Original Article

Chemical screen identifies shikonin as a broad DNA damage response inhibitor that enhances chemotherapy through inhibiting ATM and ATR

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Received 14 May 2021; received in revised form 26 July 2021; accepted 30 July 2021

KEY WORDS
Chemical screen; Shikonin; DNA damage Response; ATM; ATR; ATRIP; Protein degradation; Chemo sensitizing

Abstract DNA damage response (DDR) is a highly conserved genome surveillance mechanism that preserves cell viability in the presence of chemotherapeutic drugs. Hence, small molecules that inhibit DDR are expected to enhance the anti-cancer effect of chemotherapy. Through a recent chemical library screen, we identified shikonin as an inhibitor that strongly suppressed DDR activated by various chemotherapeutic drugs in cancer cell lines derived from different origins. Mechanistically, shikonin inhibited the activation of ataxia telangiectasia mutated (ATM), and to a lesser degree ATM and RAD3-related (ATR), two master upstream regulators of the DDR signal, through inducing degradation of ATM and ATR-interacting protein (ATRIP), an obligate associating protein of ATR, respectively. As a result of DDR inhibition, shikonin enhanced the anti-cancer effect of chemotherapeutic drugs in both cell cultures and in mouse models. While degradation of ATRIP is proteasome dependent, that of ATM depends on caspase- and lysosome-, but not proteasome. Overexpression of ATM significantly mitigated DDR

Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ATM and RAD3-related; ATRIP, ATR-interacting protein; BAF, bafilomycin A; CPT, camptothecin; CHK1/2, checkpoint kinase 1/2; DDR, DNA damage response; CIS, cisplatin; ETO, etoposide; GEM, gemcitabine; KAP1, KRAB-associated protein 1; Luc, Luciferase; PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline; qPCR, quantitative polymerase chain reaction; RNAi, RNA interference; SKN, shikonin; ULK1, Unc-51-like kinase 1; Z-V AD, Z-V AD-FMK.

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Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2021.08.025
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1. Introduction

DNA damage response (DDR) defines evolutionally conserved signaling pathways that recognize abnormal DNA structures caused by external or internal stresses. It plays a crucial role not only in the maintenance of genome integrity and cell viability, but also in the determination of some of the most used anticancer therapies, especially those that target DNA. The DDR is composed of proteins and protein complexes that form elegant signaling networks to respond to various types of DNA damage such as UV light, replicative stress, DNA double strand breaks, etc. Central to the signaling pathway are several protein kinases including the upstream initiating kinases ataxia telangiectasia mutated (ATM) and ATM and RAD3-related (ATR) and downstream effector checkpoint kinase 1 and 2 (CHK1 and CHK2). The MRE11/NBS1/RAD50 (MRN) complex and the ATR-interacting protein (ATRIP) are critical factors that bind to and activate ATM and ATR, respectively, during DDR. Phosphorylation of these protein kinases, especially ATM autophosphorylation at Ser1981 and CHK1 phosphorylation at Thr68, highlights the importance of ATM in the anti-cancer activity of shikonin in combination with chemotherapy.

2. Materials and methods

2.1. Chemicals, reagents and cell culture

All chemicals and reagents were obtained from commercial suppliers. DAPI (#D9542) was purchased from Sigma (St. Louis, MO, USA). MG132 (#HY-13259), bortezomib (HY-10227) and cisplatin (#HY-17394) were purchased from MedChemExpress (Junction, NJ, USA). (S)-(+-)-Camptothecin (#111282) was obtained from Aladdin Chemical Co. (Shangai, China). Shikonin (#S17-89-5), Z-V-AD-FMK (#S7023), etoposide (#S1225) and genemcitabine (#S1149) were from Selleckchem (Huston, TX, USA). Cells were grown in DMEM (U2OS, PANC-1, SW620 and MDA-MB-231) or RPMI-1640 (A549) supplemented with 10% fetal bovine serum (ExCell bio, China) and 1% streptomycin/penicillin. Lipofectamine RNAiMAX (#13778030) was purchased from Thermo/Fisher Scientific (Carlsbad, CA, USA). Matrigel was purchased from Corning (Glendale, AR, USA).

2.2. Antibodies

Anti-human LC3B (#NB100-2220) and anti-NBS1 (ID7, GTX70224) were purchased from Novus Biologicals (Littleton, CO, USA). Antibodies for β-actin (#4970), pCHK1 (Ser-345, #2348), pATR (Thr-1989, #58014), pATM (Ser-1981, #13050), RAD50 (#3427), RPA (4E4, #2208), ULK1 (#8054S), PARP (#9542S), pCHK2 (Thr68, #2197) and cleaved-caspase-3 (#9664S) were from Cell Signaling Technology (Beverly, MA, USA). Anti-CHK1 (#SC-56291), anti-P21 (#SC-397), anti-ATR (#SC-515173), anti-ATRIP (#SC-365383) and anti-UHRF1
Shikonin inhibits ATM/ATR

2.3. Western blotting

Total cell lysates were harvested in lysis buffer (BeyotimeInst Biotech, China). Protein concentration was determined by the Pierce® BCA Protein Assay Kit (Pierce, #23225). Equal amount of total proteins (~40 µg) were separated on 6%--10%--15% SDS-PAGE, transferred to PVDF membranes (#IPVH00010, Millipore), blocked with 5% skim milk, probed with primary antibodies overnight at 4 °C, incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, and imaged with the Taunan 5200 chemiluminescence imaging system (BioTanan, China).

2.4. Clonogenic cell survival assay

Clonogenic survival assay was used to determine the long-term survival capability of cells. Briefly, 5000 cells from control or treatment groups were seeded into 6-well plates in triplicate and cultured in drug-free full media for 10 days or until colonies were clearly visible. Cells were washed once with phosphate buffered saline (PBS) and fixed in acetic acid--methanol solution (1:7, v/v) at room temperature for 5 min. After staining with 0.1% crystal violet in methanol at room temperature for 15 min, the plates were then gently rinsed under tap water, placed upside down to air dry. The dried plates were first scanned, then incubate in 1% SDS to dissolve all colonies. The absorbance of each well was measured at 570 nmol/L using a microplate reader (Synergy TM HT, BioTEK, USA).

2.5. Annexin V analysis

Apoptosis was determined by the Annexin V fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (KeyGEN BioTECH, Nanjing, China) following the manufacturer’s instructions. Briefly, 1 × 10⁶ A549 cells were seeded into each well of 6-well plates in RPMI 1640 medium and cultured overnight. The cells were treated with 5 µmol/L shikonin, 200 µmol/L CPT or both for 24 h, harvested by trypsinization, washed twice with ice cold PBS and re-suspended in PBS at a final concentration of 10⁵ cells/mL. Ten µL cell suspension were mixed with 100 µL low melting agarose and poured onto glass slides. Lysis was performed at 4 °C overnight. Electrophoresis was carried out in alkaline buffer (1 mmol/L EDTA, 300 mmol/L NaOH; pH 13) for 20 min at 20 V and 300 mA. Slides were then dried and stained with the supplied Vista Green DNA Dye for 10 min. Images were acquired by a Zeiss LSM510 Meta Duo Scan laser scanning confocal microscope (CarlZeiss AG, Oberkochen, Germany).

2.6. Transfection with siRNA

For ATM knockdown, 4 × 10⁵ MDA-MB-231 cells were seeded into 6-well plates and cultured in full media without antibiotics overnight. Cells were at about 80% confluency when being transfected. To prepare for the transfection reagent, 150 µL serum-free Opti-MEM was added separately into SmartPool siRNAs (siLuciferase-siLuc control or siATM) (Dharmacon/Horizon Discovery, Lafayette, CO, USA) or 5 µL Lipofectamine RNAiMAX (Thermo/Fisher) and incubated at room temperature for 5 min. The siRNA and RNAiMAX solutions were then mixed and incubated for another 20 min at room temperature. The mixture was applied dropwise into cell cultures. The final concentration of siRNA was 50 nmol/L in each well. After 48 h, the cells were treated with shikonin, CPT or both and analyzed for protein expression and cell death by trypan blue exclusion assay.

2.7. Confocal fluorescence microscopy

For confocal fluorescence analysis, A549 cells were plated on glass coverslips placed in 6-well plates for 24 h, pretreated with 5 µmol/L shikonin for 2 h, added 200 nmol/L CPT for 0, 1, 4, 8 and 12 h. The cells were fixed in 3.7% formaldehyde for 15 min at room temperature, blocked with 3% BSA in 0.1% Triton X-100/ PBS at room temperature for 1 h, washed three times with 0.1% Triton X-100/PBS, and incubated with anti-pRPA antibodies at 4 °C overnight, followed by anti-rabbit secondary antibody conjugated with Alexa Flour® 488 at room temperature for 1 h in dark. After secondary antibody incubation, cells were stained with DAPI for 10 min at room temperature and washed three times with PBS. Images were acquired by a Zeiss LSM510 Meta Duo Scan laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

2.8. Comet assay

The alkaline comet assay was performed as previously described¹⁶ using the Cell Biolabs kit (#STA-351, San Diego, USA). Briefly, A549 cells were treated with shikonin (5 µmol/L), CPT (200 nmol/L) or both for 24 h, harvested by trypsinization, washed once with ice cold PBS and re-suspended in PBS at a final concentration of 10⁵ cells/mL. Ten µL cell suspension were mixed with 100 µL low melting agarose and poured onto glass slides. Lysis was performed at 4 °C overnight. Electrophoresis was carried out in alkaline buffer (1 mmol/L EDTA, 300 mmol/L NaOH; pH 13) for 20 min at 20 V and 300 mA. Slides were then dried and stained with the supplied Vista Green DNA Dye for 10 min. Comet images were acquired using confocal microscope (Carl Zeiss AG, Oberkochen, Germany) and analyzed using the CasLab software.¹⁷

2.9. Quantitative polymerase chain reaction (qPCR)

A549 were treated with 5 µmol/L shikonin for 0, 4, 8, 12 and 24 h. The cells were collected and RNA samples were extracted using the Qiagen RNeasy Plus mini kit (#74134, Qiagen). The synthesis of cDNA was carried out by the Revert Aid First Strand cDNA Synthesis Kit (#KI622, Thermo Scientific) and followed by SYBR Green qPCR kit (Qiagen) on the Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories). HPRT1 was used as an internal control for normalization. Primer sequences used are listed below:

HPRT1, forward primer, 5'-AGCTTGCTGGTAAAGGA-3', reverse primer, 5'-CCAAACCTCAACTGGAACCTCTAC-3'; ATM, forward primer, 5'-ATAGATGTTGAGTTCCCGATG-3', reverse primer, 5'-TTTCTCCTCTAGTTTCCGTG-3'.
2.10. Mouse studies

Mouse studies were carried out as previously described. Four-week-old female nude mice (#002019) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), housed in-group in cages with bedding, controlled temperature (23 ± 2 °C), humidity (50 ± 5%) and illumination (12 h light/dark cycle). Mice were given sterile food and water and libitum, and were adapted to the facility for one week before experiments. All animal experiments were performed in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised in 1996) and were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

To test the effect of shikonin and chemo drug on tumor growth, 2 × 10^6 A549 cells suspended in RPMI-1640 medium:Matrigel (1:1, v/v) without serum were injected subcutaneously into the right flank of each mouse. Tumors were allowed to grow till the volume reached approximately ~100 mm^3, and mice were randomly divided into the following groups with 5 mice per group: (1) Control; (2) Shikonin alone (2 mg/kg); (3) Gemcitabine alone (100 mg/kg); and (4) Shikonin (2 mg/kg) plus gemcitabine (100 mg/kg). Shikonin was prepared at 70 mmol/L stock solution in DMSO. The injection solution was always freshly prepared by mixing the stock solution with PEG300 (30%), Tween-80 (5%) and sterile H_2O. Gemcitabine was prepared in sterilized distilled water at a stock concentration of 66.8 mmol/L. The injection volume was adjusted based on mouse weight. Drugs were given by i.p. every four days. Tumor volume and body weight were measured at least twice a week for three weeks. Tumor volume was calculated using the following formula:

\[ V = \left( L \times W^2 \right) \times 0.52 \]

where \( V \) is volume, \( L \) is length, and \( W \) is width.

2.11. Statistical analysis

The statistical analysis was conducted by the Prism 9.0 (GraphPad) software. Pairwise comparison was performed using a two-tailed Student t-test, whereas one-way ANOVA was used to compare multiple comparisons. \( P \)-values of less than at least 0.05 were considered statistically significant.

3. Results

3.1. Shikonin strongly inhibits the DDR signaling

We recently carried out a chemical library screen to identify compounds with innovative activities in inhibiting DDR in KRAS mutant lung cancer by assessing the phosphorylation level of...
CHK1 in cells treated with camptothecin (CPT), a topoisomerase 1 poison that induces CHK1 phosphorylation and activates DDR. In addition to two known ATR inhibitors, we identified cardiac glycosides and shikonin (Fig. 1A) as top hits that significantly suppressed CPT-induced CHK1 phosphorylation. We have already characterized the cellular and molecular activities of cardiac glycosides in inhibiting DDR specifically in KRAS mutant lung cancer cells. Here, we will explore the detailed mechanisms by which shikonin inhibit the DDR and determine the significance of such regulation.

First, we confirmed the screening results by pretreating cancer cells originated from different tissues and organs including lung (A549), breast (MDA-MB-231), pancreas (PANC-1) and bone (U2-OS) with 5 μmol/L shikonin for 2 h, followed by adding 200 nmol/L CPT for another 4 h. We then measured pCHK1 levels using specific antibodies. The results show that shikonin significantly inhibited CPT-induced CHK1 phosphorylation in all cell lines tested (Fig. 1B and C). We also observed a general dose-dependent inhibition of DDR by shikonin in these cell lines (Supporting Information Fig. S1). Since cardiac glycosides specifically inhibited DDR in KRAS mutant lung cancers, these results suggest that shikonin has a much broader effect than cardiac glycosides on inhibiting DDR.

Subsequently, we asked at what stage shikonin inhibited CHK1 phosphorylation induced by chemotherapeutic drugs? To answer this question, we used A549 and PANC-1 as the representative cell lines. We treated cells with CPT and shikonin for 0, 2, 4 and 8 h, and measured the level of pCHK1. The results show that shikonin inhibited CPT-induced CHK1 phosphorylation at all time points in these two cancer cell lines (Fig. 1D and E), indicating a potent inhibitory effect of shikonin on CPT-activated DDR.

Next, we asked if shikonin could also inhibit DDR activated by other DNA damaging agents. To this end, we pretreated A549 or PANC-1 cells with 5 μmol/L shikonin for 2 h, added 200 nmol/L CPT, etoposide (ETO), gemcitabine (GEM) or cisplatin (CIS), all are currently used chemotherapeutic agents in the clinic, for another 4 h. We found that shikonin inhibited CHK1 phosphorylation induced by all agents except the lack of effect of ETO and CIS in A549 cells under these treatment conditions (Fig. 1F and G). The inhibitory effect of shikonin on pCHK1 levels might vary; however, such inhibition was consistent and highly reproducible. Hence, we conclude that shikonin strongly inhibits various chemotherapeutic agent-induced CHK1 phosphorylation, suggesting shikonin as a broad DDR inhibitor.

3.2. Shikonin enhances the anti-cancer effect of chemotherapeutic agents

Then we asked if shikonin could enhance the anti-cancer effect of chemotherapeutic drugs as it suppressed the DDR. We first tested this idea in cell cultures by determining the long-term survival of the two representative cell lines, A549 and PANC-1. The results show that treatment with shikonin or chemo drug alone only weakly suppressed the growth of A549 and PANC-1 cells (Fig. 2A–D). However, combining shikonin with chemo drugs significantly inhibited the growth of both A549 and PANC-1 cancer cells (Fig. 2A–D), suggesting that shikonin enhanced the growth inhibitory effects of various chemotherapeutic agents.

To confirm the chemo sensitizing effect of shikonin in a preclinical setting, we inoculated A549 cells into nude mice and treated the mice with 2 mg/kg shikonin, a dose that did not cause obvious mouse weight loss, 100 mg/kg GEM or both by intraperitoneal injection every 4 days. The results show that after 3 weeks of treatment, shikonin or GEM alone already significantly suppressed tumor growth compared with the vehicle control (Fig. 2E and F), consistent with our previous report and the known anti-cancer effect of shikonin. The in vivo tumor inhibitory effect of shikonin was much stronger than the in vitro cell culture data, likely because mice were injected multiple times, whereas in cell cultures cells were only treated once. The
combination further significantly inhibited the tumor growth compared with shikonin or GEM alone (Fig. 2E and F), suggesting that shikonin enhanced the anti-cancer effect of chemotherapeutic drugs in vivo.

3.3. Shikonin increased DNA damage and induced P53-independent apoptotic cell death

If shikonin inhibits the DDR, it should increase DNA damage when combined with chemo drugs. To this end, we measured both single and double strand DNA damage by the alkaline comet assay. The results show that CPT or shikonin alone induced DNA damage (Fig. 3A and B); the combination further significantly increased the level of nuclear DNA damage (Fig. 3A and B), consistent with the enhanced anti-cancer activity of shikonin.

The finding that shikonin increased DNA damage in the presence of chemo drug suggests that it may induce cell death, through which it inhibits cancer growth (Fig. 2). To this end, we co-treated PANC-1 cells with shikonin and CPT over time and measured cell death by trypan blue exclusion assay. Again, shikonin inhibited CPT-induced phosphorylation of CHK1 at all time points (Fig. 3C). In the meantime, treatment with shikonin alone for 24 h induced the cleavage of poly (ADP-ribose) polymerase (PARP), a known marker of apoptosis (Fig. 3C). Co-treatment with CPT plus shikonin induced a higher level of PARP cleavage than shikonin alone (Fig. 3C). Consistent with the PARP cleavage, shikonin alone for 24 h already induced cell death, which was further enhanced by CPT co-treatment (Fig. 3D). Similar results were obtained in A549, MDA-MB-231 (Supporting Information Fig. S2A and S2B), and colorectal SW620 cancer cells (Supporting Information Fig. S3). The cell death rate of SW620 cells in the shikonin plus CPT group at 24 h was less than that in shikonin alone (Fig. S3B), which could be due to massive cell death in the combined group that led to too little cells left for accurate counting.

To confirm if the shikonin-induced cell death was through apoptosis or not, we first performed Annexin V staining by flow cytometry. The results show that while shikonin alone triggered apoptosis, shikonin plus CPT induced more apoptosis than shikonin alone (Fig. 3D). Thus, these results suggest that shikonin alone or in combination with CPT induced apoptotic cell death independent of the status of the tumor suppressor P53.

Figure 3 Shikonin enhanced chemo drug-induced DNA damage and cell death. (A) A549 cells were treated with 5 μmol/L shikonin, 200 nmol/L CPT or both for 24 h, and DNA damage was assessed by comet assay. Representative images are shown. (B) Oliver tail moment (OTM) was measured from cells in (A). Cell numbers analyzed for DMSO, shikonin, CPT and shikonin plus CPT were 65, 49, 41, and 53, respectively. (C) PANC-1 cells were pretreated with 5 μmol/L shikonin for 24 h, added 200 nmol/L CPT for 0, 4, 8, 12 or 24 h, and protein expression was examined. Shikonin alone (SKN) cells were treated for 24 h. The arrow indicates cleaved PARP. The protein band intensity was analyzed using the Image J software and listed above the corresponding gel. (D) Cell death rate of cells in (C). Data represent average ± SD from n = 5 replicates; *P < 0.05 and **P < 0.001.
PARP, as well as caspase 3 (cCasp3), another marker of apoptosis\textsuperscript{21–23} (Fig. 4B). Co-treatment with Z-VAD greatly reduced the levels of cleaved PARP and caspase 3 by shikonin or shikonin plus CPT (Fig. 4B). Consistently, Z-VAD significantly reduced cell death induced by these agents (Fig. 4C), confirming the involvement of apoptosis in the cell death induction by shikonin and chemo drugs.

3.4. Shikonin inhibits the activation of ATM and ATR, the upstream factors of the DDR

Having confirmed the chemo sensitizing effect, we decided to determine the detailed molecular mechanisms by which shikonin inhibit the DDR. We noticed that shikonin also inhibited CPT-induced phosphorylation of ATM, a master upstream responder of DDR, in A549 cells (Fig. 3C), indicating that shikonin could inhibit the early steps of DDR. To confirm this result, we evaluated the effect of shikonin on phosphorylation of ATM and ATR, another crucial upstream factor in DDR\textsuperscript{1,2,24} in various cancer cell lines. We found that shikonin inhibited CPT-induced phosphorylation of ATM, and to a lesser degree ATR, in a time-dependent manner in PANC-1 (Fig. 5A), A549 (Fig. S2A), MDA-MB-231 (Fig. S2A) and SW620 (Fig. S3A) cells. Consistent with the inhibition on pATM and pATR, the levels of phosphorylated downstream substrates for ATM (i.e., CHK2 and KAP1) and ATR (CHK1 and RPA) in the presence of CPT were also greatly reduced by shikonin (Fig. 5A and Fig. S2A). We noticed that shikonin alone increased the level of pKAP1 (and weakly pCHK2), but not others in Fig. 5A; however, an increase in pKAP1 in cells treated with shikonin alone was not seen in other experiments (Fig. 8), suggesting that the increase in pKAP1 in Fig. 5A was case-dependent, but not due to a general activation of the DDR by shikonin. Given that shikonin caused apoptotic cell death (PARP cleavage in Fig. 5A), it is tempting to speculate that the increase in the level of pKAP1 or pCHK2 in Fig. 5A might be caused by the cell death-associated DNA fragmentation. Nonetheless, these results, as well as data presented later, strongly suggest that shikonin significantly inhibited the ATM and ATR signaling.

Then we asked how exactly shikonin inhibits ATM and ATR activation? We found that treatment with shikonin, but not CPT, time-dependently reduced the expression level of ATM, and to a much lesser degree, ATR (Figs. 3C and 5A, Fig. S3A). In the meantime, we assessed the expression levels of a number of ATM- or ATR-associating proteins that are known to be important for ATM and ATR activation including the MRN complex (NBS1, MRE11 and RAD50), TopBP1, ATRIP, ETAA1, RAD17 and RPA\textsuperscript{3}–\textsuperscript{5}. While the antibodies for RAD17 and ETAA1 did not work in our hand, we did clearly detect all other proteins. The results show that shikonin did not affect the protein level of MRE11, MRE11 and RAD50, TopBP1, ATRIP, ETAA1, RAD17 and RPA\textsuperscript{1–3}. While the antibodies for RAD17 and ETAA1 did not work in our hand, we did clearly detect all other proteins. The results show that shikonin did not affect the protein level of MRE11, RAD50, NBS1, TopBP1 or RPA (Fig. 3A); however, it greatly reduced that of ATRIP (Fig. 5A).

Consistent with the inhibition on pATR and pCHK1, shikonin significantly reduced foci formation of phosphorylated RPA in A549 cells (Fig. 5B and C), a critical step in the activation of ATR and phosphorylation of CHK1\textsuperscript{25}. Together, these data demonstrate that shikonin inhibits the activation of the upstream DDR likely by suppressing the expression of ATM and ATRIP.

3.5. Shikonin induced lysosome-/caspase- and proteasome-dependent degradation of ATM and ATRIP, respectively

To further determine how shikonin reduced the expression level of ATM or ATRIP, we treated A549 cells with increasing concentrations of shikonin over time. We found that shikonin reduced the protein levels of ATM and ATRIP, but much less clearly on ATR,
generally in both a time- and a dose-dependent manner (Fig. 6A).

To determine if the reduction in the protein levels of ATM and ATRIP by shikonin was through proteasome-dependent degradation, we co-treated A549 or PANC-1 cells with shikonin and a proteasome inhibitor, MG132, and examined expression of ATM and ATRIP. While MG132 failed to rescue shikonin-induced ATM reduction, it greatly rescued ATRIP expression (Fig. 6B). To further confirm this observation, we tested the effect of a more specific proteasome inhibitor, bortezomib. Similarly, bortezomib prevented shikonin-induced reduction in ATRIP, but not ATM (Supporting Information Fig. S4A). The increase in the protein level of P21 and LC3B confirmed the effect of MG132 or bortezomib in inhibiting the proteasome (Fig. 6B and Fig. S4A). These data suggest that while proteasomal degradation is not accountable for shikonin-induced ATM downregulation, it is involved in ATRIP degradation.

We then decided to further determine how shikonin reduced the protein level of ATM. We first performed quantitative PCR (qPCR) to evaluate the mRNA level of ATM. The results show that shikonin did not affect the mRNA level of ATM (Fig. 6C), indicating that other protein degradation mechanisms, but not transcription, were responsible for shikonin-induced ATM reduction. In addition to proteasome, caspase and lysosome can also degrade proteins. Hence, we used bafilomycin A1 (BAF) and Z-VAD to inhibit lysosomal and caspase, respectively, and evaluated their effects on shikonin-induced ATM degradation. Again, MG132 did not prevent shikonin-induced ATM degradation (Fig. 6D, lanes 2&6). BAF or Z-VAD alone had limited effect on protecting ATM from shikonin-induced degradation (Fig. 6D, lanes 2, 7&8). However, BAF and Z-VAD together greatly inhibited shikonin-induced ATM degradation (Fig. 6D, lanes 2&10). Similar protection on shikonin-induced ATM degradation was also observed when treated with additional lysosome inhibitors including hydroxychloroquine sulfate and ammonium chloride (Supporting Information Fig. S5A). These data suggest a collaborative effect of lysosome and caspase in inducing ATM degradation in the presence of shikonin.

To further confirm the roles of lysosome and caspase in shikonin-induced ATM degradation, we stably depleted UNC-51-like kinase 1 (ULK1), a critical autophagy factor involved in lysosome-induced protein degradation, or caspase 3/6/7 in A549 cells, and examined ATM expression in the presence of
The results show that depletion of ULK1 or caspase 3/6/7 together significantly reduced ATM degradation (Fig. 7A and C) and cell death (Fig. 7B and D) induced by shikonin, confirming that lysosome and caspases were the major factors that drove shikonin-induced ATM degradation.

3.6. ATM is primarily involved in cell death induced by shikonin and chemo drugs

During analysis, we noticed that MG132 or bortezomib did not reduce the level of cleaved PARP or caspase 3 (Fig. 6B and Fig. S4A), indicating that restoring the expression level of ATRIP did not inhibit or prevent apoptosis. Consistently, MG132 or bortezomib did not reduce, but actually enhanced cell death induced by shikonin (Fig. 6E and Fig. S4B). In contrast, BAF and Z-VAD significantly reduced shikonin-induced cleavage of PARP and caspase 3 (Fig. 6D), as well as cell death (Fig. 6E and Fig. S5B). We realized that adding more chemical inhibitors did not necessarily further reduce shikonin-induced cell death (Fig. S5B), probably due to cell toxicity caused by a combination of these chemicals. Importantly, knockdown of ULK1 or caspase 3/6/7 significantly reduced shikonin-induced cell death (Fig. 7B and D). Combined, of these results suggest that it was the reduction in ATM, but not ATRIP, that contributed largely to shikonin-induced apoptotic cell death.

If ATM is indeed mainly responsible for shikonin-enhanced cell death, then we would expect that depletion of ATM should also sensitize CPT. To test this idea, we used siRNA to knockdown ATM (Fig. 8A), and treated cells with shikonin in the presence or absence of CPT. Depletion of ATM reduced CPT-induced pKAP1 and pCHK2 levels, and to a lesser degree, pCHK1, compared with control cells (Fig. 8A), supporting the inhibition of the ATM signaling. Knockdown of ATM did not affect the expression level of ATRIP (Fig. 8A), suggesting that these two factors are independent. Importantly, depletion of ATM significantly enhanced CPT-induced cell death (Fig. 8B), similar to previously reported results in gastric cancer. ATM depletion also increased shikonin-induced cell death (Fig. 8B), consistent with the expression levels of ATM and PARP (Fig. 8A).

To further confirm the importance of ATM in shikonin-induced DDR inhibition and cell death, we re-constituted MDA-MB-231 cells with a FLAG-HIS-tagged ATM plasmid to compensate for the shikonin-induced loss of ATM. We then treated parental or FLAG-HIS-ATM expressing cells with shikonin in the presence or absence of CPT and monitored protein expression and cell death. In control cells, shikonin induced a dramatic reduction in the levels of ATM, pATM, pCHK2, pKAP1, pCHK1, pATR and ATRIP (Fig. 8C), reinforcing the idea that both the ATM and the ATR signaling were inhibited by shikonin. Remarkably, overexpression of ATM greatly rescued the levels of ATM and pATM, but not ATRIP, in the presence of shikonin compared with parental cells (Fig. 8C). Consistently, we observed a partial rescue on pCHK1, pKAP1 and pCHK2 levels in these cells (Fig. 8C).

Importantly, overexpression of ATM also reduced the cleavage of PARP and caspase 3 (Fig. 8C), which is consistent with the significantly reduced cell death in these cells (Fig. 8D). We noticed that the rescue of pATM, pCHK2, pKAP1 and pCHK1

Figure 6  Shikonin-induced degradation of ATM and ATRIP. (A) A549 cells were treated with 0.1, 1, 5 and 10 μmol/L shikonin for 6 and 24 h, and protein expression was analyzed. (B) A549 or PANC-1 cells were treated with 5 μmol/L shikonin with or without 10 μmol/L MG132 for 12 h, and protein expression was analyzed. Arrow indicates cleaved PARP. (C) A549 cells were treated with 5 μmol/L shikonin for 0, 4, 8, 12 and 24 h, and mRNA levels of ATM was measured by qPCR. (D) A549 cells were treated with 5 μmol/L shikonin, 5 μmol/L MG132, 0.1 μmol/L BAF and 10 μmol/L Z-VAD for 24 h, and protein expression was analyzed. Arrow indicates cleaved PARP. (E) Quantitation of cell death by trypan blue staining from cells in (D). Data represent average ± SD from n = 3 replicates; **P < 0.001.
targeting ULK1 or control, treated with 5 μmol/L shikonin. (A) A549 cells were stably infected with shRNA vectors targeting ULK1 or control, treated with 5 μmol/L shikonin for indicated time and protein expression was measured. The ATM band intensity was quantitated from two duplicates and shown above. (B) Quantitation of cell death from (A). (C) A549 cells stably depleted of caspase 3/6/7 were treated with 5 μmol/L shikonin for 12 h and protein expression was examined. ATM band intensity is quantitated from three duplicates and shown above. (D) Quantitation of cell death from (C) assessed by trypan blue exclusion assay. Arrows indicate cleaved PARP. Data represent average ± SEM from n = 5 replicates; **P < 0.001.

was partial; however, the cell death inhibition by ATM overexpression was significant, suggesting that these levels of CHK1, CHK2 and ATM phosphorylation might be sufficient to rescue cell survival in the presence of Shikonin and CPT. Again, these results strongly support the idea that ATM contributed largely to the enhanced cell death induced by Shikonin and CPT (Fig. 8E).

4. Discussion

Shikonin has been reported to inhibit cancer growth in vitro and in vivo either as a single agent or in combination with chemo drugs. However, the underlying molecular mechanisms remained elusive. In the current study, we, for the first time, presented evidence to show that shikonin strongly inhibited the DDR in cells exposed to different chemotherapeutic agents, through which it enhanced the cell death inducing effects of chemo drugs. Subsequent studies reveal that shikonin suppressed the expression of ATM and ATRIP, and to a much lesser degree ATR, all are critical upstream regulators of the DDR. While proteasome was responsible for ATRIP degradation, that of ATM relied on lysosome and caspase. Proteasome inhibitors restored the expression level of ATRIP in the presence of shikonin; however, they failed to reduce shikonin-induced cell death. On the other hand, blocking ATM degradation by lysosome/caspase inhibitors or overexpressing ATM significantly mitigated cell death induced by shikonin. Hence, we conclude that ATM, but not ATR/ATRIP, primarily contributed to the increased cell death induction by shikonin and chemotherapy (see model in Fig. 8E).

Unlike known ATM inhibitors that inhibit the catalytic activity of the enzyme, here we show that shikonin induced degradation of ATM through caspase and lysosome systems, but not the proteasome. These studies not only illustrate novel molecular mechanisms of shikonin, but also reveal a new layer of regulation of ATM by small molecules. To the best of our knowledge, this is the first report of a non-enzymatic inhibitor of ATM from natural resource that causes its degradation. These results also suggest a novel strategy in cancer treatment by inducing ATM degradation, but not through inhibiting its catalytic activity as proposed in conventional approaches. ATM mutations are the cause of a rare recessive autosomal disorder called Ataxia Telangiectasia (A-T), also known as Louis—Barr syndrome. ATM mutations in AT patients often result in a reduction in its protein level, leading to hypersensitivity of AT cells to ionizing radiation. In contrast, overexpression of ATM full-length cDNA restored normal radiosensitivity in AT cells, which were similar to our observations where shikonin-induced ATM degradation enhanced the sensitivity of cancer cells to chemotherapy. These results suggest a potential application of shikonin in inhibiting cancer through inducing ATM degradation.

The chemical structure of shikonin indicates that it might be a promiscuous DNA binding agent and therefore may non-specifically cause the effects reported here. If so, we would expect that shikonin alone should also activate ATM and/or ATR, just like CPT that stabilizes the DNA-topoisomerase 1 complex. Therefore, we should expect to observe increases in the levels of pCHK1, pATR, pATM, etc., in cells treated with shikonin alone; further, shikonin should increase the levels of these proteins in the presence of chemo drugs. However, these were not the cases. First, shikonin alone did not increase the basal levels of pCHK1, pRPA, pATR or pATM in all cell lines tested even for up to 24 h; in contrast, CPT alone substantially increased the levels of these proteins. Although shikonin alone did result in an increase in pKAP1 (and slightly on pCHK2) in Fig. 5A, the lack of increase in pATM or pCHK1 argues against a general activation of the DDR by shikonin. Also, such an effect was not repeated in Fig. 8A, suggesting that the increase in pKAP1 in Fig. 5A was a specific case for this particular experiment. Given that shikonin alone caused apoptotic cell death, we speculate that the increase in pKAP1 in Fig. 5A might have resulted from cell death-induced DNA fragmentation, but not through a general activation of the ATM signaling. Second, we have provided compelling evidence to show that shikonin significantly reduced chemo drug-induced phosphorylation of all DDR signaling proteins, the opposite to the prediction if shikonin indeed interferes with DNA. Further, we showed that shikonin induced specific degradation of ATM and ATRIP through distinct mechanisms, which cannot be explained if shikonin non-specifically binds DNA. Together, these findings suggest that shikonin exerts the specific function described herein through targeting the ATM/ATR signaling, but not through non-specific DNA-binding.

5. Conclusions

This study unveils several lines of novel findings. First, we demonstrate that shikonin is a broad DNA damage response inhibitor. Second, we show that shikonin inhibits the upstream event of the DDR by inducing degradation of ATM and ATRIP, which accounted for the inhibition of the ATM and ATR signaling, respectively. Third, we present evidence to show that shikonin induces degradation of ATM through lysosome- and caspase-, but not proteasome-, dependent pathways, whereas it degrades ATRIP.
through the proteasome. Last but not least, we show that ATM is the key factor that contributed to the enhanced anti-cancer effect of chemotherapy by shikonin. Our results, for the first time, illustrate a previously uncharacterized cellular function of shikonin that could greatly improve the anti-cancer effects of chemotherapeutic drugs, holding the promise of developing shikonin and likely its chemical derivatives as either tool compounds in DDR research or even possibly potential chemo sensitizers in cancer treatment in the future.

Acknowledgments

This work was supported by Guangdong Basic and Applied Basic Research Foundation (2021A1515011244, China) to Jinshan Tang and the National 111 Project of China (No. B13038, China) to Xinsheng Yao.

Author contributions

Jinshan Tang, Xinshen Yao and Youwei Zhang conceived and designed the project. Fangfang Wang conducted the majority of experiments and provided statistical analysis. Sora Jin conducted the caspase and ATM knock down experiments. Franklin Mayca Pozo performed mouse tumor studies. Danmei Tian developed the chemical screen method. Xiyang Tang and Yi Dai assisted the measurement of cell toxicity. Jinshan Tang and Youwei Zhang wrote the paper.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.08.025.

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