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Triptolide induces DNA breaks, activates caspase-3-dependent apoptosis and sensitizes B-cell lymphoma to poly(ADP-ribose) polymerase 1 and phosphoinositide 3-kinase inhibitors

JIAWEI GUAN[1*], QIAN ZHAO[1*], JIAN LV[1], ZHIWEI ZHANG[1], SHIJIE SUN[2] and WEIFENG MAO[1]

Departments of [1]Biotechnology and [2]Immunology, College of Basic Medical Sciences, Dalian Medical University, Dalian, Liaoning 116044, P.R. China

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Abstract. Triptolide is the primary compound isolated from *Tripterygium wilfordii*, which has been reported to inhibit nucleotide excision repair as well as exhibit anti-inflammatory and antitumor activities. However, the action of triptolide in DNA breaks remains unknown. The present study investigated the effects of triptolide in the induction of DNA breaks and apoptosis in a murine B-cell lymphoma cell line, CH12F3. An MTT assay revealed that X-ray repair cross-complementing protein 1 (XRCC1)$^{-/-}$ CH12F3 cells were more sensitive to 6 nM triptolide compared with the wild-type CH12F3 cells, which suggests that low levels of triptolide induce DNA breaks in a manner that is dependent on the XRCC1-mediated repair pathway. Flow cytometric analysis identified that 50 nM triptolide increased the phospho-histone H2AX level, demonstrating that triptolide induces double-strand breaks. Western blot analysis revealed that triptolide up-regulated Rad51 and nuclear proliferating cell nuclear antigen. Annexin V/propidium iodide staining identified that triptolide promoted apoptosis and western blot analysis confirmed that triptolide activated caspase-3-dependent apoptosis. The results of the present study also demonstrated that 5 nM triptolide sensitized CH12F3 lymphoma cells to poly(ADP-ribose) polymerase 1 and phosphoinositide 3-kinase inhibitors, suggesting that triptolide may be a potent antitumor drug for sensitizing lymphoma cells to chemotherapeutic agents.

Correspondence to: Dr Weifeng Mao, Department of Biotechnology, College of Basic Medical Sciences, Dalian Medical University, 9 West Lvshun South Road, Dalian, Liaoning 116044, P.R. China
E-mail: maoweifeng@dmu.edu.cn

Dr Shijie Sun, Department of Immunology, College of Basic Medical Sciences, Dalian Medical University, 9 West Lvshun South Road, Dalian, Liaoning 116044, P.R. China
E-mail: sjsun@126.com

*Contributed equally

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Introduction

Triptolide is a bioactive ingredient isolated from *Tripterygium wilfordii* (1) known to exhibit immune-suppressive and anti-inflammatory activity (2-7). A number of studies have identified that triptolide also exhibits antitumor activity, inhibiting proliferation and migration of cancer cells (8-12). A previous study has demonstrated that triptolide inhibited ATPase activity of the basal transcription factor transcription factor II H (TFIIH) and RNA polymerase II-mediated transcription in nucleotide excision repair (NER) (13). Triptolide also interfered with other transcription factors including p53, nuclear factor-κB and heat-shock factor protein 1 (6,14). Considering the important role it serves by interfering with the transcription of tumor-associated factors, triptolide was demonstrated to be a potent antitumor drug. However, the underlying molecular mechanisms of triptolide action in DNA damage repair are not well defined.

The DNA repair protein X-ray repair cross-complementing protein 1 (XRCC1) serves a key role in base excision repair (BER) and single strand break (SSB) repair as a scaffolding protein which recruits a number of DNA repair-associated factors in the repair pathway, including ligase III, poly(ADP-ribose) polymerase 1 (PARP1), apurinic/apyrimidinic endonuclease 1, proliferating cell nuclear antigen (PCNA) and DNA polymerase-β (15-17). Ligase IV serves to join together the double-strand breaks (DSBs) in non-homologous end-joining (NHEJ) repair (18). Rad51 serves vital roles in homologous recombination (HR), in which the Rad51 filament seeks a homologous sequence for DNA replication (19). PCNA is a DNA sliding clamp, functioning in DNA replication (20). The present study used XRCC1$^{-/-}$ and ligase IV$^{-/-}$ CH12F3 cells to analyze the DNA breaks induced by triptolide while also detecting cellular Rad51 and nuclear PCNA levels following triptolide treatment to investigate the effect of triptolide in cellular DNA repair.

PARPs are a family of proteins that transfer mono(ADP-ribose) or poly(ADP-ribose) (PAR) groups onto their target proteins (21). As the PARylation of DNA repair

factors is required for recruitment to DNA breaks, PARPs are essential for DNA repair (22-25). The function of PARP1 in DNA repair contributes to cancer cell survival in response to genotoxic agents, thus PARP1 is a promising therapeutic target in cancer therapy, particularly for breast cancer 1-deficient cancer cells. The phosphoinositide 3-kinase (PI3K) pathway is a critical signaling pathway frequently activated in numerous types of cancer, and a number of PI3K-targeted compounds are used therapeutically in the clinic (26-28). The present study investigated the combination of triptolide with PARP1 inhibitors and PI3K inhibitors to analyze the potential use of triptolide in treatment of lymphoma. The present study contributes to the current understanding of the role served by triptolide in DNA damage and apoptosis, as well as in clinical therapeutics in combination with chemotherapeutic agents to treat patients with lymphoma.

Materials and methods

Antibodies and reagents. Triptolide, PARP inhibitor, PI3K inhibitor, protease inhibitor and phosphatase inhibitor were purchased from Selleck Chemicals (Shanghai, China). Anti-Rad51 (catalog no. 14961-1-AP) and anti-H3 (catalog no. 17168-1-AP) antibodies were purchased from ProteinTech Group, Inc. (Chicago, IL, USA). RPMI-1640 medium, fetal bovine serum (FBS) and penicillin and streptomycin were purchased from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). Dimethyl sulfoxide (DMSO) and β-mercaptoethanol (BME) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). MTT was purchased from Tiangen (Beijing, China). The Cell Cytoplasmic and Nuclear Protein Extraction kit was purchased from Sangon Biotech Co. Ltd. (Shanghai, China). Antibodies against phospho-histone H2AX (γH2AX; Ser^{139}) were purchased from CST Biological Reagents Company Limited (Shanghai, China; catalog no. 2577). Antibodies against caspase-3 (catalog no. 19677-1-AP), caspase-9 (catalog no. 10380-1-AP), PCNA (catalog no. 10205-1-AP), PARP1 (catalog no. 13371-1-AP) and β-actin, used as the reference gene (catalog no. 60008-1), were purchased from Wuhan Sanying Biotechnology (Wuhan, China) and all used at a dilution of 1:300 in western blot analysis. Goat anti-Rabbit immunoglobulin G (IgG) secondary antibody conjugated to Alexa Fluor-488 (catalog no. A-11034) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). HRP-conjugated goat anti-rabbit and anti-mouse IgG secondary antibody (catalog nos. SA00001-2 and SA00001-1, respectively) were from purchased from Wuhan Sanying Biotechnology, Inc. (Wuhan, China).

Cell culture. The murine B-cell lymphoma cell line CH12F3 was obtained from Professor T. Honjo (Kyoto University, Kyoto, Japan). Ligase $IV^{-/-}$ and $XRCC1^{-/-}$ CH12F3 cells were obtained from Dr Kefei Yu (Michigan State University, East Lansing, MI, USA). All cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 ng/ml streptomycin and 50 nM BME, and incubated at 37˚C with 5% CO_2.

Western blot analysis. The 6-well plates were plated with $1x10^6$ cells and treated with distinct concentrations of triptolide for 24 h. Cellular whole protein or nuclear protein was extracted using the cytoplasmic and nuclear protein extraction kit according to the manufacturer's protocol. Protein concentrations were determined using a Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Inc.). A total of 50 μg protein from each sample was separated using SDS-PAGE in a 10 or 15% gel for 2 h at 120 V and transferred onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were blocked with 5% skimmed milk powder (diluted in Tris-Buffered Saline-Tween 20) for 1 h at room temperature prior to incubation with the mentioned primary antibodies (1:300 dilution) overnight at 4˚C. HRP-conjugated goat anti-rabbit and anti-mouse IgG secondary antibody with appropriate dilution (1:2,000) were used against primary antibodies (dilution, 1:300) for 1 h at room temperature. Finally, the proteins were detected using enhanced chemiluminescent substrate (Thermo Fisher Scientific, Inc.). Histone 3 (H3) was used as the control.

Flow cytometric analysis. CH12F3 cells were treated with triptolide (0, 10, 20, 30, 40 and 50 nM) for 4 h before collection, and 0.5 ml 4% formaldehyde was added for 10 min at 37˚C. Cells were collected by centrifugation (1,000 x g, 3 min, room temperature) and ice-cold 100% methanol was slowly added until a final concentration of 90% methanol was achieved. Cells were incubated for 30 min on ice. A total of $0.5x10^6$ cells of each sample were washed with 2 ml wash buffer including 1X PBS with 0.5% bovine serum albumin (Sangon Biotech Co. Ltd.). Cells were suspended in 100 μl of anti-rabbit antibody against γH2AX (1:200 dilution in 1X PBS) and incubated for 1 h at room temperature. Cells were washed in 2 ml wash buffer. Cells were suspended in 100 μl goat anti-rabbit IgG secondary antibody Alexa Fluor 488 for 30 min at room temperature prior to a repeat wash with wash buffer as aforementioned. Finally, cells were suspended in 0.5 ml PBS for flow cytometry (FCM) and the results were analyzed using BD CellQuest 5.1 software (BD Biosciences, San Jose, CA, USA).

Cell viability assay. A total of 5,000 cells were placed in a 96-well plate and incubated in triptolide (10, 20, 30, 40 and 50 nM) at 37˚C for 24 h. Following incubation, MTT was added to each well at a final concentration of 0.5 mg/ml. Cells were incubated at 37˚C for 4 h before collection by centrifugation (1,000 x g for 3 min) at room temperature. A total of 200 μl DMSO was added to each well and the plate was incubated for 15 min at 37˚C. Finally, the absorbance values were detected at 589 nm using a microplate photometer (Thermo Fisher Scientific, Inc.).

Apoptosis assay. Cellular apoptosis was detected using FCM. Annexin V and propidium iodide (PI) were used to stain cells that were treated with 5 nM triptolide for 12 h and incubated for 15 min at room temperature. The apoptotic cells were quantified using FCM and the results were analyzed using BD CellQuest 5.1 software (BD Biosciences).

Statistical analysis. SPSS software (version 6.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. A Student's t-test was performed to compare differences between groups.

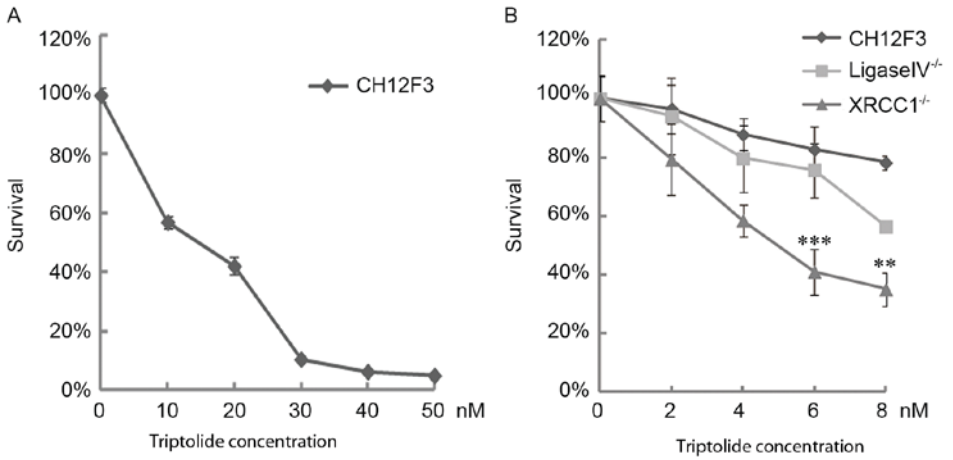

Figure 1. Triptolide suppresses CH12F3 cell proliferation. (A) CH12F3 cells were treated with the indicated concentrations of triptolide for 24 h and analyzed using an MTT assay. (B) XRCC1$^{-/-}$, ligase IV$^{-/-}$ and wild-type CH12F3 cells were treated with the indicated concentrations of triptolide for 24 h, and the cell viability was analyzed using an MTT assay. Results are presented as the mean ± standard deviation of three independent experiments. **P<0.01 and ***P<0.001 vs. CH12F3 cells. XRCC1, X-ray repair cross-complementing protein 1.

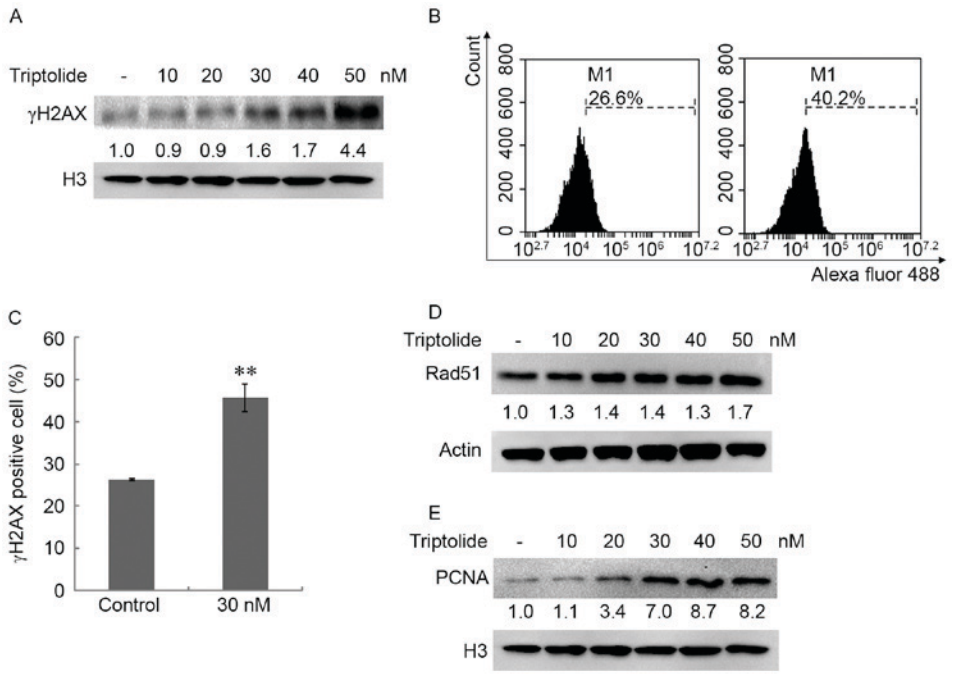

Figure 2. Triptolide induces DNA damage. Cells were treated with the indicated concentrations of triptolide for 4 h. (A) γH2AX expression was detected using western blot analysis. (B) γH2AX expression was detected using FCM. (C) Quantification of FCM results. Results are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. corresponding control. (D) Cells were treated with the indicated concentrations of triptolide for 4 h. The Rad51 level was detected using western blot analysis. (E) Cells were treated with the indicated concentrations of triptolide for 4 h. Nuclear proteins were extracted and the nuclear PCNA level was detected using western blot analysis. H3 was used as the control. FCM, flow cytometry; PCNA, proliferating cell nuclear antigen; H3, histone 3; γH2AX, phospho-histone H2AX.

P<0.05 was considered to indicate a statistically significant difference, with P<0.01 considered to be highly significant.

Results

Triptolide suppresses CH12F3 cell viability. The effects of triptolide on CH12F3 cell viability were analyzed using an MTT assay which revealed that triptolide suppressed CH12F3 cell proliferation. Following treatment with triptolide doses ranging between 0 and 50 nM for 24 h, cell viability was almost completely inhibited at 30 nM (Fig. 1A). To assess the effects of triptolide on DNA damage, the viability of ligase IV$^{-/-}$ and XRCC1$^{-/-}$ CH12F3 cells was analyzed. Results of the MTT assay demonstrated that 6 nM triptolide suppressed XRCC1$^{-/-}$ viability to 40%; however, the viability of ligase IV$^{-/-}$ and control CH12F3 cells were increased, compared with XRCC1$^{-/-}$ cells at the same dose of triptolide (Fig. 1B). As XRCC1 is vital for the BER SSB pathway and ligase IV is essential for NHEJ DSB repair, these results suggested that triptolide induces DNA damage, primarily dependent on XRCC1-mediated repair.

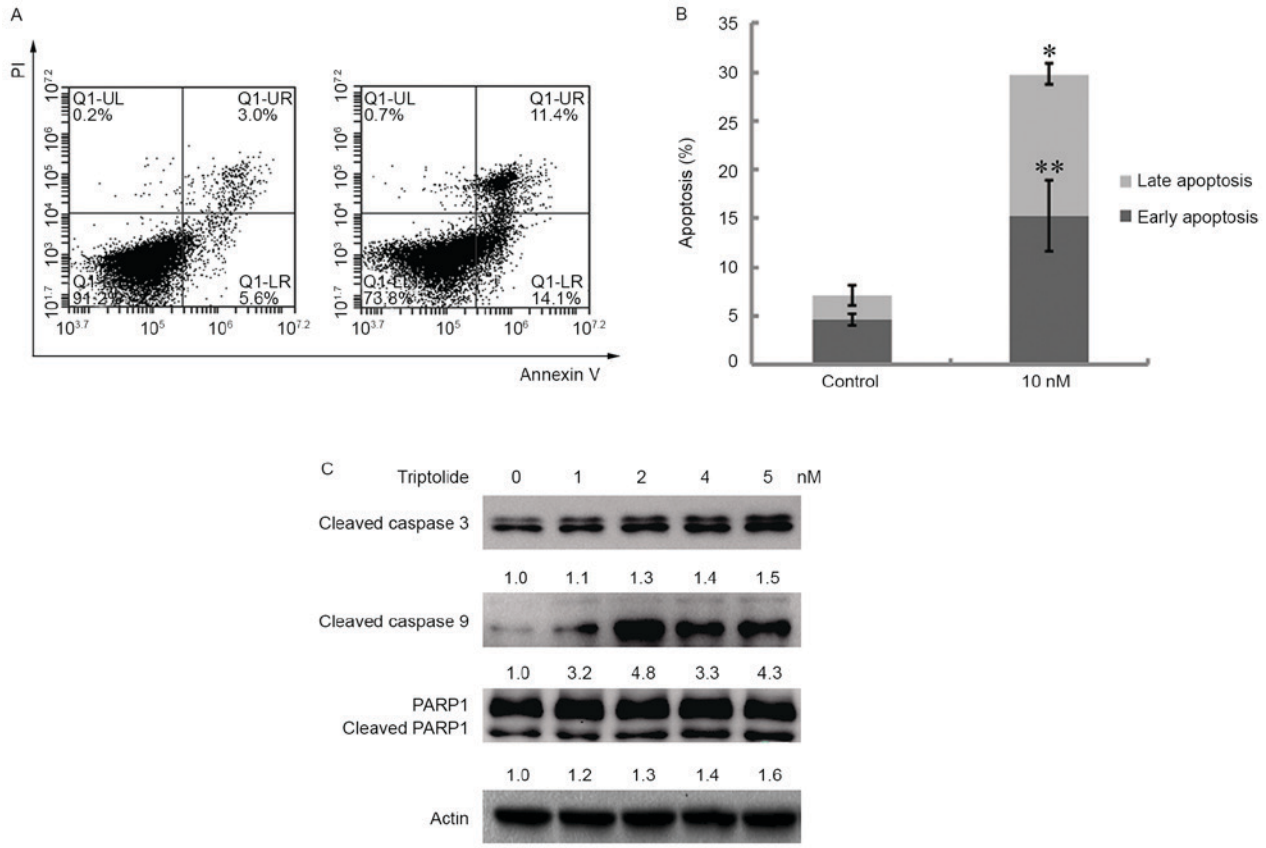

Figure 3. Triptolide induces caspase-3-dependent apoptosis. (A) Cells were treated with 10 nM triptolide for 12 h. Apoptosis was analyzed using flow cytometry with annexin V and PI staining. (B) Early and late apoptosis rates were quantified. Results are presented as the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. control. (C) Following treatment with triptolide for 12 h, cellular total proteins were extracted and the apoptotic proteins caspase-3, caspase-9 and PARP1 were detected by western blot analysis. PI, propidium iodide; PARP1, poly(ADP-ribose) polymerase 1.

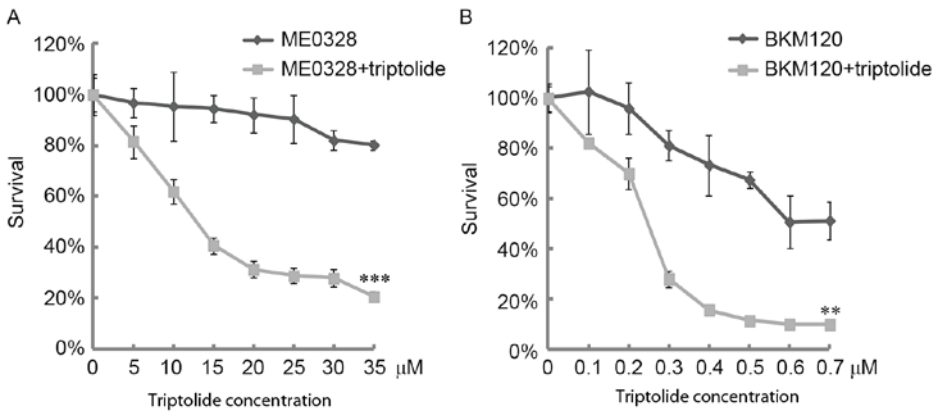

Figure 4. Triptolide sensitizes cells to poly(ADP-ribose) polymerase 1 inhibitor (ME0328) and phosphoinositide 3-kinase inhibitor (BKM120). (A) Cells were treated with 5 nM triptolide in combination with the indicated concentrations of ME0328 for 24 h and the viability of cell was detected using an MTT assay. (B) Cells were treated with 5 nM triptolide in combination with the indicated concentration of BKM120 for 24 h and the viability of cell was detected using an MTT assay. Results are presented as the mean ± standard deviation of three independent experiments. **P<0.01 and ***P<0.001 vs. control.

Triptolide induces DNA breaks and regulates Rad51 and PCNA levels. The present study investigated the DNA damage of CH12F3 cells following triptolide exposure. The CH12F3 cells were treated with triptolide (0, 10, 20, 30, 40 and 50 nM) for 4 h and the nuclear proteins were extracted. γH2AX was detected using western blotting (Fig. 2A). The expression of nuclear γH2AX was upregulated in a dose-dependent manner following triptolide treatment, which suggested that a high dose of triptolide induced DSBs (29,30). To confirm this result, the γH2AX level was detected using FCM, which revealed that triptolide increased the γH2AX level (Fig. 2B and C). These results further suggested that a high dose of triptolide resulted in cellular DSBs.

To illustrate the cellular response to triptolide, the expression of Rad51 and nuclear PCNA was detected in cells

treated with triptolide. Following treatment with triptolide (0, 10, 20, 30, 40 and 50 nM) for 4 h, Rad51 levels were increased (Fig. 2D) and nuclear PCNA was markedly upregulated by triptolide (Fig. 2E). PCNA is a DNA sliding clamp that functions in DNA replication. These results suggest the effect of PCNA in DNA replication is important for repairing DNA damage caused by triptolide.

Triptolide induces caspase-3-dependent apoptosis. Apoptosis of cells treated with triptolide was analyzed. CH12F3 cells were treated with 10 nM triptolide for 12 h. The apoptotic cells were analyzed through annexin V/PI staining. Triptolide caused apoptosis at early (annexin V-positive and PI-negative) and late (annexin V-positive and PI-positive) stages (Fig. 3A and B). The underlying molecular mechanism of apoptosis was revealed through analyzing the expression of apoptotic proteins. Cells were treated with triptolide at 1, 2, 4 and 5 nM for 12 h. The whole cellular lysate was extracted for western blot assay. Cleaved caspase-3, cleaved caspase-9 and cleaved PARP1 were up-regulated in a dose-dependent manner (Fig. 3C). These results demonstrated that triptolide induces caspase-3-dependent apoptosis.

Triptolide sensitizes CH12F3 cells to PARP1 and PI3K inhibitors. Following the aforementioned results demonstrating that triptolide induced DNA damage and apoptosis in CH12F3 cells, the sensitivity of CH12F3 cells to triptolide in combination with PARP1 and PI3K inhibitors was analyzed. At a dose of 5 nM triptolide, the cell viability was >80%; however, this dose of triptolide significantly sensitized CH12F3 cells to PARP1 (ME0328) and PI3K inhibitors (Fig. 4A and B). These results demonstrate that triptolide sensitizes lymphoma to PARP1 and PI3K inhibitors, which supports the use of triptolide as an antitumor drug to sensitize lymphoma cells to genotoxic agents.

Discussion

Triptolide was previously reported to inhibit cancer cell growth and induce apoptosis in different types of tumor (1-3). However, the majority of its antitumor activity was observed at high concentrations, at which adverse effects were noted (31). The present study investigated the effects of low doses of triptolide on DNA breaks and apoptosis as well as in combination with chemotherapeutic agents to treat B lymphoma cells. The results of the present study demonstrated that triptolide inhibits CH12F3 cell viability and low doses of triptolide (6 nM) suppressed XRCC1$^{-/-}$ CH12F3 cells, indicating that triptolide induces DNA breaks dependent on the XRCC1-mediated repair pathway. Additionally, γH2AX expression in CH12F3 cells treated with various triptolide doses demonstrated that triptolide induced DSBs in a dose-dependent manner. The present study also identified that triptolide induced caspase-3-dependent apoptosis.

A previous study demonstrated that triptolide specifically binds to TFIIH basal transcription factor and, through which, inhibits NER (13). This result suggested that triptolide exhibits DNA damage effects; however, the underlying molecular mechanism of triptolide in DNA damage repair are not well defined. The present study demonstrated that low doses of triptolide inhibited XRCC1$^{-/-}$ CH12F3 cells, but not ligase IV$^{-/-}$ CH12F3 cells or wild-type CH12F3 cells. XRCC1 is a scaffolding protein which recruits DNA repair-associated factors involved in BER and SSB repair. Thus, the results of the present study suggest that triptolide induces base damage or SSBs. Analyzing the expression of Rad51 and PCNA in cells treated with triptolide, the results of the present study revealed an 8-fold increase in PCNA expression following triptolide treatment. Rad51 serves a role in HR and PCNA is a sliding clamp that functions in DNA replication. This result suggests that triptolide may highly regulate the transcription of PCNA in response to DNA breaks. The present study analyzed the apoptosis induced by triptolide in CH12F3 cells and identified that low doses of triptolide induced caspase-3-dependent apoptosis. Considering that low doses of triptolide induced DNA breaks, it may be concluded that the DNA damage induced by triptolide triggers caspase-3-dependent apoptosis. PARP1 inhibitors are promising clinical therapeutic agents in the treatment cancer cells, particularly for HR-deficient cancer cells. Activation of PI3K signaling contributes to cancer cell proliferation, thus PI3K inhibitors are used clinically to treat tumors with aberrant PI3K signaling. Following the results of the present study revealing that low doses of triptolide induced DNA breaks and apoptosis, the application of low doses of triptolide in combination with PARP1 and PI3K inhibitors to treat lymphoma cells was analyzed. The present study demonstrated that 5 nM triptolide may efficiently sensitize CH12F3 cells to PARP1 and PI3K inhibitors. Therefore, triptolide may be a potent antitumor drug to sensitize lymphoma cells to chemotherapeutic agents. Further studies are required to explore the underlying molecular mechanism of triptolide function in regulating cellular DNA repair and sensitizing tumor cells to antitumor genotoxic agents.

Acknowledgements

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