Arnebia* plant which is used in cooking and also considered as a nutraceutical ingredient. As a nutraceutical preparation, shikonin has shown alleviative effects in multiple disorders such as neurodegenerative diseases[12], cancer[13] and metabolic disorders[14].

Shikonin is also known as a pleotropic medicinal agent and exhibited its beneficial role in multiple therapeutic areas. In oncology, shikonin has shown compelling anticancerous effect through several cellular mechanisms such as inhibition of pyruvate kinase-M2[15], NF-κB[16], matrix metalloproteinase-9[17], p-PI3K and p-Akt expression[18], STAT3 and IGF-IR phosphorylation[19] and many more. Various teams across the scientific community now are focusing to elucidate the possible mechanism of action of shikonin in other therapeutic areas like inflammation, metabolic and cardiovascular disorders and pain. In a very recent study, shikonin was shown to have promising analgesic effect in multiple *in vivo* pain models through sodium channel modulation[20].

To elucidate the mode of action of shikonin in biological system, most common approach of researchers were to investigate its effect on cellular signaling events using mammalian wild type cell lines. In spite of many years of investigation, limited data of shikonin action is available on cell surface receptors particularly on GPCR and enzyme targets. In the current study, the aim of this study is to explore the effect of shikonin on specific targets, mostly on over expressed receptors and recombinant enzymes involved in the cancer and inflammation.

To evaluate the therapeutic potential of shikonin in cancer, few recent and relevant oncology targets like HDAC, MERTK, FLT3, TrkA, BRD4, autotaxin and A2B which were not tested previously for the effect of shikonin were analyzed in this study. In the area of inflammation, possible role of shikonin was studied on inflammatory disease targets like CXCR2, cannabinoid receptor type 2 (CB2), prostaglandin E2 and E4 (PGE2 and PGE4), adenosine 2B (A2B), histamine 1 (H1), chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2) autotaxin (ATX), pantetheinase 1, nuclear respiratory factor 2 (NRF2) and TNFα converting enzyme (TACE) in this segment of study. This study has also investigated the effect of shikonin on multiple cancerous cell viability and human neutrophil secondary signaling mechanism.

MATERIALS AND METHODS

Molecular biology and cell culture reagents were obtained from Thermo Fisher Scientific (USA) unless otherwise mentioned. All other chemicals were procured either from Sigma Aldrich (USA) or Tocris (USA). Shikonin (No. S7576) was procured from Sigma Aldrich (USA). Human Autotaxin enzyme (No. E-4000) and FS-3 substrate (No. L-2000) were obtained from Echelon Biosciences Inc (USA). Assay kits such as cyclic adenosine monophosphate (cAMP) homogeneous time resolved fluorescence (No. 62AM4PEC), HDAC (No. 17-372) and NRF2 LiveBLAzer-FRET B/G Loading Kit (No. K1096) were purchased from Cisbio (USA), Millipore (USA) and Thermo Fisher Scientific (USA), respectively. Wild type mammalian cell lines were obtained from American Type Culture Collection (USA).

Recombinant cell line generation: The RNA was isolated from mammalian cell lines and utilized for the preparation of cDNA through reverse transcription PCR. The PCR amplified products were cloned in respective expression vectors and transfected in native mammalian cell lines. Recombinant cells were cloned and characterized in multiple *in vitro* assays[20].

Intracellular Ca^{2+} mobilization assay: Intracellular calcium mobilization was measured using the Fluo-4 dye in FLIPR TETRA as reported previously[20]. Briefly, cells were seeded in 96 well plates and grown to 90% confluence overnight. Next day, cells were loaded with Fluo-4 AM dye and then desired concentration of shikonin and standard inhibitors were added for 30 min. Plate was placed in FLIPR together with a separate plate containing specific agonists to stimulate Ca^{2+} influx. The FLIPR was programmed to transfer the agonists to cell plate just after 30 sec of commencement of recording. Freshly isolated human neutrophils at concentration of 1×10^5 cells per well were seeded on the day of assay and assayed using same protocol.

cAMP accumulation assay: Mammalian cells expressing target receptor were detached and resuspended in assay buffer and seeded in 384 half well black assay plate (Greiner Bio One No. 784076) at a density of 1×10^4 cells per well. Desired concentration of shikonin/standard antagonist dilutions were added and incubated for 15 min at 37°C in 5% CO_2. Cells were stimulated with specific agonist, incubated for 30 min and then lysed and the cAMP content was measured using the cAMP dynamic HTRF detection kit. In case of Gi pathway specific receptors, forskolin was added along with agonist to produce cAMP[20].

Radioligand binding assay: The CRTh2 receptor binding assay was performed in SPA (scintillation proximity assay) format utilizing CRTh2 over expressed cell membrane. About 25 µL of PVT-WGA SPA beads (Perkin Elmer, USA No. RPNQ 0001) were added at a final concentration of 0.2 mg per well in a non binding surface flat clear bottom white 96 well plate. About 25 µL of various dilutions of shikonin/standard antagonist and 25 µL of cell membrane (Europhins, USA No. HTS031M) at concentration of 10 µg per well were added. About 25 µL of [3H] hPGD2 radioligand (5 nM) was then added to the assay plate. Plate was sealed and incubated for 1 h at ambient temperature in the dark. Final concentration of DMSO was maintained at 0.5% in the assay. All the reagents were prepared in binding buffer (50 mM Hepes pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl, 10 µg mL^{-1} saponin and 0.2% BSA). Radioactive counts were determined in the Wallac MicroBeta Trilux counter.

Enzyme assays: For the enzyme assays, optimized concentration of an enzyme was incubated with specific substrate in presence or absence of shikonin or standard inhibitor for 30 min. The HDAC, MERTK, FLT3 and TrkA enzyme assays were performed according to manufacturer's kit protocol. The BRD4 assay was based on Fluorescence Polarization (FP) technology using Cy5 probe. Autotaxin, pantetheinase 1 and TACE assays were performed using recombinant enzyme and respective fluorescent substrate. In all the assays, fluorescence readings were obtained in a multimode fluorescence plate reader at specific wave length of excitation and emission. Autotaxin, TrkA kinase and FLT3 Z-LYTE biochemical assays were performed according to Gupta *et al.*[20].

ARE-bla HepG2 reporter gene assay: Effect of shikonin was evaluated on NRF2 protein in gene blazer assay, using manufacturer's protocol (Thermo Fisher Scientific, USA). In brief, 15×10^3 cells (CellSensor® ARE-BLA HepG2 cell line contains a β-lactamase reporter gene) were plated in a 384 well plate and incubated for 5 h at 37°C in 5% CO_2. Varying dilutions of shikonin and standard compound (tBHQ) were added to the plate and incubated for 18 h at 37°C in 5% CO_2. About 5 µL of β-lactamase dye solution was then added to the cell plate and incubated for 2 h at room temperature in dark. Cell plate was read on envision multimode reader for determination of blue/green (460/535) emission ratio.

Migration assay: Neutrophils were washed once and resuspended in chemotaxis buffer (HBSS+0.1% bovine serum albumin). Cells were plated at concentration of 1×10^5 in

25 µL volume in the upper chamber of 96 well chemotaxis plate (5 µm pore size). Varying dilutions of shikonin or test compounds were added in the same chamber in 25 µL volume and incubated for 30 min at 37°C in 5% CO_2. About 1 nM of IL-8 was placed as an agonist in lower chamber in 150 µL volume. Plate was incubated for 1 h at 37°C in a 5% CO_2 cell culture incubator to allow cell migration. On completion of incubation, 100 µL of cells were transferred from lower chamber to a separate 96 well white tissue culture plate and mixed with 25 µL of cellTitre-Glo (Promega No. G7570). Results were quantified in terms of luminescence read in envision.

MTS cell viability assay: The CellTiter 96® AQueous assay is based on the reduction of the tetrazolium salt, MTS, to a colored formazan compound by viable cells in culture. This assay was performed using manufacturer's protocol (Promaga No. G3580). Briefly cells were incubated for 30, 60 min, 4, 24, 48 and 72 h with varying dilutions of shikonin at 37°C and then 10 µL of MTS solution was added to the cell plate. Absorbance read was measured at 490 nm. Mammalian cancerous cell lines: MCF-7, HeLa, HEPG2, K562, PC3 and COLO205 were assayed in this study to analyze the cytotoxic effect of shikonin.

Statistical analysis: The IC_{50} (50% inhibitory concentration) values were determined from 10-point concentration-response curves fitted to the sigmoid modified Hill equation:

$$y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10 \wedge [(\text{LogEC}_{50} - x)] \times \text{Hill slope}}$$

where, x is the logarithm of the concentration, y is the response, all curve fittings were performed using GraphPad (San Diego, CA) Prism 5.0 or excel based Macros software developed in house at TCG Life Sciences (Kolkata, India).

RESULTS

Effect of shikonin on oncology targets

Cell viability assay: To determine the cytotoxic effect of shikonin, multiple mammalian cancerous cell lines of varying lineage were studied in MTS assay. Serial dilutions of shikonin were tested on these cells at different incubation period. The MCF-7 a human breast cancer cell line showed dose dependent sensitivity for shikonin in MTS assay. At 1 h incubation, MCF-7 cells showed LD_{50} of ~19.7 µM (LD_{50} = 50% lethal dose) and the LD_{50} decreased to 6 µM at 24 h, 3.1 µM at 48 h and 1.1 µM at 72 h, respectively. The HeLa is human cervical carcinoma cells showed no sensitivity toward shikonin upto 1 h but at 4 h it exhibited LD_{50} of 17.9 µM which reduced with longer incubation period. At 24, 48 and 72 h incubation, calculated LD_{50} values were 4.5, 1.2 and 1 µM, respectively. The HEPG2 cells (human liver carcinoma cells) exerted more sensitivity towards shikonin: At 1 h incubation LD_{50} was 13.6 µM which decreased to 8.4 µM, 252 and 213 nM at 24, 48 and 72 h, respectively. The PC3 cells (human prostate cancer cell) also showed dose dependent sensitivity towards shikonin and demonstrated LD_{50} of 48, 15.6, 3.9 µM, 320 and 179 nM at 1, 4, 24, 48 and 72 h incubation, respectively. Shikonin demonstrated high cytotoxicity on human colon cancer cells (COLO205) and showed 6.4, 3.8 µM, 170 and 41 nM LD_{50} at 1, 24, 48 and 72 h incubation. On K562 cells (Myelogenous Leukemia) shikonin showed no cytotoxic effect upto 4 h but post 24 h it showed very high cell death with LD_{50} of 16 µM, 200 and 12 nM at 24, 48 and 72 h exposure, respectively. Cell viability data has been compiled in Table 1 and Fig. 1a-f.

Intracellular calcium mobilization and enzyme assay: To analyze the effect of shikonin on oncology targets, GPCRs: A2B and CXCR2, enzymes: HDAC, MERTK, FLT3, TrkA, autotaxin and proteins: NRF2 and BRD4 were studied. However shikonin didn't show significant inhibitory effect on these tested targets of cancer except CXCR2 and NRF2 protein.

Effect of shikonin on inflammation targets: To evaluate the efficacy of shikonin in inflammation mediated diseases, 11 biological targets that are predominantly involved in inflammation were tested employing multiple *in vitro* assays. The CXCR2, CB2, PGE2, PGE4, A2B, H1, CRTh2 autotaxin, pantetheinase 1, TACE and NRF2 were chosen to determine the anti-inflammatory effect of shikonin.

The PGE2, PGE4, CB2 and CRTh2 receptors were over expressed in endogenous cells and tested in intracellular cAMP accumulation assay (Fig. 2a, b). The A2b, H1 and CXCR2 receptors were tested in intracellular calcium mobilization assay (Fig. 3a, b). Human neutrophils were also evaluated in

Table 1: Cytotoxic effect if shikonin on cancerous cells

Incubation time	LD_{50} (µM)					
	MCF7	HeLa	HEPG2	PC3	COLO205	K562
30 min	22.00	No lethal	21.10	53.00	6.9	No lethal
1 h	19.70	No lethal	13.60	48.00	6.4	No lethal
4 h	18.00	17.90	10.40	15.60	3.20	45.00
24 h	6.00	4.50	8.40	3.90	3.8	16.30
48 h	3.10	1.20	0.25	0.32	0.17	200.00
72 h	1.10	1.00	0.21	0.18	0.04	11.70

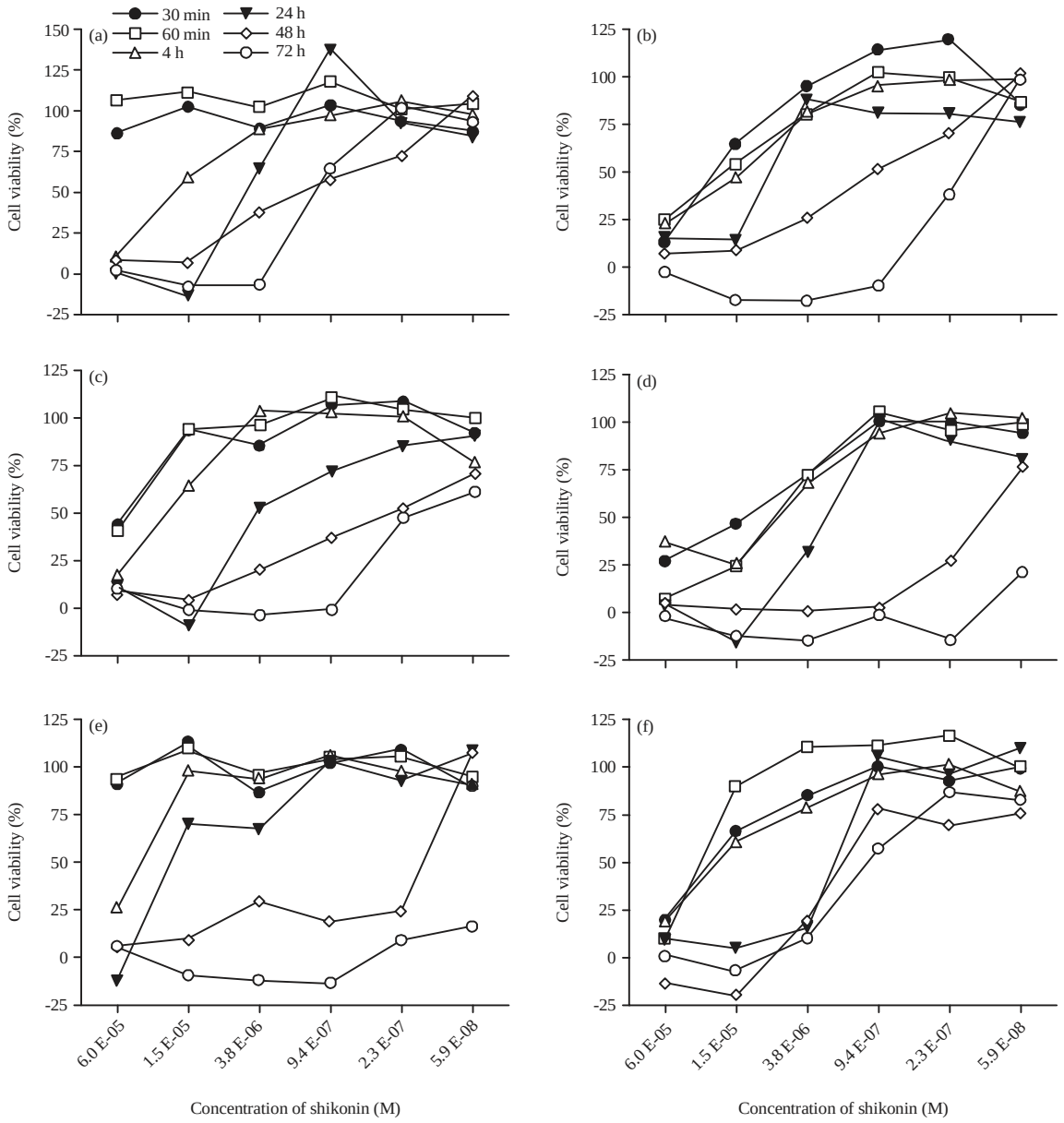

Fig. 1(a-f): Cytotoxic effect of shikonin on mammalian cancerous cell lines at varying incubation time in MTS cell viability assay, shikonin displayed a strong dose dependent cell killing effect on (a) HeLa, (b) HEPG2, (c) PC3, (d) COLO205, (e) K562 and (f) MCF7 cells and data are expressed as Means±SEM of four replicates

calcium assay to determine the effect of shikonin on endogenously expressed CXCR2 receptors (Fig. 3c). Autotaxin, pantetheinase 1 and TACE targets were tested in enzyme inhibition assay whereas NRF2 was evaluated in ARE-bla reporter gene based assay (Fig. 4). Shikonin was also tested in cell migration assay using human neutrophils that endogenously express CXCR2 receptors (Fig. 5).

Shikonin displayed differential and specific effect on studied targets (Table 2). (1) Shikonin didn't exert any inhibitory effect on autotaxin, pantetheinase 1 and TACE enzymes. (2) There was very feeble effect of shikonin (>10 μM IC_{50}) was found on CB2, PGE2 and PGE4 receptors. (3) On A2B and H1 receptors, shikonin demonstrated moderate inhibition with IC_{50} of 5.6 and 8 μM (Fig. 3a). (4) Very promising effect of shikonin was observed on NRF2 target with EC_{50} of 7 μM (Fig. 4) in ARE-bla reporter gene assay, compared to standard compound (tBHQ) which gave EC_{50} of ~8 μM in same assay conditions. (5) Shikonin showed high potency on CRTh2 receptor and demonstrated IC_{50} of ~1 μM in cell based intracellular cAMP accumulation assay (Fig. 2b).

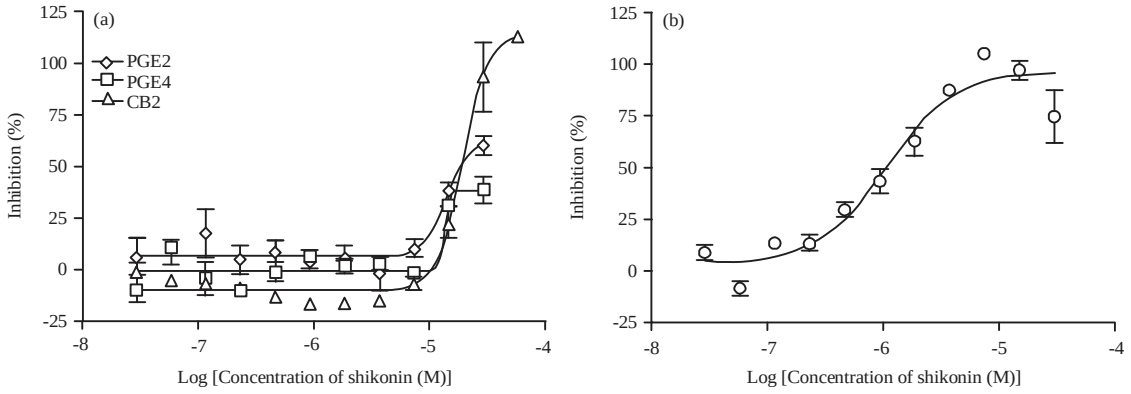

Fig. 2(a-b): Effect of shikonin on inflammation targets evaluated in intracellular cAMP assay, (a) PGE2, PGE4 and CB2 over expressed cells and (b) CRTh2 over expressed cells, shikonin showed significant inhibition of CRTh2 receptors with IC_{50} of ~1 μM and data are expressed as Means±SEM of four replicates

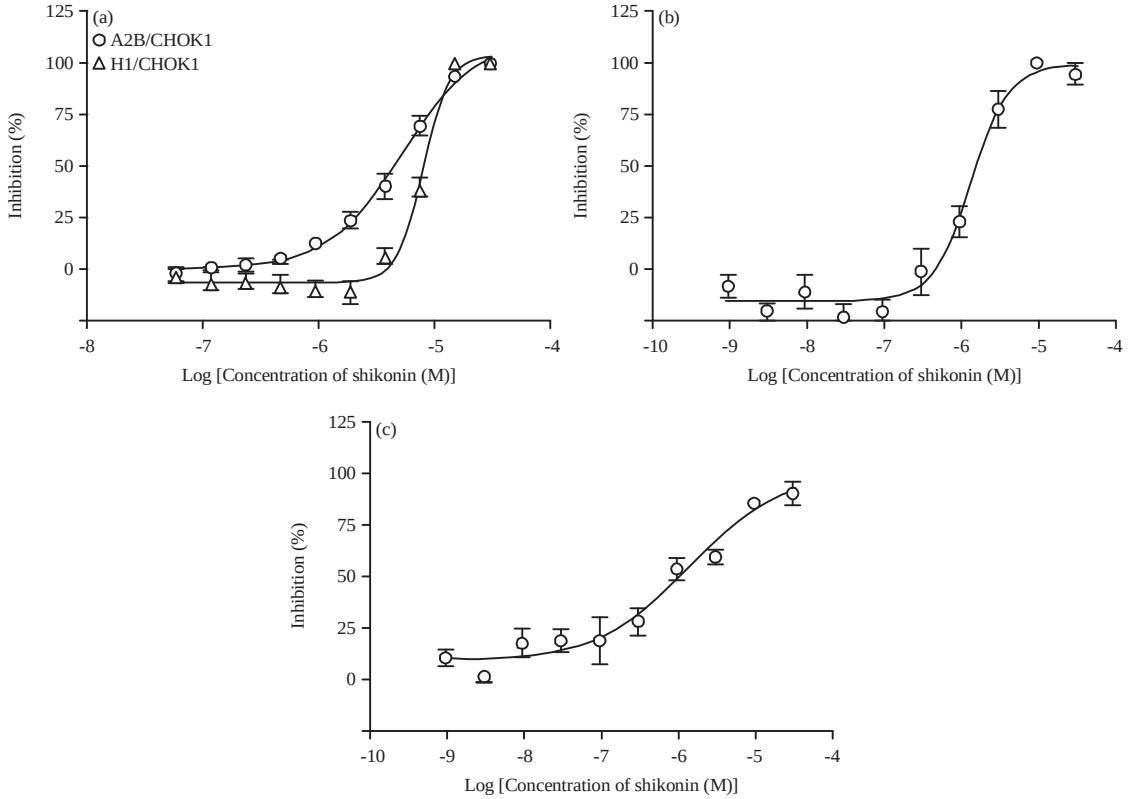

Fig. 3(a-c): Effect of shikonin on inflammation targets evaluated in intracellular calcium mobilization assay, (a) A2B and H1 over expressed cells, (b) CXCR2/U937 cells and (c) Human neutrophils, shikonin showed significant inhibition of CXCR2 receptors on over expressed cells and human neutrophils with IC_{50} of 1.3 and 1.4 μM, respectively and data are expressed as Means±SEM of four replicates

In contrast, when shikonin was tested in CRTh2 radioligand binding assay, it didn't show any inhibition of [3H] PGD2 binding to the CRTh2 receptor. (6) This study also found significant antagonistic effect of shikonin on CXCR2 receptor in intracellular calcium mobilization assay and cell migration assay. With recombinant CXCR2 cells as well as human neutrophils, shikonin demonstrated notable inhibition of hIL-8 and showed IC_{50} of 1.3 μM (Fig. 3b) and 1.4 μM (Fig. 3c) in

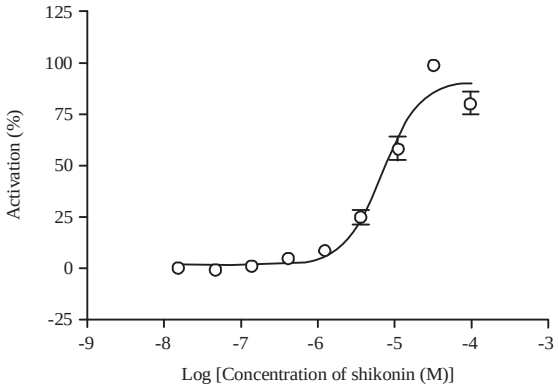

Fig. 4: Effect of shikonin in ARE-bla and HepG2 reporter gene assay, shikonin showed prominent activation of NRF2 with EC_{50} of 7 μM compared to its standard stimulator which showed 8 μM EC_{50} (data not shown) and data are expressed as Means±SEM of eight replicates

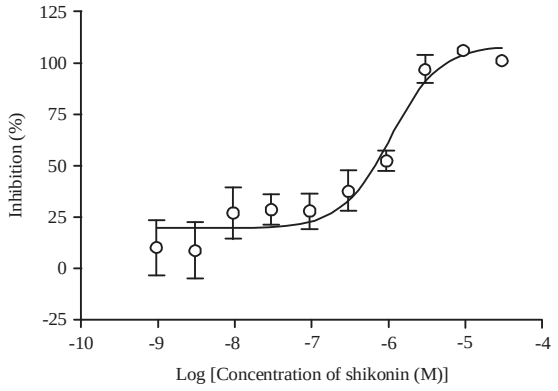

Fig. 5: Effect of shikonin on freshly isolated human neutrophils in IL-8 induced migration assay, shikonin showed marked inhibition of CXCR2 mediated human neutrophil migration with IC_{50} of ~1 μM and data are expressed as Means±SEM of four replicates

Table 2: Effect of shikonin on therapeutic targets in inflammation

Therapeutic targets	IC_{50} (μM)
Autotaxin	No inhibition
Panthetheinase 1	No inhibition
TACE	No inhibition
CB2	>10
PGE2	>10
PGE4	>10
A2B	5.6
H1	8
NRF2	7
CRTh2 (cAMP assay)	1
CXCR2 (calcium assay)	1.3
Human neutrophils (calcium assay)	1.4
Human neutrophils (migration assay)	1

calcium mobilization assay. In subsequent experiment, shikonin showed marked inhibitory effect in IL-8 induced neutrophil migration assay with IC_{50} of ~1 μM (Fig. 5).

DISCUSSION

Target-identification is a crucial step in small molecule drug discovery paradigm. Development of a new drug is a complex and very lengthy process and involves 10-12 years from target identification to its launch in the market. The whole process of drug discovery includes several steps such as target identification and validation, synthesis, preclinical screen, clinical study (phase 1-3), approval, marketing and post marketing examination. In modern drug discovery, selecting a specific disease target followed by identification of a potent drug able and safe compound is of prime interest. Thus, identified compound if shows a specific effect on the target with a modulation of the signaling pathway is a differentiator among the same class of lead molecule.

Shikonin is a well known medicinal agent and possess diverse pharmacological properties[21]. Its effect was evaluated largely in anticancerous and modestly in few anti-inflammatory signaling pathways but its target specific effect is still sketchy. Inhibitory role of shikonin on GPCRs, ion channels, relevant enzyme targets and proteins has not well established yet.

In this study, the aim was to identify the specific disease target of shikonin primarily in the area of oncology and inflammation. To establish the target specific effect, chosen number of recent and disease relevant targets and evaluated shikonin for its activity as inhibitor or activator especially on GPCRs, enzymes and protein.

The initial effort was to determine the cytotoxic potency of shikonin on multiple cancerous cells from diverse origin followed by limited panel screening on few recent oncology targets.

Shikonin was tested on several cancerous cell lines of different origin such as liver cancer (HEPG2) breast cancer (MCF-7), cervical carcinoma (HeLa), prostate cancer (PC3), colon carcinoma (COLO205) and myelogenous leukemia (K562) for its cytotoxic effects at varying incubation time from 30 min to 72 h. Shikonin displayed specific and differential cell killing effect at varying doses and incubations on these mammalian cells of diversified origin Fig. 1(a-f).

In this study, myelogenous leukemia cells were found to be most sensitive towards shikonin at longer incubation and exhibited 200 and 12 nM LD_{50} at 48 and 72 h, respectively. Myelogenous leukemia is a malignant cancer of white blood

cells and is a huge burden for society with high mortality. So far, many drugs have been approved and tested for the treatment of myelogenous leukemia. These drugs are efficacious. However, these have safety concerns and also induced drug resistance. Shikonin might be a new and interesting choice of lead compound in future considering its potent and lethal effect on these cells. Colon carcinoma cells also showed high sensitivity towards shikonin even at shorter incubation (~7 μM LD_{50} at 30 min). Shikonin showed huge death of COLO205 cells at 48 and 72 h with LD_{50} of 170 and 40 nM, respectively. Liver carcinoma and prostate cancer cells showed moderate sensitivity for shikonin at early incubation but demonstrated substantial cell killing effect at 48 and 72 h: HEPG2 cells showed LD_{50} of 250 and 210 nM where as PC3 cells exhibited LD_{50} of 320 and 180 nM at 48 and 72 h, respectively. Breast cancer and cervical carcinoma cells displayed lesser sensitivity for shikonin and showed ~1 μM LD_{50} at 72 h. Similar study was published in 2006, where scientists found LD_{50} of shikonin ~3.9 μM on MCF-7 cells at 72 h incubation[22].

To the best of the knowledge, this is the first comprehensive report to establish the cell killing effect of shikonin on several cancerous cell lines at six different incubations (30 min to 72 h) in a dose dependent manner. The results confirm the substantial and significant cell killing ability of shikonin on broad range of cancerous cells. These results imply that shikonin could be developed as an effective anticancerous compound in future.

Numerous studies reported compelling effect of shikonin in cancer, which works via diverse signaling mechanism: Inhibition of pyruvate kinase-M2[15], NF-κB[16], matrix metalloproteinase-9[17], p-PI3K and p-Akt[18], STAT3, IGF-IR phosphorylation[19], exosome release[23], ERK1/2 signaling pathway[24] and modulation of the androgen receptor[25]. In current scenario, considering the complex and intricate cellular signaling, a single drug might not be efficacious against cancer and it could also induce drug resistance. Taking this in account, combinatorial chemotherapeutic treatment for cancer could be a possibly effective treatment option[26].

In spite of significant study in this area, there is scope to identify novel oncology targets that were not previously screened towards the effect of shikonin. In current study, evaluated the potency of shikonin on a panel of recent oncology targets such as HDAC, MERTK, FLT3, TrkA, BRD4, autotaxin, A2B, CXCR2 and NRF2. All these enzymes, receptors and proteins are the current targets for oncology drugs of major pharmaceutical and biotech companies. Few targets like HDAC, BRD4 and NRF2 are epigenetic targets that are currently drawing attention of scientific community. In the study, significant effect of shikonin on CXCR2 receptor and NRF2 protein.

Inhibitory role of shikonin on inflammatory disorders is not well understood. Few scientific groups have shown broad involvement of shikonin in cellular signaling pathways like inhibition of CCR1[27], TNFα[28], MMP-1[29], COX2[30], NOS[31], Syk kinase and histamine release[32]. However, specific mechanism of action is not clearly well defined. As mentioned previously, this provides scope to screen shikonin on many novel anti-inflammatory drug discovery targets that were not evaluated earlier for the effect of shikonin. This study has examined some of these new and relevant targets such as CXCR2, CB2, PGE2, PGE4, A2B, H1, CRTh2, autotaxin, pantetheinase 1, TACE and NRF2 for analyzing the shikonin efficacy in inflammation. The results demonstrated the promising effect of shikonin on CXCR2, CRTh2 receptors and NRF2 protein (Table 2).

The CXCR2 is a chemokine GPCR coupled to Gi/o, binds to proinflammatory chemokine (hIL-8) with high affinity. This signaling event induces leukocytes recruitment and their activation at the sites of inflammation. Chemokine mediated cell migration is an important event in the process of inflammation and CXCR2 is best known for its ability to control leukocyte migration. The CXCR2 receptor has critical role in several cellular processes such as angiogenesis, inflammation, tumor cell proliferation and invasion[33]. Role of CXCR2 receptor has also been well established in psoriasis, atherosclerosis[34], asthma, arthritis, chronic obstructive pulmonary disease (COPD)[35], pulmonary fibrosis[36] and pancreatitis[37]. Few CXCR2 receptor antagonists have also been reported in clinical trial[38]. This study demonstrated a notable effect of shikonin on CXCR2 receptor, both on over expressed cells as well as on human neutrophils. Neutrophils are the primary cells which express CXCR2 receptor and upon binding to IL-8, migrate from blood to the site of injury during inflammation. In this study, shikonin displayed strong inhibitory effect on recombinant CXCR2 cells and neutrophils in intracellular calcium mobilization assay with IC_{50} of 1.3 and 1.4 μM (Fig. 3b, c), respectively. This study tested shikonin in IL-8 driven neutrophil migration assay, where it showed specific effect on CXCR2 receptor and exhibited very promising effect with IC_{50} of ~1 μM (Fig. 5).

The CRTh2 a GPCR is expressed on Th2 cells and a current target of study in drug discovery for inflammation associated diseases. Role of CRTh2 is validated in many inflammatory diseases like asthma, allergic rhinitis[39] and colitis[40]. There are many CRTh2 antagonists in clinical development for asthma,

such as AMG-853, OC000459 and[41] MK-2746. In this study shikonin displayed promising efficacy on CRTh2 over expressed cells with IC_{50} of ~1 μM (Fig. 2b) in a functional assay. When tested in radioligand binding assay using cell membrane, shikonin couldn't displace radiolabelled hPGD2. This study couldn't establish the explanation of difference in potency between functional and binding assay but there might be few possible reasons, (a) Shikonin might be acting as allosteric inhibitor in functional assay and affecting PGD2 signaling from a distant site, but in binding assay as an allosteric inhibitor, it is not able to displace the bound radioligand and (b) There could also be possibility of partial displacement of PGD2 upon binding of shikonin which is more visible in function assay.

The NRF2 is a transcription factor and had emerged as a regulator of cellular homeostasis. It protects the cells from oxidative stress through activation of antioxidant pathway. The NRF2 is a high valued therapeutic target for Chronic Obstructive Pulmonary Disease (COPD)[42], oncology[43] and kidney diseases[44]. In this study, promising effect of shikonin on NRF2 in ARE-bla gene blazer assay with EC_{50} of 7 μM (Fig. 4). Moreover this effect was better than the standard compound tBHQ which showed EC_{50} of ~8 μM in same assay. Few scientific groups have evaluated the effect of shikonin on NRF2 protein and found similar results[45].

CONCLUSION

It is concluded that shikonin possess strong anti-inflammatory effect through CXCR2, NRF2 and CRTh2 targets. The CXCR2 and NRF2 targets are also play crucial role in pathogenesis of cancer. This study substantially demonstrates that shikonin has all the potential to be developed as an anti-inflammatory and anticancerous drug. There is a need of developing highly potent and selective analogues of shikonin through rational drug designing and structural activity relationship. This study opens up further scope of study of shikonin in the area of COPD, asthma and cancer.

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