ANTICANCER RESEARCH *40*: 6869-6877 (2020)
doi:10.21873/anticanres.14709

Maslinic Acid Induces DNA Damage and Impairs DNA Repair in Human Cervical Cancer HeLa Cells

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Abstract. *Background/Aim: Maslinic acid, a natural plant-derived triterpenoid compound, exhibits pharmacological activities, including anti-cancer. In the present study, we investigated the cytotoxic effects of maslinic acid on human cervical cancer HeLa cells in vitro and further investigated the molecular mechanism of maslinic acid-induced DNA damage and repair. Materials and Methods: Cell viability was measured by flow cytometry. DNA condensation (apoptotic cell death), DNA damage, and DNA fragmentation (DNA ladder) were assayed by DAPI staining, comet assay, and agarose gel electrophoresis, respectively. The expression of DNA damage and repair proteins was assayed by western blotting. Results: Maslinic acid decreased total cell viability and induced DNA condensation, damage, and fragmentation in HeLa cells. Furthermore, maslinic acid elevated the levels of p-ATM^{Ser1981}, p-ATR^{Ser428}, p53, p-p53^{Ser151}, p-H2A.X^{Ser139}, BRCA1 and PARP at 30-40 μM. However, it decreased the levels of DNA-PK and MGMT. Conclusion: Maslinic acid reduced the number of viable HeLa cells by inducing DNA damage and altering the expression of proteins involved in DNA damage and repair.*

Cervical cancer is the 8th most common cancer worldwide, with an estimated 569,847 new cases reported worldwide in 2018 (1). It is a highly preventable cancer and its incidence and mortality have declined in many developed countries (2-4) based on effective population-based screening programs, allowing diagnosis at earlier stages (5, 6). Human papilloma virus (HPV) (7), smoking (8), co-infection with HIV and some sexually transmitted infections (9), and genetic changes are significant risk factors and can lead to the development of cervical cancer (10). Currently, the treatments for cervical cancer include surgery, radiotherapy, and chemotherapy. Targeted therapy for cervical carcinoma is primarily focusing on EGFR (11) and COX-2 (12), which are associated with adverse events. Therefore, the search for new targets and new compounds for cervical cancer treatment is urgent.

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Key Words: Maslinic acid, human cervical cancer, HeLa cells, DNA damage, DNA condensation and fragmentation.

DNA damage (oxidation and alkylation of DNA bases, base mismatches, pyrimidine dimers, and single- and double-strand breaks) is caused by genotoxic agents and anti-cancer drugs (13, 14). These damages may cause mutations in the genome, and cells must maintain genomic stability by continually repairing such damages in a timely manner (15). Thus, cells trigger DNA repair responses for cell survival or apoptosis to eliminate cells that have developed genomic instability (16). Anti-cancer drugs may induce excessive DNA-damage that may result in cell apoptosis and/or necrosis (17, 18), or prolong blockage of proliferation (19, 20). Numerous anti-cancer drugs have been obtained from natural products and some of these drugs have been shown to cause DNA damage in cancer cells or to affect the DNA repair systems that may be related to their anti-cancer activities.

Maslinic acid (2-α, 3-β-dihydroxyolean-12-en-28-oic acid), a plant-derived natural triterpenoid compound, is isolated from *Olea europaea* L. (21), and has been found in a variety of medicinal plants (21, 22). Maslinic acid has shown a wide range of biological activities (22, 23), including anti-malarial (24), anti-protozoan (25), anti-virus (26), anti-oxidant (26), anti-diabetogenic (27), anti-inflammatory (28), and anti-tumor (22). Maslinic acid may also prevent oxidative stress and the production of pro-inflammatory cytokines (28, 29). Furthermore, oral administration of maslinic acid has been shown to induce anti-inflammatory and anti-arthritis effects in animal studies (30). Recently, we have also found that maslinic acid enhanced immune responses by elevating macrophage phagocytosis and natural killer cell activities in WEHI-3 cell generated leukemic mice (31).

Many studies have shown that maslinic acid causes apoptosis in many human cancer cells *in vitro,* but not in human cervical cancer cells (32, 33). The effects and the molecular mechanism of maslinic acid on DNA damage responses in human cervical cancer cells are still unclear. Therefore, in the present study, we investigated and further explored the mechanism through which maslinic acid affects expression of proteins involved in DNA damage and repair in human cervical HeLa cells *in vitro*.

Materials and Methods

Chemicals and Reagents. Maslinic acid, dimethyl sulfoxide (DMSO), propidium iodide (PI), and trypsin-EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Maslinic acid was dissolved in DMSO. Dulbecco's Modified Eagle's Medium (DMEM) medium, fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

Anti- p-ATM^{Ser1981} and -p-H2A.X^{Ser139} were obtained from GeneTex Inc. (Irvine, CA, USA). Anti-p-ATR^{Ser428}, -p-p53^{Ser15}, -BRCA1, and -PARP were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-p53 and -β-actin were obtained from Sigma-Aldrich. Anti-DNA-PK was purchased from Calbiochem (San Diego, CA, USA). Anti-MGMT was purchased from Millipore (Billerica, MA, USA).

Cell line and culture. The human cervical cancer cell line (HeLa) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). About 1x10^6 cells/ml were cultured in 90% DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, and penicillin-streptomycin (100 Units/ml penicillin and 100 μg/ml streptomycin) onto 75 cm^2 tissue culture flasks in an incubator with 90% humidity, at 37˚C and 5% CO_2 atmosphere as previously described (34).

Cell viability. HeLa cells (2×10^5 cells/well) were plated in 12-well culture plates overnight in a 5% CO_2 incubator and incubated with 0, 25, 30, 35, 40, or 45 μM of maslinic acid for 48 h. At the end of incubation, cells were harvested, washed with PBS, and then resuspended in PI solution (5 μg/ml) for measuring the total percentage of viable cells by flow cytometry as described previously (34).

4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. HeLa cells (2×10^5 cells/well) were plated in 12-well plates overnight and exposed to 0, 30, 35, or 40 μM of maslinic acid for 48 h. After treatment, cells were washed with PBS, fixed with 3.7% paraformaldehyde (v/v) in PBS for 15 min, and permeabilized by 0.1% Triton X-100 in PBS for 5 min. Subsequently, all samples were stained with DAPI (2 μg/ml) for 15 min, washed with PBS, and observed and photographed using a fluorescence microscope (Carl Zeiss, Axiovert 25, Oberkochen, Germany) at 200×, as described previously (35).

Comet assay. Single-cell electrophoresis (comet assay) was one of the methods to measure DNA damage in cells after exposure to reagents or compounds. HeLa cells (2×10^5 cells/well) in 12-well plates were treated with 0, 30, 35, or 40 μM of maslinic acid for 48 h. All cells were subjected to the comet assay and photographed, and their comets were randomly captured at a constant depth of the gel. Individual comet tail length following treatment was measured and quantified by using the TriTek Comet Score^{TM} software image analysis system (TriTek Corp, Sumerduck, VA, USA) as described previously (36).

DNA gel electrophoresis. HeLa cells (1×10^6 cells) in 10-cm dishes were incubated with 0, 30, 35, or 40 μM of maslinic acid for 48 h. After incubation, cells from were collected, washed with PBS, and were lysed in ice-cold lysis buffer. DNA was extracted, quantitated, and electrophoresed on a 2% agarose gel and photographed as previously described (37).

Western blotting. HeLa cells (1×10^6 cells) in 10-cm dishes were treated with 0, 30, 35, or 40 μM of maslinic acid for 48 h. After treatment, cells were collected for protein extraction. Cells were lysed by RIPA buffer [50 mM Tris-HCI (pH 7.4), 125 mM NaCl, 0.1% Triton X-100, and 5 mM EDTA containing both 1% protease inhibitor and 1% phosphatase inhibitor mixture II] (Sigma-Aldrich) as described previously (37). Extracted proteins were quantitated using the Bradford method (BSA as a protein control). Thirty μg of protein samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred

onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories; Richmond, CA, USA). Then, the membranes were blocked with 1% BSA, probed with primary antibodies against p-ATM^{Ser1981}, p-ATR^{Ser428}, p53, p-p53^{Ser15}, p-H2A.X^{Ser139}, BRCA1, DNA-PK, MGMT, PARP, and β-actin, followed with goat anti-mouse IgG coupled to HRP. The bound antibodies in the membranes were detected by chemiluminescence kits (NEN Life Science Products, Inc, Boston, MA, USA) and quantified by densitometry as described previously (37).

Statistical analysis. The data are shown as the mean values with a standard deviation (S.D.) (mean±S.D.) of three independent experiments and were analyzed using one-way ANOVA. $p<0.05$ was considered to indicate significant differences between the maslinic acid-treated and untreated (control) groups.

Results

Maslinic acid decreased cell viability of HeLa cells. Before investigating the effects of maslinic acid on DNA damage, we examined the effects of maslinic acid on cell viability. HeLa cells were treated with 0, 25, 30, 35, 40, or 45 μM maslinic acid for 48 h. Then, cells were collected and the total viable cell numbers (cell viability) were measured by flow cytometric assay. Treatment with 30-45 μM of maslinic acid led to a dose-dependent decrease in cell viability from 10.67 to 91.04% (Figure 1).

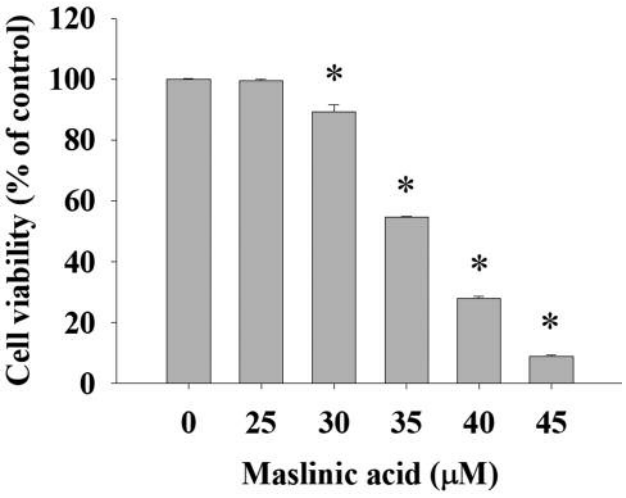

Figure 1. *Maslinic acid decreased the number of viable HeLa cells. Cells were incubated with 0, 25, 30, 35, 40, or 45 μM of maslinic acid for 48 h and collected for measuring the percentage of viable cells by flow cytometry as described in Materials and Methods. Experiments were performed in triplicate Data represents mean±S.D. *p<0.05 significant difference between maslinic acid-treated and control groups.*

Maslinic acid induced DNA condensation in HeLa cells. To investigate whether the decrease in cell viability in HeLa cells after exposure to maslinic acid was mediated by DNA condensation, HeLa cells were incubated with 0, 30, 35, or 40 μM of maslinic acid for 48 h. Subsequently, cells were stained with DAPI solution and the nucleus morphology was monitored under fluorescence microscopy. Maslinic acid-treated HeLa cells showed a lighter DAPI staining (Figure 2A) and fluorescence intensity (Figure 2B) than the control groups. Cells treated with 30-40 μM of maslinic acid for 48 h showed condensed chromatin structures (apoptotic cell death) when compared to the control group.

Maslinic aid induced DNA damage in HeLa cells. To confirm that the decrease in the number of viable HeLa cells by maslinic acid was mediated through the induction of DNA damage, cells were incubated with 0, 30, 35, or 40 μM of maslinic acid for 48 h and the comet assay was performed. As shown in Figures 3A and B, 30-40 μM of maslinic acid significantly increased the length of the comet tail when compared to that of control group (Figure 3A). These observations indicated that treatment with maslinic acid for 48 h induced DNA damage in a dose-dependent manner (Figure 3B).

Maslinic acid induced DNA fragmentation in HeLa cells. Furthermore, we examined the effect of maslinic acid on DNA fragmentation by DNA agarose gel electrophoresis (laddered DNA) in HeLa cells. Cells were incubated with 0, 30, 35, or 40 μM of maslinic acid for 48 h. The cellular DNA was isolated and electrophoresed by agarose gel, and the results are presented in Figure 4. The typical ladder pattern of oligonucleosomal fragments (DNA ladder) was observed at three doses of maslinic acid, which indicated that HeLa cells underwent apoptotic cell death.

Maslinic acid affected the levels of proteins associated with DNA damage and repair in HeLa cells. To further investigate the molecular mechanism of maslinic acid-induced effects described above, cells were exposed to maslinic acid and the levels of proteins involved in DNA damage and repair were investigated by western blotting. Treatment with 30-40 μM of maslinic acid augmented the expression of p-ATM^{Ser1981}, p-ATR^{Ser428}, p53, and p-p53^{Ser15} (Figure 5A), p-H2A.X^{Ser139}, BRCA1, and PARP (Figure 5B); however, decreased that of DNA-PK and MGMT (Figure 5B). Based on these observations, maslinic acid induced cell death by affecting the expression of proteins involved in DNA damage.

Discussion

In our bodies, cells suffer from DNA damage events every day (38) and various mechanisms have evolved to deal with these damaged cells. The evaluation of anti-cancer drugs and carcinogens is focused on the alteration of DNA damage and repair systems (39, 40). Maslinic acid exerts multiple

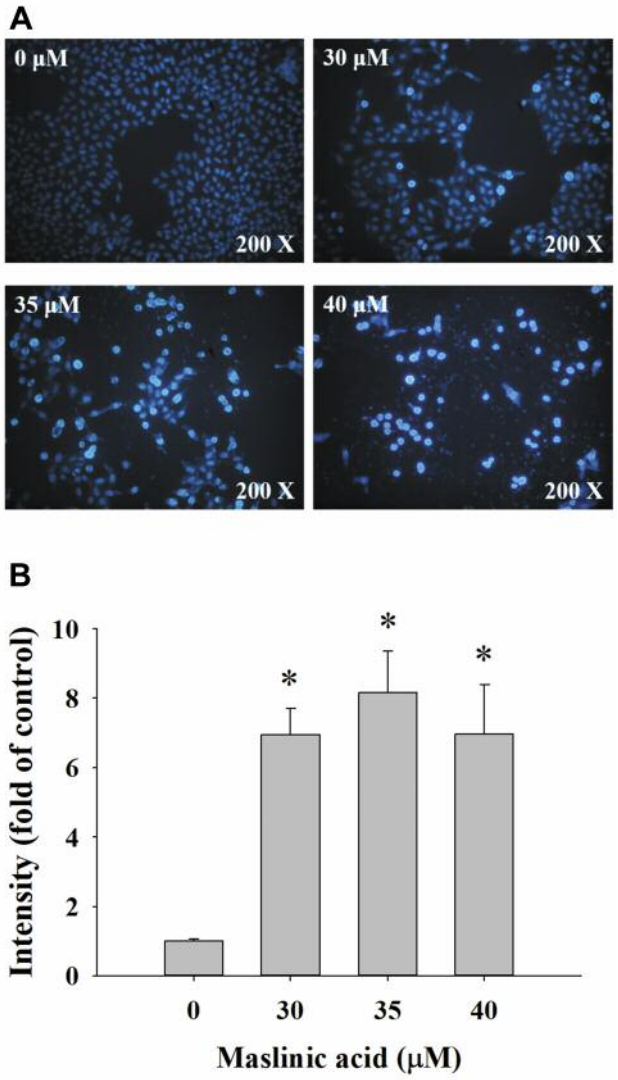

Figure 2. *Maslinic acid induced chromatin condensation (apoptotic cell death) in HeLa cells. Cells (2×10^5 cells/well) were plated in 12-well plate and were incubated with 0, 30, 35, or 40 μM of maslinic acid for 48 h. Cells were fixed, stained, examined, and photographed using a fluorescence microscope at 200× (A) and the intensity of fluorescence was measured (B) as described in Materials and Methods. Data represents mean±S.D. *p<0.05 significant difference between maslinic acid-treated and control groups.*

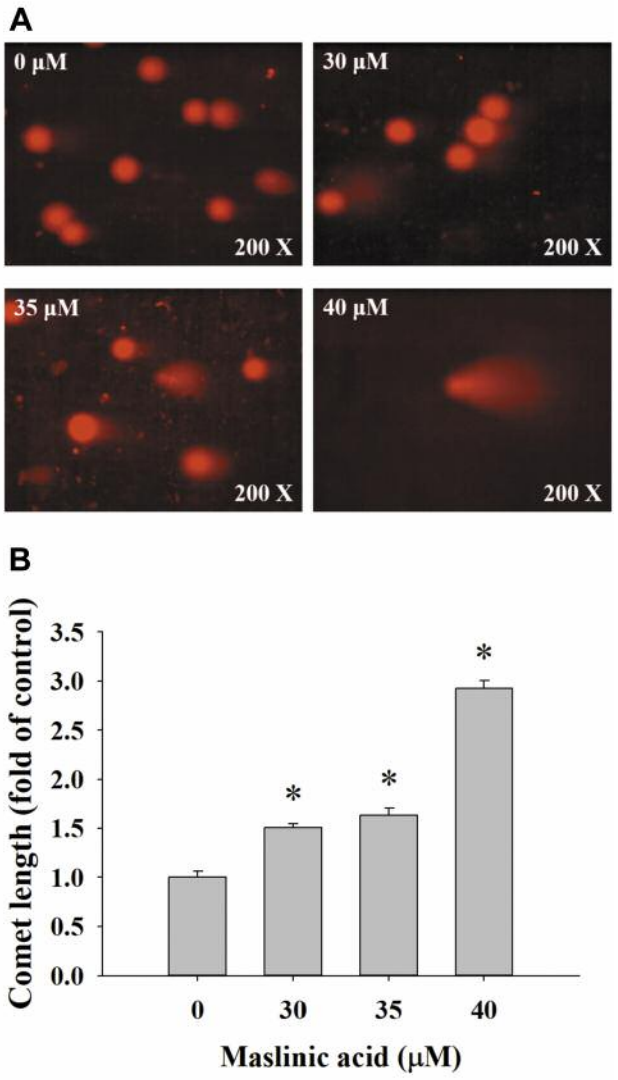

Figure 3. *Maslinic acid induced DNA damage in HeLa cells. Cells were incubated with 0, 30, 35, or 40 μM of maslinic acid for 48 h and were examined by the comet assay (A) and evaluated the length of comet tail (B) as described in Materials and Methods. Data represents mean±S.D. *p<0.05 significant difference between maslinic acid-treated and control groups.*

biological and pharmacological activities (22). Oral administration of maslinic acid did not have a profound effect including side effects, compared to untreated mice (41). There is no available information concerning the effects of maslinic acid on the induction of DNA damage in human cervical cancer cells. Therefore, in the present study, we investigated the effects of maslinic acid on DNA damage (apoptotic cell death) and their related mechanism in human cervical cancer HeLa cells *in vitro*.

At first, we investigate the effects of maslinic acid on the viability of HeLa cells *in vitro* and the results indicated that maslinic acid decreased cell viability in a dose-dependent manner (Figure 1). These findings are consistent with other reports showing that maslinic acid induced cytotoxic effects in different human cancer cell lines (42-44).

Second, we further confirmed that the reduction of cell viability by maslinic acid was mediated through the induction of cell damage by DAPI staining. The results

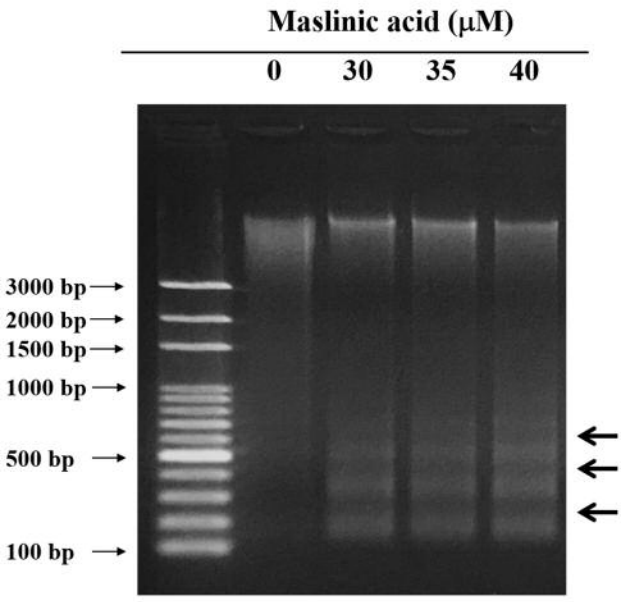

Figure 4. *Maslinic acid induced DNA fragmentation in HeLa cells. Cells were incubated with 0, 30, 35, or 40 μM of maslinic acid for 48 h. Cells were collected and lysed and DNA was extracted for agarose gel electrophoresis as described in Materials and Methods.*

showed that maslinic acid induced chromatin condensation, a marker of cell apoptosis (Figure 2A and B). These findings are in agreement with other reports showing that maslinic acid causes apoptosis in human colon cancer HT29 (21) and human leukemia HL-60 cells (44).

Third, we used the comet assay to confirm DNA damage of HeLa cells exposed to maslinic acid and the results indicated that maslinic acid caused DNA damage in a dose-dependent manner (Figure 3A and B). The higher amount of maslinic acid the longer the comet tail compared to the control group. The comet tail length seems to be the most sensitive parameter for examining the response to DNA damage (45). The comet assay is suitable for this purpose because it is relatively simple, sensitive, inexpensive and can be performed within few hours with a low number of cells (46).

Fourth, to further examine whether maslinic acid causes DNA fragmentation, cells were exposed to maslinic acid and then, laddered DNA was examined by agarose electrophoresis (47). The results showed that maslinic acid induced DNA fragmentation (Figure 4), which indicated apoptosis of HeLa cells *in vitro*.

Finally, we examined the effects of maslinic acid on the expression of proteins involved in DNA damage and repair in HeLa cells by western blotting. The ataxia-telangiectasia mutated (ATM), Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK) proteins are involved in the cellular response to DNA double-strand breaks (48). The results presented in Figure 5A indicate that maslinic acid increased the expression of p-ATM, p-ATR, p53, and p-p53. In mammals, after DNA damage, p53 is phosphorylated by DNA-PK and is stabilized and activated by DNA-damage checkpoint signaling (48). p53 is linked to the DNA-damage-response signaling pathway, and is activated after DNA damage (49). DNA double-strand breaks further activate ATM and ATR to phosphorylate p53 and to activate DNA repair pathways to remove double-strand breaks (DSBs) (50, 51). It is well known that the activation of ATM kinase results in p53 activation (52, 53). p53 is phosphorylated (p-p53) *via* post-transcriptional mechanisms and plays a crucial role in DNA repair (54, 55). DNA damage response is the predominant tumor suppression pathway (56), and the mechanism through which chemotherapy and radiotherapy exert their anti-tumor effects.

Treatment of Hela cells with 30-40 μM maslinic acid for 48 h increased the levels of p-H2A.X^{Ser139}, BRCA1, and PARP, however, it decreased the levels of DNA-PK and MGMT (Figure 5B). BRCA1, p-H2A.X^{Ser139}, and PARP are known to be involved in DNA damage repair systems (57). The phosphorylation of H2A.X has been verified to be a highly specific and sensitive molecular marker for DNA damage (58).

Herein, our results showed that maslinic acid promoted p-ATM. The phosphorylation of H2A.X activates ATM that may phosphorylate downstream targets, such as p53 and histone H2A.X (γH2A.X) (58), and lead to further activation of the ATM kinase, generating a positive feedback loop (59). BRCA1 protein directly participates in the repair of double-strand DNA (dsDNA) breaks (60) and deficiency of BRCA1 results in increased chromosomal instability and genomic alterations (61). Deficiency of DNA damage repair system by gene mutations has been found in various human cancers. MGMT is frequently methylated in various tumors such as gliomas (40%) (62) and patients bearing methylated glioma show high sensitivity to alkylating agents (63). It is well documented that poly ADP ribose polymerase (PARP) is involved in DNA damage, repair responses and genome stability. It participates in the cellular processes of DNA damage detection, DNA repair, and carcinogenesis (64). The activation of PARP contributes to cell survival during DNA damage (65).

In conclusion, our results showed that maslinic acid decreased viability of HeLa cells *in vitro via* the induction of DNA damage that was confirmed by DAPI staining, comet assay, and agarose gel electrophoresis. Furthermore, maslinic acid also altered the levels of DNA repair associated protein such as p-ATM^{Ser1981}, p-ATR^{Ser428}, p53, p-p53^{Ser15}, BRCA1, p-H2A.X^{Ser139}, DNA-PK, MGMT, and PARP in HeLa cells *in vitro* (Figure 6).

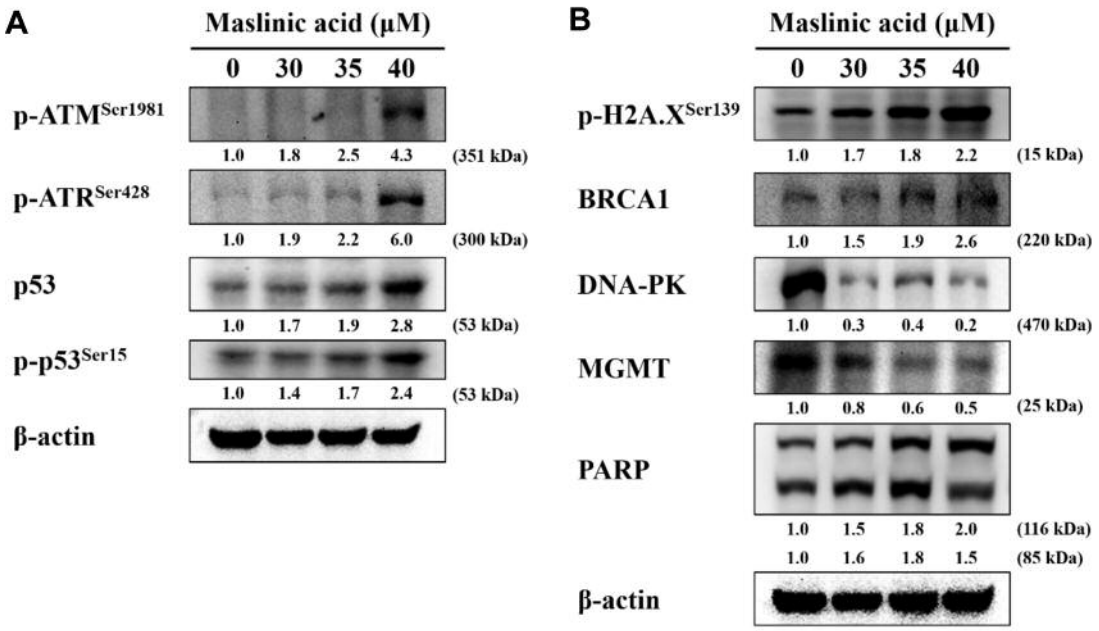

Figure 5. *Maslinic acid affected the expression of DNA damage and repair associated proteins in HeLa cells. Cells were incubated with 0, 30, 35, or 40 μM of maslinic acid for 48 h, lysed for western blotting and the resultant protein extracts were used to probe with anti-p-ATM^{Ser1981}, -p-ATR^{Ser428}, -p53 and -p-p53^{Ser15} (A), -p-H2A.X^{Ser139}, -BRCA1, -DNA-PK, -MGMT, and -PARP (B) as described in Materials and Methods. β-actin was used as an internal control.*

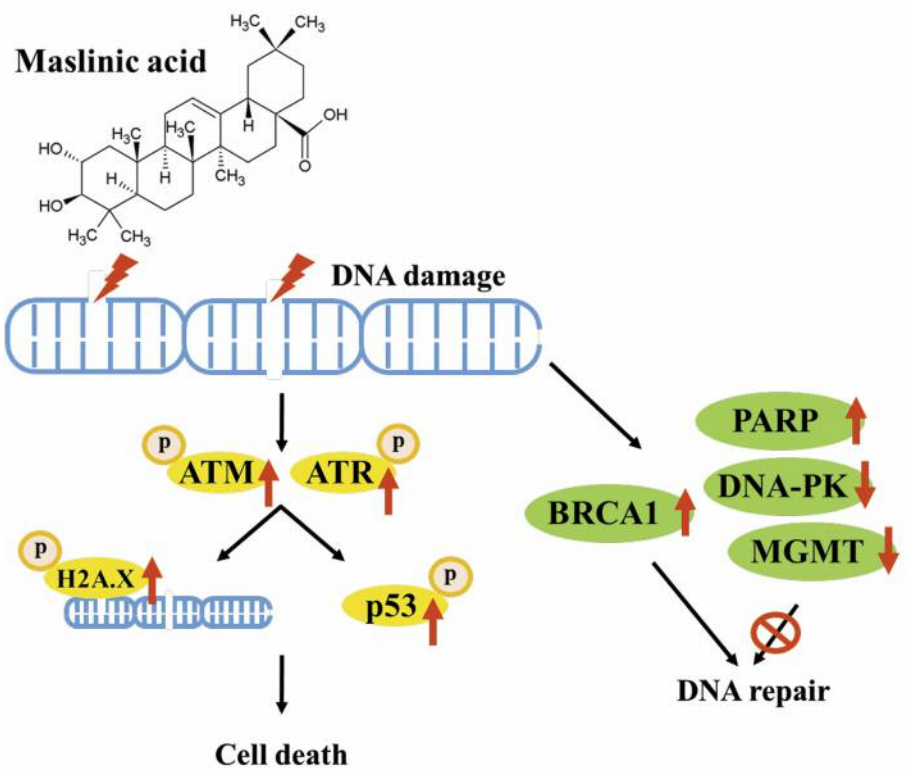

Figure 6. *Proposal of possible signaling pathways for maslinic acid-induced DNA damage and altered repair associated protein expressions in HeLa cells in vitro.*

Conflicts of Interest

The Authors confirm that there are no conflicts of interest regarding this study.

Authors' Contributions

K.W. Lu, M.D. Yang, K.C. Liu and Y.Y. Tai conceived and designed the experiments; K.W. Lu, M.D. Yang, S.F. Peng, J.C. Chen, P.Y. Chen and H.Y. Chen performed the experiments; K.W. Lu, T.J. Lu and F.S. Chueh analyzed the data; J.C. Lien and K.C. Lai contributed reagents/materials/analysis tools; K.C. Liu and Y.Y. Tai wrote the paper.

Acknowledgements

This work was supported by grant RD106069 from Chang Bing Show Chwan Memorial Hospital, Changhua, Taiwan, and by grant CMU106-ASIA-01 from China Medical University, Taichung, Taiwan. Experiments and data analysis were performed in part through the use of the Medical Research Core Facilities Center, Office of Research & Development at China Medical University, Taichung, Taiwan.

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Received July 28, 2020
Revised October 7, 2020
Accepted October 9, 2020