

Shikonin circumvents cancer drug resistance by induction of a necroptotic death

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Abstract

Defect in apoptotic signaling and up-regulation of drug transporters in cancer cells significantly limits the effectiveness of cancer chemotherapy. We propose that an agent inducing non-apoptotic cell death may overcome cancer drug resistance and showed that shikonin, a naturally occurring naphthoquinone, induced a cell death in MCF-7 and HEK293 distinct from apoptosis and characterized with (a) a morphology of necrotic cell death; (b) loss of plasma membrane integrity; (c) loss of mitochondrial membrane potentials; (d) activation of autophagy as a downstream consequence of cell death, but not a contributing factor; (e) elevation of reactive oxygen species with no critical roles contributing to cell death; and (f) that the cell death was prevented by a small molecule, necrostatin-1, that specifically prevents cells from necroptosis. The characteristics fully comply with those of necroptosis, a basic cell-death pathway recently identified by Degterev et al. with potential relevance to human pathology. Furthermore, we proved that shikonin showed a similar potency toward drug-sensitive cancer cell lines (MCF-7 and HEK293) and their drug-resistant lines overexpressing P-glycoprotein, Bcl-2, or Bcl-x_L, which account for most of the clinical cancer drug resistance. To our best knowledge, this is the first report to document the induction of necroptosis by a small molecular compound to circumvent cancer drug resistance. [Mol Cancer Ther 2007;6(5):1641–9]

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Introduction

Cancer drug resistance is a major problem in chemotherapy and relevant to many intrinsic and extrinsic factors, including the potency of anticancer drugs, the response of cancer cells to the drugs, the tumor microenvironment, and the heterogeneity of cancer cells. The potency of anticancer drugs is largely determined by their efficacies in selectively killing cancer cells and simultaneously inducing drug resistance in cancer cells. Conventional anticancer agents, regardless of their targets and mechanisms, mostly induce apoptosis. Cancer cells are usually sensitive to apoptotic induction initially, but become resistant eventually through dysregulation of apoptotic machinery, manifested by overexpression of antiapoptotic proteins and defect in apoptotic signaling (1–3). Numerous apoptotic inducers are also inducers and substrates of drug transporters, including P-glycoprotein, multidrug resistance-associated protein 1, and breast cancer resistance protein (4–6). Because these drug transporters recognize many structurally and functionally unrelated anticancer agents and efficiently expel intracellular drugs out of cells, the overexpression of these proteins confers cancer cells with a multidrug resistance. Some drug transporters, such as P-glycoprotein, also protect cancer cells from caspase-dependent cell death (7, 8).

There are several potential pharmacologic approaches to overcome cancer drug resistance. To overcome drug transporter (e.g., P-glycoprotein)-mediated drug resistance is theoretically achievable because the targets are few and mechanisms are clear. However, clinically, the efficacies of the specific inhibitors to these drug transporters are yet not conclusive (9). To overcome the drug resistance relevant to apoptotic defect is much more complicated than to drug transporters. Apoptotic machinery is composed of at least dozens of antiapoptotic and proapoptotic proteins. The balance of antiapoptotic and proapoptotic proteins contributes to the balance of cell growth and cell death. Many lines of evidence have shown an imbalance with elevated antiapoptotic and reduced proapoptotic activities in cancer cells one way or another, including overexpression of antiapoptotic proteins (Bcl-2, Bcl-x_L, Mcl-1, c-FLIP, inhibitors of apoptosis, and heat shock proteins), mutations of proapoptotic proteins (p53, Apaf-1, Bax, FAS, Fas-associated protein with death domain, and caspase), and loss of caspases (caspase-3 and caspase-8; refs. 1, 2, 10, 11). In addition, the apoptotic pathways in cancer cells are affected by many oncogenic signals (12). Therefore, it is highly difficult to treat cancers with apoptotic resistance because of so many potential targets. Nevertheless, it is likely to circumvent apoptotic resistance of cancer cells if the action of chemotherapeutic agents is to induce non-apoptotic cell death.

In this report, we showed that shikonin, a naturally occurring compound (13–18), could induce a cell death

through a necroptotic pathway. Necroptosis is a programmed cell death pathway recently identified by Degterev et al. (19), which is distinct from apoptosis, and is characterized by necrotic cell death morphology and activation of autophagy. Necroptosis contributes to the delayed mouse ischemia brain injury *in vivo* and is a basic cell-death pathway with potential relevance to human pathologies. Because shikonin induces a dominant necroptosis in cell death in MCF-7 and HEK293, we examined if shikonin could circumvent drug resistance in MCF-7 and HEK293 overexpressing P-glycoprotein, Bcl-2, and Bcl-x_L. The results showed that the anticancer activities of shikonin are not affected by these proteins, suggesting a potential application of necroptotic inducer as a choice for cancer chemotherapy.

Materials and Methods

Cell Lines and Reagents

MCF-7, HeLa, and HL60 cells were maintained in RPMI 1640 containing 10% FCS. MCF-7/Adr cells were grown in RPMI 1640 containing 10% fetal bovine serum and 1 µg/mL doxorubicin. HEK293 were grown in DMEM supplemented with 10% FCS and 1 mmol/L glutamine (Life Technologies). Plasmid pSFFV-Neo, pSFFV-Bcl-2, and pSFFV-Bcl-x_L were kindly provided by Dr. Steven Grant (Medical College of Virginia). Shikonin was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) with purity of >99%. Necrostatin-1 (Nec-1) and Nec-1 inactive control (Nec-1i) was kindly provided by Dr. J. Yuan (Department of cell Biology, Harvard Medical School, Boston, 02115 MA). Etoposide, doxorubicin, taxol, staurosporine, vitamin E, butylated hydroxyanisole, 3-methyladenine, cyclosporin A, bongkrekic acid, N-acetyl-L-cysteine, and Hoechst 33342 were purchased from Sigma. The broad-spectrum caspase inhibitor z-VAD-fmk was from Calbiochem.

Antibodies

Primary antibodies used were mouse monoclonal antibodies anti-caspase-8 (Cell Signaling Technology), anti-Bcl-2 and anti-Bcl-x_L (Calbiochem), anti-β-actin (Sigma), anti-Bax (clone 6A7; BD Biosciences Pharmingen), anti-microtubule-associated protein 1 light chain 3 (LC3; Medical and Biological Laboratories), rabbit polyclonal antibodies, anti-caspase-9, anti-apoptosis-inducing factor (AIF; Cell Signaling Technology), and goat polyclonal antibody anti-autophagy-related 7 homologue (Atg7; Santa Cruz Biotechnology). The secondary antibodies were FITC or horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG (Santa Cruz Biotechnology).

Flow Cytometry Analysis

The integrity of the plasma membrane was determined by propidium iodide (PI; Sigma) exclusion assay. After drug treatment, cells were mixed with 30 µg/mL PI, and dead cells permeable to PI were counted by a FACS Calibur (Becton Dickinson).

Cell death was estimated by flow cytometry using an Annexin V (AV)-FITC/PI kit (Sigma) according to the manufacturer's instructions, and flow cytometry was

conducted on a FACS Calibur. Statistical analysis was done using WinMDI software version 2.8. Alternatively, the apoptotic cells were determined by PI staining as described previously (20).

For reactive oxygen species detection, cells were plated in six-well plates and treated with shikonin in the presence or absence of Nec-1 for appropriate intervals. The treated cells were incubated with phenol red-free RPMI 1640 (Invitrogen) containing 1 µmol/L 6-carboxy-2',7'-dichlorodihydrofluoresceindiacetate (Molecular Probes) in the dark for 30 min at 37°C. Then, the cells were harvested and analyzed by FACS Calibur.

Mitochondrial membrane potential was probed by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1, a potential-sensitive probe; Molecular Probes) using FACS Calibur according to the manufacturer's instruction. JC-1 is a dye that oligomerizes in normal mitochondria with red fluorescence. Loss of red fluorescence indicates loss of mitochondrial membrane potential.

Animal Studies

Cells (6×10^6 MCF-7 or 2.5×10^6 MCF-7/Adr) were injected (s.c.) into one flank of 5-week-old nude female mice (Shanghai SLAC Laboratory Animal Co. Ltd.). On day 7 after inoculation, mice received vehicle control or shikonin (2.5 mg/kg/d, 5 days, i.p.) suspended in 20% Intralipid (Sino-Swed Pharmaceutical Corp. Ltd.) in a total volume of 0.2 mL. Mice were sacrificed 2 days after the last injection of shikonin. The tumors were weighed and subjected for death morphology by electron microscopy.

Electron Microscopy

The tumors excised from nude mice were cut into 1-mm³ pieces and fixed in 2.5% glutaraldehyde. The samples were treated with 1.5% osmium tetroxide, dehydrated with acetone, and embedded in durcupan resin. Thin sections were post-stained with lead citrate and examined in the TECNAI 10 electron microscope (PHILIPS) at 60 kV.

Cultured cells were washed once in PBS and fixed for 30 min in 2.5% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4), and the samples were processed as above.

Measurement of Caspase-3/DEVDase Activity

Caspase-3 activity was measured using a Caspase-3/CPP32 Colorimetric Assay kit (BioVision Research Products) according to the manufacturer's instructions. Briefly, cell lysate from 1.0×10^6 cells was incubated at 37°C for 2 h with 200 µmol/L DEVD-ρNA substrate. Samples were read at 400 nm with a model ELX800 Micro Plate Reader (Bio-Tek Instruments, Inc.) and expressed as fold increase on the basal level (DMSO-treated cells).

Immunofluorescent Confocal Laser Microscopy

For AIF detection, 2 to 4×10^4 cells were trypsinized, collected, fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 3% bovine serum albumin in PBS for 1 h. Cells were incubated with anti-AIF for 2 h, washed with PBS, incubated for another 45 min with FITC-conjugated goat anti-rabbit IgG, and washed well with

PBS. Nuclei were counterstained with PI for 10 min. Samples were examined under an Olympus FV1000 microscope (Olympus) equipped with a $\times 40$ (numerical aperture, 1.2) oil immersion lens. Image was processed with a FV10-ASW software (Olympus). The procedure was carried out at room temperature.

For Bax activation and translocation, mitochondria was firstly labeled by incubation with 500 nmol/L Mito-Tracker Red (Molecular Probes), a mitochondrial reporter dye, for 30 min at 37°C. Cells were collected, fixed and permeabilized with 1% CHAPS buffer (150 mmol/L NaCl, 10 mmol/L HEPES, 1.0% CHAPS) at room temperature for 10 min, incubated at 4°C overnight with anti-Bax, and then processed as the procedure applied for AIF.

Western Blot Analysis

Cells were lysed in a lysis buffer containing 625 mmol/L Tris-HCl (pH 6.8), 10% SDS, 25% glycerol, 5% β -mercaptoethanol, and 0.015% bromphenol blue followed by sonication and heat denaturation. The protein was applied to a 10% to 15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and then detected by the proper primary and secondary antibodies before visualization by chemiluminescence kit (Pierce).

Generation of MCF-7/Bcl-2, HEK293/Bcl-2, MCF-7/Bcl-x_L, and HEK293/Bcl-x_L Stable Transfectants

MCF-7 or HEK293 cells (5×10^4) were added into a 24-well plate and incubated overnight. Plasmid DNA (2 μ g) was diluted into 0.1 mL of Opti-MEM (Invitrogen) and combined with 5 μ L of LipofectAMINE (Invitrogen) in 0.1 mL of Opti-MEM. After incubation for 20 min, 0.8 mL of Opti-MEM was added, and the mixtures were overlaid onto cells. After 4 h, 1 mL of Opti-MEM containing 20% FCS was added to the cultures. The next day, medium was replaced with a complete culture medium. Transformants were selected by growth for 14 days in a complete medium containing 0.5 mg/mL G418 (Sigma). Polyclonal populations were grown and assayed for stable transgene expression by Western blotting.

Atg7 Short Hairpin RNA Constructs, Transfection, and Expression

The human Atg7 short hairpin RNA sequence (GGAGT-CACAGCTCTTCCTT) was cloned into *Bam*HI and *Eco*RI sites of the pGSU6 vector (Gene Therapy Systems). Control scrambled and short hairpin RNA plasmids (2 μ g) were transfected into MCF-7 and MCF-7/Adr cells plated at 50% confluence in 24-well plates using LipofectAMINE (Invitrogen). Transfected cells were selected by growth for 14 days in a complete medium containing 0.5 mg/mL G418 (Sigma). Polyclonal populations were grown and assayed for stable transgene expression by Western blotting.

Results

Shikonin Induces a Non-Apoptotic Cell Death

Apoptotic cells exhibit morphologic and biochemical characteristics including condensation of cytoplasm,

chromatin marginalization, nuclear fragmentation, externalization of phosphatidyl-serine on the cell surface, and activation of caspases, etc. We examined if shikonin-induced cell death had the characteristics of apoptosis.

After treating MCF-7 cells with shikonin (2.5–20 μ mol/L), cells permeable to PI increased proportionally to the increment of both concentration of and incubation time with shikonin, indicating the loss of plasma membrane integrity (Fig. 1A and B). Shikonin-treated cells did not exhibit apoptotic nuclear fragmentation (Fig. 1C; Supplementary Fig. S1A).³ Instead, the cells had a typical necrotic cell death morphology (Fig. 1D), as manifested by extensive vesiculation of cytoplasmic organelles, dilation of the endoplasmic reticulum elements, cytoskeletal degradation, and rupture of the plasma membrane. Mitochondria were also severely damaged, showing increased matrix density, swelling of cristae, and wrapping by a single rough endoplasmic reticulum cistern. The cell nuclei were less affected.

Shikonin-induced cell death neither was inhibited by a pan-caspase inhibitor z-VAD-fmk (Fig. 2A), nor involved translocation of AIF (Fig. 2B; ref. 21), a critical protein mediating caspase-independent apoptosis (22), nor externalization of phosphatidyl-serine on the cell surface (Supplementary Fig. S1B),³ an early indicator of apoptosis. After treated with shikonin for 3 h, 42% cells were permeable to PI, but AV⁺PI⁻ cells only constituted 1.8% of the total cells, comparable with that of vehicle control. After treated with shikonin for 6 h, the percentage of the AV⁺PI⁻ cells remained the same, although the AV⁺PI⁺ cells increased dramatically. Considering the reduced percentage of the AV⁻PI⁺ cells and the constant percentage of the AV⁺PI⁻ cells, the AV⁺PI⁺ cells were obviously converted from the AV⁻PI⁺ cells whose plasma membrane was further severely damaged.

Previous studies indicated that although MCF-7 was caspase-3 deficient (23), both caspase-dependent and caspase-independent apoptotic pathways were functionally active in this cell line (3). Consistent with previous data (24), we showed that etoposide induced a typical apoptosis in MCF-7 cells characterized with phosphatidyl-serine externalization, which was not inhibited by Nec-1 (Supplementary Fig. S1C).³ Therefore, shikonin-induced non-apoptotic death in MCF-7, to a large extent, is not because it lacks caspase-3. To further prove this, we tested if shikonin could induce a similar cell death in HEK293, which has full apoptotic machinery and expresses caspase-3. The results indicated that shikonin induced a death in HEK293 morphologically the same as MCF-7, and functionally, shikonin-induced death of HEK293 did not involve activation of caspase-3, caspase-8, and caspase-9 and was effectively prevented by the specific necroptotic inhibitor Nec-1 (Supplementary Fig. S2).³

Taken together, the cell death of MCF-7 induced by shikonin is distinct from apoptosis and is characterized with a loss of plasma membrane integrity and morphology of necrotic cell death.

³ Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

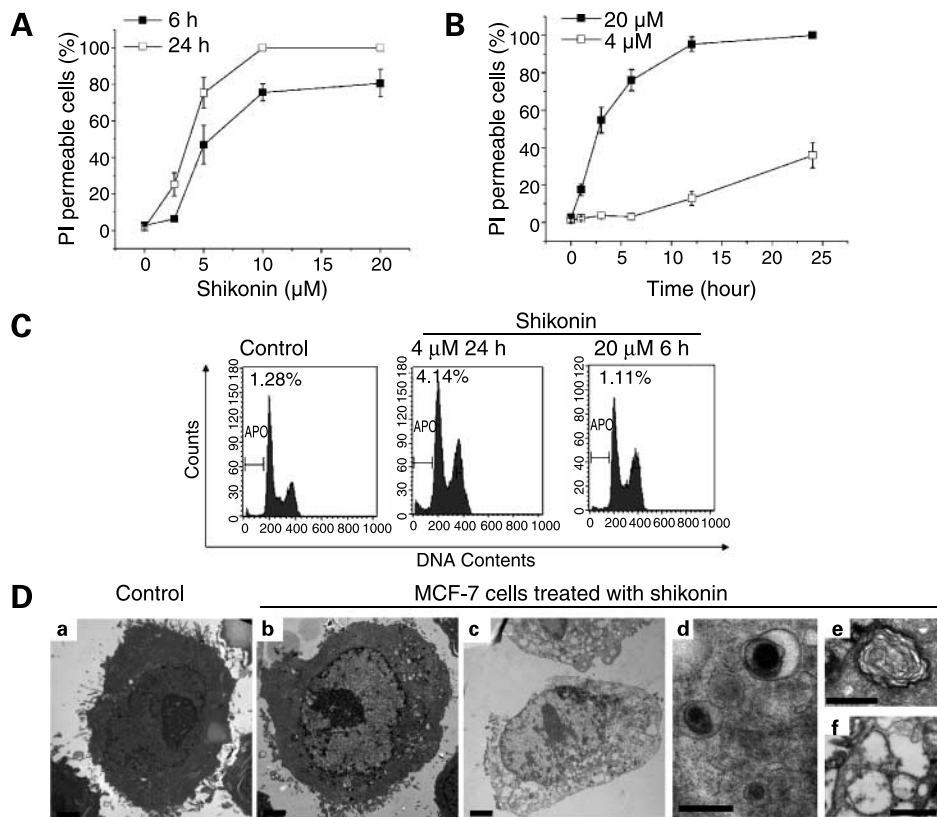


Figure 1. Shikonin-induced cell death is characterized by a loss of plasma membrane integrity and morphology of necrotic death. **A** and **B**, dose- and time-dependent loss of plasma membrane integrity. MCF-7 cells were incubated with varying concentrations of shikonin for 6 or 24 h, or incubated with 4 or 20 μmol/L shikonin for appropriate intervals. The integrity of plasma membrane was examined by a PI exclusion assay. **C**, representative FACS profiles of shikonin-treated MCF-7 cells, showing an insignificant apoptotic peak. The numbers are percentages of apoptotic cells. Cells were treated with 4 μmol/L shikonin for 24 h or 20 μmol/L shikonin for 6 h and subjected to a DNA content analysis by a FACS Calibur. **D**, transmission electron micrograph of shikonin-treated MCF-7 cells, showing numerous swollen mitochondria (**b**), plasma membrane rupture (**c**), autophagosomes (**d**), autophagic vacuoles containing membranous whorls (**e**), severe damage of mitochondria with disrupted internal structures (**f**). Bar, 2 μm (**a–c**) and 1 μm (**d–f**). Points, mean or representative of at least three independent experiments; bars, SD.

Shikonin Induces Necroptosis

Necroptosis is a programmed cell death with the following characteristics (19): (*a*) a morphology of necrotic cell death; (*b*) loss of plasma membrane integrity; (*c*) autophagy as a common downstream consequence of necroptosis other than a contributing factor to necroptosis; (*d*) elevation of reactive oxygen species in some necrotic cells, with no critical roles contributing to necroptosis; (*e*) loss of mitochondrial membrane potential that plays a critical role in necroptosis; and (*e*) most importantly, necroptosis is defined as a basic cell death pathway that can be specifically inhibited by a small molecule, Nec-1, that targets a critical yet unidentified stage of necroptosis. We examined if shikonin-induced cell death complied with the characteristics of necroptosis.

As shown in Fig. 1, shikonin-induced cell was PI permeable and had a typical necrotic death morphology, similar to necroptosis. Nec-1, but not Nec-1i, prevented shikonin-induced loss of plasma membrane integrity (Fig. 3A and B). Under a microscope, the cells treated with shikonin became round and detached from their anchorage, whereas Nec-1 clearly prevented these cellular changes (Supplementary Fig. S1D).³ On the other hand, Nec-1 did not prevent etoposide-induced MCF-7 apoptosis (Supplementary Fig. S1C).³

Shikonin caused a loss of mitochondrial membrane potential, which was proportionally correlated with the loss of plasma membrane integrity. When cells were treated

with shikonin in the presence of Nec-1, cells with low mitochondrial membrane potential and positive PI were significantly reduced (Fig. 3C and D), indicating the critical roles of mitochondrial dysfunction in shikonin-induced cell death. This mitochondrial dysfunction was not associated with Bax or mitochondrial PT pore (Supplementary Fig. S3,³ refs. 25, 26).

Although shikonin induced an autophagosome formation (Fig. 1D) and an elevation of LC3-II, which could be inhibited by Nec-1 (Fig. 4B), the death of MCF-7 was not inhibited in the presence of 3-methyladenine, an inhibitor of autophagy (Fig. 4A), but inhibited in the presence of Nec-1 (Fig. 3), which did not inhibit autophagy (19). In addition, although Atg7 expression was reduced by 70% in MCF-7 by RNA interference, shikonin-induced death was not significantly inhibited (Fig. 4C). More importantly, the death rates of MCF-7 cells after knockdown of Atg7 was effectively prevented by Nec-1 similarly to those of vehicle control cells, strongly suggesting that autophagy was a consequence downstream of shikonin-induced death. Our data were consistent with those separating necroptosis from autophagy reported by Degterev et al. We did not detect the intermediate LC3-I species in cells as also reported by Degterev et al., suggesting that LC3-I might be rapidly processed into LC3-II in some context. Similar results were obtained with MCF-7/Adr (Supplementary Fig. S4).³

Shikonin-treated cells had an elevation of intracellular reactive oxygen species (Supplementary Fig. S5A).³ If

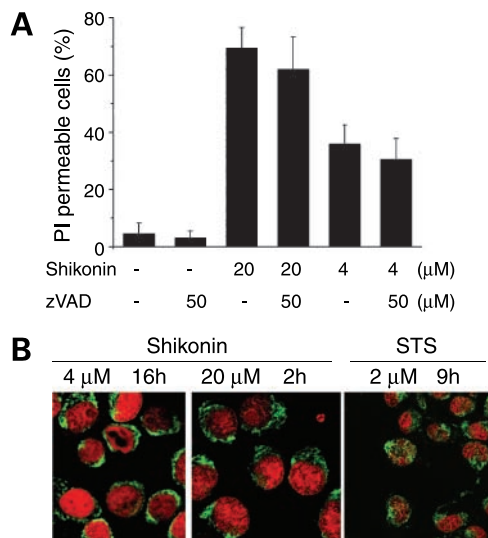


Figure 2. Shikonin-induced cell death is neither prevented by z-VAD-fmk (**A**) nor involves a translocation of AIF (**B**). **A**, MCF-7 cells were preincubated with or without 50 μmol/L z-VAD-fmk (zVAD) before treatment of 20 μmol/L shikonin for 6 h or 4 μmol/L shikonin for 24 h and examined for cell death by a PI exclusion assay. **B**, MCF-7 cells were treated with 4 μmol/L shikonin for 16 h or 20 μmol/L shikonin for 2 h followed by detection of AIF translocation as described in Materials and Methods. HeLa cells treated with 2 μmol/L staurosporine (STS) for 9 h were used as a positive control for AIF translocation (21). *Green*, AIF; *red*, nuclei counterstained with PI. *Columns*, mean or representative of three independent experiments; *bars*, SD.

elevated reactive oxygen species contributed to shikonin-induced cell death, reactive oxygen species scavenger should prevent it. Our results indicated that although antioxidants butylated hydroxyanisole and vitamin E

suppressed DCFH oxidation, they did not prevent shikonin-induced cellular PI permeability, whereas Nec-1, without effect on DCFH oxidation, effectively prevented cell death (Supplementary Fig. S5).³

Shikonin-induced death in the drug-resistant lines derived from MCF-7 and HEK293 was effectively prevented by Nec-1 (Supplementary Fig. S6).³ Thus far, Nec-1 is the best discriminator to separate necroptosis from other cell deaths. The unique activities of Nec-1 preventing cells from only necroptosis but not other deaths have been established with many stringent tests on inhibitory specificities toward the known cell death pathways and effects on cellular and molecular biology of cells (19).

Taken together, all the above evidence supports that shikonin induces a dominant necroptosis in MCF-7, HEK293, and their drug-resistant derivatives.

Shikonin-Induced Necroptosis Circumvents P-Glycoprotein-Mediated Drug Resistance

MCF-7/Adr is characterized with overexpression of P-glycoprotein and highly resistant to a panel of anticancer drugs including anthracyclines, taxanes, and *Vinca* alkaloids (27, 28). Figure 5A showed that MCF-7/Adr was more resistant to shikonin than MCF-7 in a 6-h treatment. However, a time-dependent experiment indicated that the ultimate effects of shikonin on MCF-7 and MCF-7/Adr were the same. As shown in Fig. 5A, although initially, MCF-7/Adr showed a significant higher survival rate than MCF-7, when the incubation time extended to 48 h or beyond, nearly 100% cells were killed, and the difference of survival rates between MCF-7 and MCF-7/Adr was abolished. When the cells were incubated with shikonin for 72 h, the IC₅₀ values of MCF-7 and MCF-7/Adr were not significantly different from each other (Supplementary

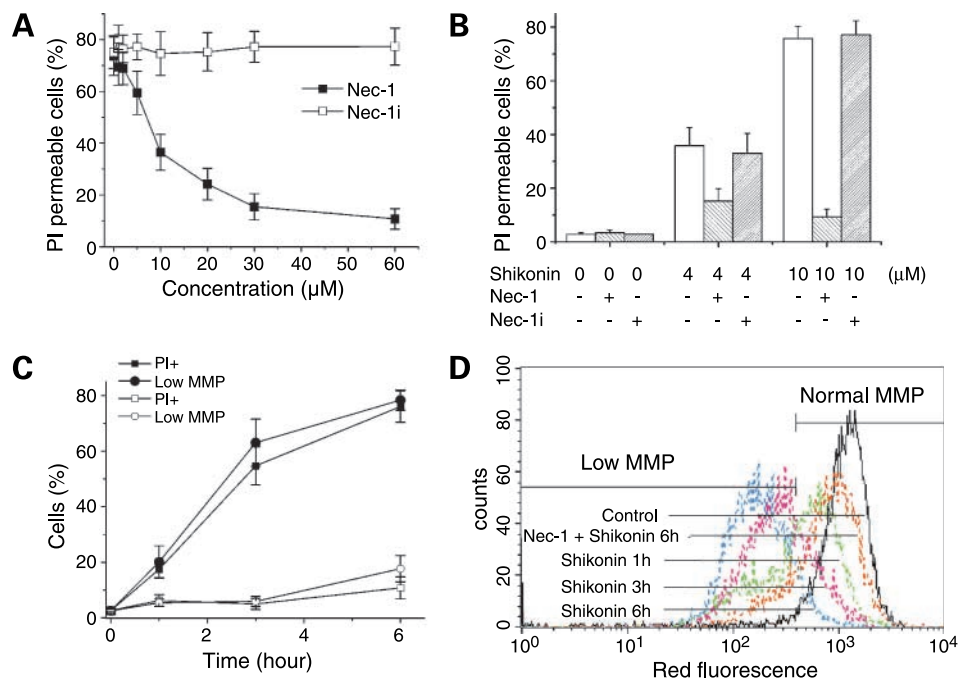


Figure 3. Nec-1 prevents shikonin-induced cell death and loss of mitochondrial membrane potential. **A**, dose-dependent protection of MCF-7 cells against shikonin (20 μmol/L, 6 h) by Nec-1 or Nec-1i. **B**, time-dependent protection of MCF-7 cells against shikonin (4 μmol/L for 24 h or 10 μmol/L for 6 h) by Nec-1 or Nec-1i at 60 μmol/L. *Columns*, mean of at least three independent experiments; *bars*, SD. **C**, Nec-1 prevents shikonin-induced loss of mitochondrial membrane potential. **D**, representative profiles of the mitochondrial membrane potential (MMP) of MCF-7 treated with 20 μmol/L shikonin in the absence or presence of 60 μmol/L Nec-1.

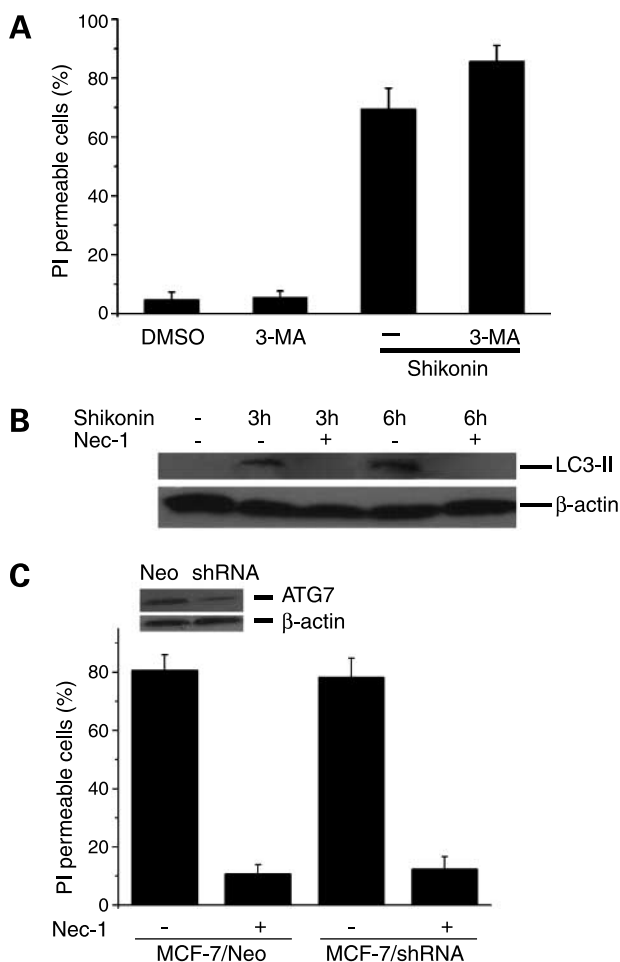


Figure 4. Roles of autophagy in necroptosis induced by shikonin. **A**, 3-methyladenine (3-MA) does not prevent shikonin-induced cell death. MCF-7 cells were treated with 20 $\mu\text{mol/L}$ shikonin for 6 h in the presence or absence of 1.5 mg/mL 3-methyladenine and subjected for analysis of cell death by a PI exclusion assay. *Columns*, mean of at least three independent experiments; *bars*, SD. **B**, LC3-II in MCF-7 cells treated with 20 $\mu\text{mol/L}$ shikonin for 3 or 6 h in the presence or absence of Nec-1. **C**, knockdown of Atg7 in MCF-7 by RNA interference did not affect shikonin-induced cell death. *shRNA*, short hairpin RNA.

Table S1).³ In contrast, when cells were treated with taxol or doxorubicin, the difference of survival rates between MCF-7 and MCF-7/Adr was not abolished simply by extending the incubation time (27, 28). The results implicates that the “resistance” of MCF-7/Adr against shikonin in Fig. 5A probably reflects a difference of responding time to shikonin between MCF-7 and MCF-7/Adr.

To further prove if the difference of survival rates between MCF-7 and MCF-7/Adr was associated with a responding time of necroptosis rather than a “drug resistance,” we carried out a wash-out experiment. After incubation of MCF-7 and MCF-7/Adr with shikonin or taxol at a series concentrations for 4 h, drugs were removed, and the survival of cells were examined in a 7-day period. If such treatment could kill 100% of cells,

there would be no viable cell, and the ultimate result would be no cell growth, or vice versa. The results indicated that whereas taxol at 1 $\mu\text{mol/L}$ completely killed the MCF-7 cells, the same drug even at 64 $\mu\text{mol/L}$ could not kill 100% of MCF-7/Adr (i.e., the cells grew back eventually in 7 days; Fig. 5B). On the other hand, shikonin showed an equal potency in killing MCF-7 and MCF-7/Adr (i.e., whereas shikonin at ≤ 2 $\mu\text{mol/L}$ allowed both cells finally grew back, shikonin at ≥ 4 $\mu\text{mol/L}$ eliminated both cells; Fig. 5B). The results indicated that once the necroptosis of cells was triggered by shikonin, the ultimate fate of cell was death, although the onset of necroptosis could vary from cell to cell. The varying onset of necroptosis does not simply reflect a sensitivity or resistance of cells against shikonin and is distinct from drug resistance. Overall, these results indicated that shikonin-triggered necroptosis was not significantly affected by P-glycoprotein-mediated drug resistance.

Figure 5C and D showed that shikonin was similarly effective on the growth inhibition of MCF-7 and MCF-7/Adr in a pilot animal study, indicating that the potency of shikonin was not significantly affected by P-glycoprotein, consistent with the *in vitro* data. The dead cancer cells in the xenograft were morphologically similar to necroptosis (Fig. 5D).

Shikonin-Induced Necroptosis Circumvents Bcl-2- and Bcl-x_L-Mediated Apoptotic Resistance

The potency of shikonin against Bcl-2- and Bcl-x_L-mediated apoptotic resistance was assayed using MCF-7 and HEK293 cells stably transfected with Bcl-2 or Bcl-x_L. The transfected cells overexpressed either Bcl-2 or Bcl-x_L in comparison with the vector only controls (Fig. 6A and B). After cells were treated with shikonin for 6 h, shikonin showed similar activities against MCF-7/Bcl-x_L and MCF-7/Neo (vector only) but a slightly “weaker” activity against MCF-7/Bcl-2. On the basis of data obtained from MCF-7/Adr, we expected that MCF-7/Bcl-2 may respond to shikonin not as fast as MCF-7/Neo, and this was confirmed by a further assay with an incubation time of 72 h (Supplementary Table S1).³ Similar results were obtained with HEK293/Bcl-2 and HEK293/Bcl-x_L (Fig. 6B).

Discussion

Noting a fact that apoptotic inducers are to a significant extent correlated with cancer drug resistance, it is reasonable to assume if an agent that kills cancer cells through non-apoptotic pathways may circumvent conventional drug resistance (8, 29–32). In search of such agents, we found that shikonin, a naturally occurring compound, could induce a cell death with the characteristics of necroptosis (19) in MCF-7 cells.

It seemed that shikonin-triggered necroptosis had a faster kinetics than that reported by Degterev et al., who used models in which intracellular apoptotic signaling from Fas/TNFR was blocked. In most cases, Fas/TNFR signals relayed to apoptosis rather than necroptosis. Only when apoptosis was blocked, Fas/TNFR signals would pass to

necroptosis. Therefore, in models reported by Degterev et al., a delayed necroptosis with an apparent slow kinetics was well rationalized. However, if an agent directly targets members in necroptosis signaling pathway rather than Fas/TNFR, a faster kinetics than Fas/TNFR-mediated necroptosis would be expected.

Could shikonin, an inducer of necroptosis of MCF-7/Adr, circumvent cancer drug resistance? Because clinical cancer drug resistance was relevant to P-glycoprotein and Bcl-2 family members, we examined if the potency of shikonin could be significantly affected by these proteins.

Although highly resistant to taxol, daunorubicin, vincristine, etc. (27, 28), MCF-7/Adr showed sensitivities toward shikonin comparable with its drug-sensitive parental cells. To date, although the major pharmacologic approach to overcome P-glycoprotein-mediated cancer drug resistance is to use specific inhibitors (9, 33); unfortunately, not a single P-glycoprotein inhibitor is clinically available. The alternative way is to use agents whose anticancer activities are not significantly affected by drug transporters (34, 35). Because the potency of shikonin is not significantly affected by P-glycoprotein as proved by *in vitro* and *in vivo* assays, it

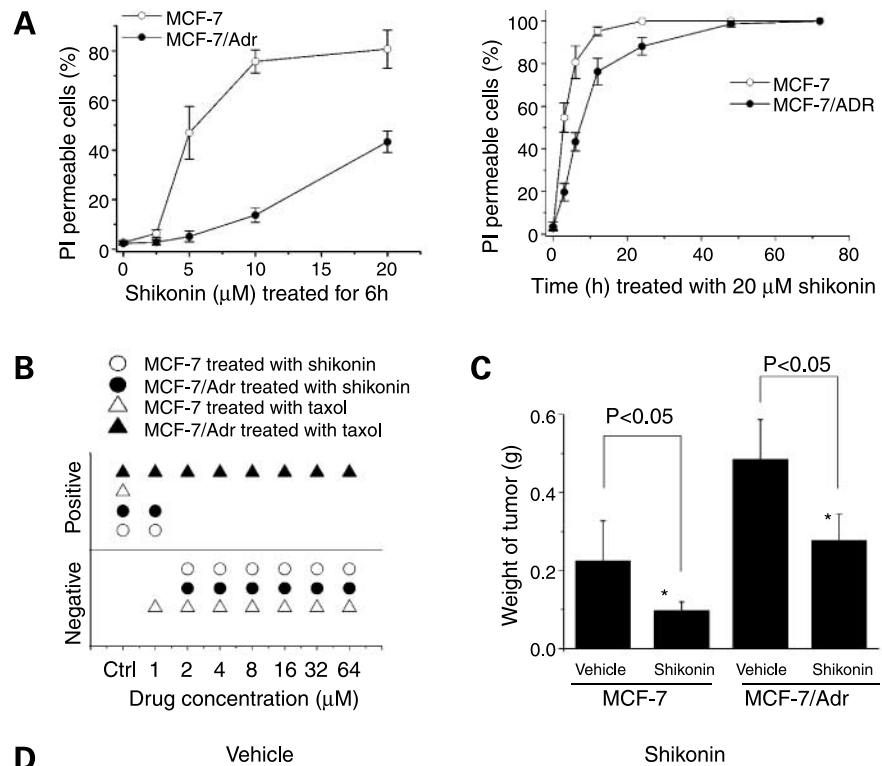


Figure 5. Shikonin-induced necroptosis circumvents P-glycoprotein-mediated drug resistance. **A**, death rates of MCF-7 and MCF-7/Adr cells treated with shikonin of varying concentrations for 6 h or treated with 20 μmol/L shikonin for appropriate intervals. **B**, pulsed treatments of MCF-7 and MCF-7/Adr with shikonin or taxol. Cells were treated with shikonin or taxol for 4 h, washed thoroughly to remove the drug, incubated in a drug-free medium for 7 d, and examined for growth under a microscope. From at least three independent experiments. **C**, effect of shikonin on *in vivo* growth of MCF-7 and MCF-7/Adr in nude mice ($n = 4$ in each group). **D**, morphology of MCF-7 and MCF-7/Adr xenograft treated with or without shikonin, showing no apoptotic features (**b** and **e**) and swollen mitochondria (**c** and **f**). Bar, 2 μm (**a**, **b**, **d**, and **e**) and 0.5 μm (**c** and **f**).

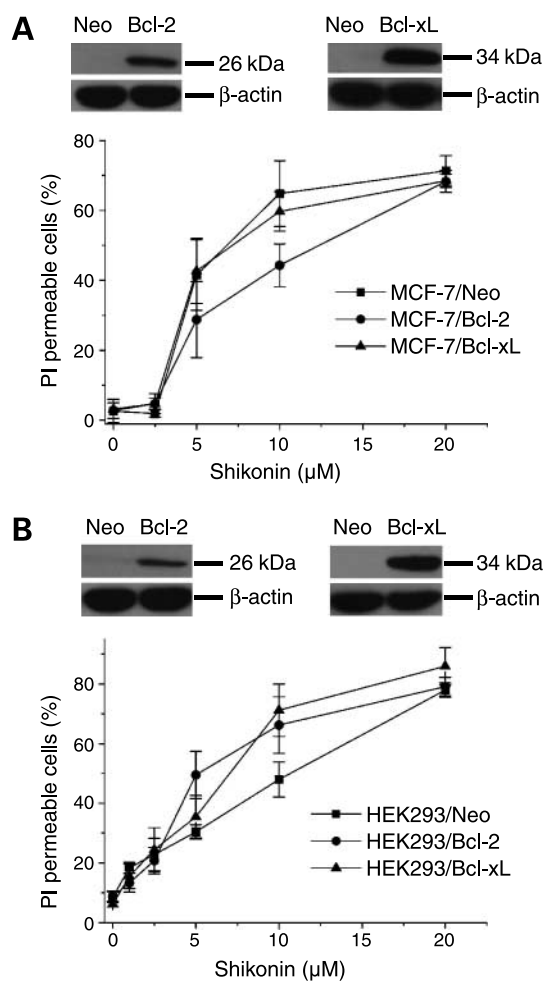


Figure 6. Shikonin-induced necroptosis circumvents Bcl-2- and Bcl-x_L-mediated apoptotic resistance. Expression of Bcl-2 and Bcl-x_L was determined by Western blot. The death rates at 6 h after shikonin treatment were determined by a PI exclusion assay. Points, mean or representative of at least three independent experiments; bars, SD.

may be a candidate in this class of agents. It is not surprising that shikonin-induced cell death is not inhibited by overexpression of either Bcl-2 or Bcl-x_L because shikonin induces a dominant necroptotic death, which is a separate death pathway from apoptosis (19).

The anticancer activities of shikonin and its derivatives were also reported independently from other laboratories (13–18). Notably, a study indicated that shikonin mixture was effective in treatment of 19 patients of later-stage lung cancer who were not suitable for operation, radiotherapy, and chemotherapy. The authors pointed out that shikonin mixture significantly inhibited the growth of lung cancer with the effective rate of 63.3%, remission rate of 36.9%, and 1-year survival rate of 47.3%. In addition, after treatment

with shikonin mixture, the life quality of patients was improved on the aspects of increased body weight and appetite and reduced symptoms of cough, bloody sputum, and chest pain caused by lung cancer, without obvious toxicities (36).

Previous reports indicated that shikonin induced an apoptotic cell death (13, 15, 17, 18). We treated HL60, MCF-7, and HEK293 with shikonin (4 or 20 μmol/L). At low concentration, shikonin induced apoptosis in HL60 (data not shown), but necroptosis in MCF-7 and HEK293, whereas at high concentrations, shikonin induced necroptosis in all the cells. The mechanisms whereby shikonin induced both apoptosis and necroptosis in HL60, whereas only necroptosis in MCF-7 and HEK293, was not understood. We found that after treating HL60 and HEK293 with shikonin, procaspase-8 was significantly down-regulated (Supplementary Fig. S2).^{3,4} It was reported that inhibition of expression of and low constitutive activities of caspase-8 was associated with an increased susceptibility to cell death such as autophagy rather than apoptosis, whereas other peptide caspase inhibitors and RNA interference suppression of caspase-1, caspase-2, caspase-3, caspase-9, and caspase-12 had no ability to induce autophagy (37). The autophagy associated with the inhibition of caspase-8 was somehow associated with activation of the receptor-interacting protein (a serine-threonine kinase) and Jun NH₂-terminal kinase. Interestingly, necroptosis also involved a participation of receptor-interacting protein (19). In a similar way, shikonin-induced down-regulation of procaspase-8 may serve as a bifurcation point to separate necroptosis from apoptosis, which needs an in-depth investigation.

In summary, this study showed, for the first time, that shikonin induced a dominant necroptosis to circumvent cancer drug resistance mediated by P-glycoprotein, Bcl-2, and Bcl-x_L in MCF-7/Adr, MCF-7/Bcl-2, MCF-7/Bcl-x_L, HEK293/Bcl-2, and HEK293/Bcl-x_L. The findings in this study raised a possibility that a necroptotic inducer might be considered as an alternative choice to treat drug-resistant cancer, which is a major obstacle for chemotherapy without a solution yet.

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⁴ Unpublished data.

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